Characterization of microbial reductive dehalogenation using novel compound-specific stable isotope analyses

Charakterisierung der mikrobiellen Dehalogenierung mit der komponenten-spezifischen stabilen Isotopenanalyse

vorgelegt von
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Abstract

In recent decades, concepts involving compound-specific stable isotope analysis have evolved which allow the assessment of organohalide (bio)transformation *in situ* as well as to evaluate complex (bio)chemical reactions. However, isotope analysis of halogenated compounds is still hampered by the difficulty of measurements of hydrogen (H) and chlorine (Cl) by available standard methods.

This research project was focused on the development of alternative methods for H and Cl isotope analysis and the application of the compound-specific stable isotope analysis (CSIA) for characterization of microbial reductive dehalogenation reaction.

The development of a novel method for isotope analysis of H and Cl was realized by constructing an analytical set-up with a simultaneously operating dual-detection system, including ion trap mass spectrometry (MS) and isotope ratio mass spectrometry (IRMS). Application of two simultaneously operating detector systems offered the opportunity for a characterization of the conversion process (via MS) prior to isotope analysis (via IRMS) in great detail.

On-line Cl - isotope analysis was established by coupling gas chromatography (GC) to a high-temperature conversion (HTC) at 1450 – 1500 °C and subsequent isotope ratio mass spectrometry (IRMS) as GC-HTC-IRMS system. This approach was successfully applied for Cl - isotope analysis for different compound classes, including chloroethenes, chloroethanes, chloromethane, hexachlorocyclohexane and chloroacetic acids. The on-line H - isotope analysis could be significantly improved with a novel chromium-based reactor system operating in at 1100 – 1500 °C. The improvement of H - isotope analysis via hot chromium reduction was extended to various heteroatom (N, Cl, S) containing compound classes and was able to demonstrate its accuracy and precision.

The characterization of reductive dehalogenation by compound-specific stable isotope analysis was applied for abiotic and enzymatic reactions. The bottlenecks of single-element (carbon) isotope analysis for microbial systems could be identified and mainly addressed to limitation in micro-scale mass transfer though the membranes, as well as at the reductive dehalogenase enzyme itself. In contrast to the single-element approach, dual-element isotope analysis of carbon and chlorine was able to elucidate reductive dehalogenation reaction mechanism in much more detail. Conclusively,

compound-specific stable isotope analysis C and Cl supported a similar reaction mechanism of reductive dehalogenation in abiotic (mediated by corrinoids) and enzymatic (mediated by RDases) system. Furthermore, the effect of different corrinoid cofactors on the reaction mechanism of RDase enzyme could be excluded.

Zusammenfassung

Im Verlauf des letzten Jahrzehnts haben sich verschiedene Konzepte für die Anwendung in der komponenten-spezifischen stabilen Isotopenanalyse entwickelt, die einen Zugang zur Evaluierung von der in situ (Bio-)Transformation, wie auch (bio-) chemischen Reaktionsmechanismen bieten. Die Isotopenanalyse von halogenierten Komponenten war jedoch aufgrund der Schwierigkeit der Analyse von Wasserstoff (H) und Chlor (Cl) mit derzeitig verfügbaren Routinemethoden weitgehend eingeschränkt.

Das Ziel dieses wissenschaftlichen Projektes ist die Entwicklung von alternativen Analysemethoden für H und Cl, sowie die Anwendung der komponenten-spezifischen stabilen Isotopenanalytik auf die Charakterisierung der mikrobiellen reduktiven Dehalogenierungsreaktion.

Die Entwicklung von neuartigen Methoden für die Isotopenanalyse von H und Cl konnte durch einen Aufbau mit zwei simultan operierenden Detektorsystemen, der IonTrap Massenspektrometer (MS) und Isotopenverhältnis-Massenspektrometer (IRMS), realisiert werden. Die Anwendung von zwei Detektoren erlaubte eine detaillierte Charakterisierung des Umwandlungsprozesses (MS) vor der nachfolgenden Isotopenanalyse (IRMS).

Die On-line Cl-Isotopeneanalyse wurde durch die Kopplung von Gaschromatographie (GC) mit der Hochtemperatur-Umwandlung (HTC) bei 1450 - 1500 °C, sowie der anschließenden Isotopenverhältnis-Massenspektrometrie (IRMS) als GC-HTC-IRMS System verwirklicht. Diese Methode wurde erfolgreich für die Cl-Isotopenanlyse verschiedener Komponentenklassen eingesetzt, z. B. für Chloroethene, Chloroethane, Chloromethane, Hexachlorcyclohexane und Chloroacetate. Die H-Isotopenanalyse wurde durch die Entwicklung von neuen Reaktorsystemen erweitert, die auf Chrom basieren und eine Einsatztemperatur von 1100 – 1500 °C zulassen. Die Verbesserung der H-Isotopenanalyse durch Reduktion am heißen Chrom konnte anhand verschiedener heteroatomhaltiger Komponenetenklassen (N, Cl, S) erfolgreich demonstriert werden.

Die Charakterisierung der reduktiven Dehalogenierung durch die komponenten-spezifische stabile Isotopenanalyse wurde auf abiotische und enzymatische Reaktionen angewendet. Die Probleme der Single-Element (Kohlenstoff) Isotopenanalyse in mikrobiellen Systemen konnte identifiziert und auf

die Limitierung des Massentransfers durch die Membranen, sowie der Interaktion am reduktiven Dehalogenase Enzym zurückgeführt werden. Im Gegensatz zu der Single-Element (Kohlenstoff) war die Dual-Element (Kohlenstoff und Chlor) Isotopenanalyse in der Lage, den Reaktionsmechanismus der Dehalogenierung im Detail aufzudecken. Die Ergebnisse der komponenten-spezifische Isotopenanalyse von C und Cl stützen einen einheitlichen Reaktionsmechanismus in der reduktiven Dehalogenierung, sowohl in abiotischen, als auch in enzymatischen Systemen. Ein Effekt unterschiedlicher Corrinoid-Typen auf den Reaktionsmechanismus des RDase-Enzyms konnte ausgeschlossen werden.

List of Publications and Declaration on the Author Contribution

Publication I

Renpenning, J.; Keller, S.; Cretnik, S.; Shouakar-Stash, O.; Elsner, M.; Schubert, T.; Nijenhuis, I., Combined C and Cl isotope effects indicate differences between corrinoids and enzyme (*Sulfurospirillum multivorans* PceA) in reductive dehalogenation of tetrachloroethene, but not trichloroethene. *Environ Sci Technol* **2014**, 48, (20), 11837-45.

Contribution:

I. Nijenhuis developed the concept of this study. *Sulfurospirillum multivorans* cells containing PceA-*norpseudo*-B_{12} and PceA-*nor*vitamin B_{12} RDase were provided by S. Keller and T. Schubert. Chlorine isotope analysis was done with the help of S. Cretnik in the isotope laboratories of M. Elsner, using chlorine isotope analysis method of O. Shouakar-Stash. All experiments were conducted by me, including the purification of corrinoid cofactors, enzymatic and abiotic dehalogenation reactions, carbon and chlorine isotope analysis, data evaluation, as well as manuscript writing.

Publication II

Renpenning, J.; Hitzfeld, K. L.; Gilevska, T.; Nijenhuis, I.; Gehre, M.; Richnow, H. H., Development and Validation of an Universal Interface for Compound-Specific Stable Isotope Analysis of Chlorine ($^{37}Cl/^{35}Cl$) by GC-High-Temperature Conversion (HTC)-MS/IRMS. *Anal Chem* **2015**, 87, (5), 2832-9.

Contribution:

M. Gehre and H. Richnow developed the concept of the former setup (GC-HTC-qMS). This concept was partly accomplished by K. Hitzfeld in 2010. The novel setup extended the analytical scope, combining the dual-detection system of ion trap MS/IRMS. This setup was constructed and optimized by me. The characterization of the high-temperature conversion process and the measurements of chlorine isotope composition of reference material were conducted by me with additional support of T. Gilevska and I. Nijenhuis. The manuscript was written by me.

Publication III

Renpenning, J.; Rapp, I.; Nijenhuis, I., Substrate Hydrophobicity and Cell Composition Influence the Extent of Rate Limitation and Masking of Isotope Fractionation during Microbial Reductive Dehalogenation of Chlorinated Ethenes. *Environ Sci Technol* **2015**, 49 (7), pp 4293–4301

Contribution:

I. Nijenhuis and me developed the concept of this study. The practical work was done by me and Insa Rapp (master student). While Insa Rapp was mainly focused on experiment with *S. multivorans* and *D. hafniense* PCE-S, further experiments including all microorganisms (*S. multivorans, D. hafniense, G. lovely, D. restrictus and D. michiganensis*), data validation and manuscript writing was my personal contribution, as well as the writing of the manuscript.

Publication IV

Gehre, M.; **Renpenning, J**.; Gilevska, T.; Qi, H.; Coplen, T. B.; Meijer, H. A. J.; Brand, W. A.; Schimmelmann, A., On-line hydrogen-isotope measurements of organic samples using elemental chromium—an extension for high temperature elemental-analyser techniques. *Anal. Chem.* **2015**, 87, 5198-5205.

Contribution:

M. Gehre developed the concept of this study and was the main writer of the manuscript. I established the technical setup for the combination of dual-detection systems, combining the elemental analyser (EA or TC/EA) with MS/IRMS for simultaneous monitoring and characterization of the conversion process via ion trap MS and subsequent analysis of hydrogen isotopes via IRMS. Other co-authors performed inter laboratory isotope analysis using a similar method (conversion in a chromium-based reactor system), in order to validate the accuracy and precision of the developed method.

Book chapter

Book title: Organohalide Respiring Bacteria

Chapter: Evaluation of the microbial reductive dehalogenation reaction using compound specific stable isotope analysis (CSIA) by **Julian Renpenning** and Ivonne Nijenhuis

Editor names: Frank E. Löffler and Lorenz Adrian

Springer-Verlag GmbH, Heidelberg

Contribution:

The chapter "Evaluation of the microbial reductive dehalogenation reaction using compound specific stable isotope analysis (CSIA)" was written by me and Ivonne Nijenhuis and summarizes the state of the art of isotope analysis in microbial reductive dehalogenation research.

The scientific results were presented in the following international conferences

Renpenning, J.; Keller, S.; Cretnik, S.; Elsner, M.; Schubert, T.; Nijenhuis, I., Effect of different corrinoid cofactors on the reaction mechanism of Sulfurospirillum multivorans PceA using 2D-CSIA ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$). Oral presentation at DehaloCon 2014 – Jena, Germany

Renpenning, J.; Keller, S.; Cretnik, S.; Elsner, M.; Schubert, T.; Nijenhuis, Effect of Corrinoid Cofactor Type on the Reductive Dehalogenation and Protonation Reaction. Oral presentation at Goldschmidt 2014 – Sacramento, USA

Submitted patents

Title (German): Verfahren und Reaktor zur quantitativen Gewinnung von molekularem Wasserstoff aus Substanzen sowie deren Verwendung zur massenspektrometrischen online Bestimmung des Isotopenverhältnisses von Wasserstoff

Patent 1 – EA-Cr/HTC-IRMS: Anmeldenummer 102015102126.9 – submitted (13.02.2015)

Patent 2 – GC-Cr/HTC-IRMS: Anmeldenummer 102015102710.0 – submitted (25.02.2015)

List of Content

Abbreviation

B_{12}	Cobalamin
CF-IRMS	Continues-Flow Isotope Ratio Mass Spectrometry
CSIA	Compound-Specific Stable Isotope Analysis
DCE	Dichloroethene
DI	Dual-inlet
GC	Gas Chromatography
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HTC	High Temperature Conversion
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IRMS	Isotop-Ratio Mass Spectrometry
KIE	Kinetic Isotope Effect
LC	Liquide chromatography
m or Λ	Dual-element slope
MS	Mass Spectrometry
MTBE	Methyl *tert*-butyl ether
PCE	Tetrachloroethene
PceA	*S. multivorans* reductive dehalogenase
qMS	Quadrupole Mass Spectrometry
RDase	Reductive dehalogenase
SMOC	Standard Mean Ocean Chloride
TC/EA	High Temperature Conversion Elemental Analyser
TCE	Trichloroethene
TIMS	Thermal Ionization Mass Spectrometry
VC	Vinyl chloride
VPDB	Vienna Pee Dee Belemnite
VSMOW	Vienna Standard Mean Ocean Water
ε	Isotope enrichment factor

1. Introduction

In recent decades, concepts involving compound-specific stable isotope analysis have evolved which allows the assessment of organohalide (bio)transformation *in situ*, as well as the evaluation of complex (bio)chemical reactions. The stable isotope composition can provide information on the source and fate of a specific chemical. In addition, changes of isotope composition over time can allow to assess degradation pathways and reaction mechanisms involved during the (bio)transformation. Chlorinated hydrocarbons have a history of extensive use and application in the industrial society, as for instance cleaning of machinery, electronic parts, dry cleaning, but also application in chemical industry. Due to improper storage, handling and disposal chlorinated solvents were released into environment and are now one of the most frequent groundwater and soil contaminants worldwide (Doherty 2000). Chlorinated hydrocarbons are threatening to human health due to their toxicity, potential carcinogenicity and persistence in groundwater and soil. The fate of these pollutants was therefore a major concern during the last four decades. A surprising observation was the disappearance of those pollutants due to microbial degradation process, which is known today as the organohalide respiration (Bouwer and McCarty 1983, Leys et al. 2013).

Compound-specific stable isotope analysis was one of the techniques that were successfully applied for monitoring and evaluation of *in situ* (bio)-remediation. Furthermore, isotope analysis was applied in order to access and understand the reductive dehalogenation reaction of several microorganisms that were found to be capable of organohalide respiration. However, success of carbon isotope analysis was limited for chlorinated hydrocarbons. Further, though analytical methods for isotope analysis of hydrogen is established as routine application for hydrocarbons, analysis of halogenated compounds was still hemmed by the difficulty of measurements of hydrogen in presence of halogens. Moreover, for chlorine isotope analysis no routine application is available thus far. Therefore, development of suitable methods is essential in order to access compound-specific stable isotope analysis of all elements in halogenated hydrocarbons.

2. Review of literature

2.1. What are isotopes?

The translation of the word *isotopes* (Greek: iso- 'equal' and topos 'place') reflects that the corresponding elements share the same place in the periodic table. First indication of the existence of multiple masses for the same elements was found together with the discovery of radioactivity by Henri Becquerel, Pierre and Marie Curie, rewarded with the Nobel Prize 1903 in Physics. Despite a giant step forward of the understanding of radioactivity, scientists still understood little of the structure of the atom. This understanding awaited the work of Ernest Rutherford. In 1911, Rutherford conducted a series of experiments in which he bombarded a piece of gold foil with positively charged α-particles emitted by radioactive material. His results suggested that the gold foil matrix primarily consists of empty space surrounding a well-defined central core, which he called 'nucleus'. However, it was Frederick Soddy who hypothesized 1913 that some elements can exist with different atomic mass with chemically inseparable and indistinguishable properties (Soddy 1913). Conclusively, those elements supposed to reside in the same place in the periodic table. For his identification of isotopes Frederick Soddy was rewarded with the Nobel Prize in Chemistry 1921 (Jordan 1942).

Isotopes of the same element have an identical number of protons. However, they differ in the number of neutrons. All elements exist in different energetic state and can be divided in stable isotopes (non-radioactive) - as for instance or ^{12}C and ^{13}C, and unstable (radioactive) isotopes - as for instance ^{14}C. Isotopes of an element can be denoted by an atomic formula, i.e. carbon-12 $^{12}_{6}C$, with a total atomic mass of 12, including 6 protons (Z). The remaining difference is the number of neutrons (N) harbored by an isotope, in case of carbon-12 the number of neutrons is 6. Generally, isotopes with a similar number of protons and neutrons ($N/Z \leq 1.5$) tend to be stable. Around 300 stable isotopes are known thus far, however, the number of non-stable radioactive isotopes is about four times higher (Hoefs 1987).

A unique isotopic composition can be found in a wide variety of materials, which provides an insightful technique to elucidate their origin, history, formation or degradation based on the isotopic profile of its atoms and molecules. Isotope analysis can therefore be used in a wide range of

applications, i.e. in the field of physical and organic chemistry, geochemistry and hydrology (Thiemens 2006).

2.2.Definition and Delta-notation

The stable isotope composition of a substance is normally reported in delta-notation (δ) in parts per thousand (per mil, ‰) relative to a known reference material. Alternatively to per mil (‰) the SI unit Urey (Ur) was introduced by Brand and Coplen (2012) as a new unit for the derived quantity isotope δ, whereby milli-urey (symbol 'mUr') is equal to per mil. The isotopic abundance of an element is usually determined relative to an international reference standard, which ensures the comparability of the isotope analysis in different laboratories on an international scale (Coplen 2011). For an element *E* isotope composition (δ) can be calculated using the isotope ratio R_{sample} for the measured compound and isotope ratio $R_{refernce}$ for the reference material, as presented equation 1 and 2.

$$R = \frac{{}^{H}E}{{}^{L}E} \qquad [1]$$

$$\delta(E)_{sample} = \left(\frac{R_{sample} - R_{reference}}{R_{reference}}\right) \times 1000 \qquad [2]$$

Corresponding reference material is provided by two organizations, the International Atomic Energy Agency (IAEA, www.iaea.org) and the National Institute of Standards and Technology (NIST, www.nist.gov). The standardization allows comparison of isotope signatures between different laboratories and experiments. Since international standards are expensive for routine use, laboratories apply one or more internal working standards, which are compared against the international standard. Additionally, to prevent potential analytical problems, compound specific standards are often used in order to ensure quality control.

2.3.Application of isotope analysis

After the natural abundance of the elements was determined, variation in isotope composition in material linked to the material sources was noted. Naturally-occurring variations in the abundances of the stable isotopes of carbon and other elements can be used to understand the dynamics of natural processes in chemistry, biochemistry, biology, medicine, ecology and other fields as geology and astronomy. Variation of isotope composition due to natural processes can provide information on biogenic relation and origin of inorganic as well as organic compounds. Prominent examples are the respiration in tree and the corresponding division of plants into C3, C4 and CAM plants, diary studies of ancient humans, nitrification rates in forests, biodegradation and climate history of earth (Oleary et al. 1992, Brenna et al. 1997, Meier-Augenstein 1999). Furthermore, an extensive range of application in forensics is accusable, including isotope finger print for source identification of chemicals, pharmaceuticals, fuels, food and many more possible (Lichtfouse 2000).

Another widespread application of isotopes are trace studies, as for instance quantitative studies of biochemical processes such as assimilation and incorporation of biogenic material, turnover rates of biologically important molecules and quantification of protein synthesis. Trace techniques facilitate a number of applications in biomedical sciences and revolutionized clinical diagnostics (Meier-Augenstein 1999, Lichtfouse 2000).

In recent decades, concepts involving compound specific stable isotope analysis have evolved which allow the assessment of *in situ* biotransformation of groundwater and soil contaminants, as well as to evaluate complex (bio)chemical reactions. Chlorinated solvents, as for instance chlorinated ethenes, are common groundwater contaminants of great concern due to their large environmental releases, microbial formation, human toxicity, potential carcinogenicity and tendency to persist in and migrate with groundwater to drinking water supplies, exposing health risk to human and groundwater dependent eco-systems (Semprini 1995, Field and Sierra-Alvarez 2004). The stable isotope composition is able to provide information on the source and fate of a specific chemical. Changes of isotope composition over time or space can allow to assess degradation pathways and reaction mechanisms involved during (bio)transformation (Sherwood Lollar et al. 1999, Slater 2003, Meckenstock et al. 2004). This topic is introduced in detail in chapter 2.7 '*Reductive dehalogenation*'.

2.4.Isotopes of interest in chlorinated hydrocarbons

Currently compound-specific stable isotope analysis (CSIA) of hydrogen, carbon and nitrogen are the most common routine applications (Elsner and Hofstetter 2011). Additional techniques are available for isotopes analyses of a wide range of elements, as for instance oxygen, sulfur, chlorine, bromine, but also noble gases and metals. This chapter, however, will focus on three relevant elements for organohalide-respiration of chlorinated hydrocarbons: hydrogen ($^{2}H/^{1}H$), carbon ($^{13}C/^{12}C$) and chlorine ($^{37}Cl/^{35}Cl$) isotopes (Table 1).

Table 1: Relative abundance of hydrogen, carbon and chlorine isotopes modified after Coplen (1994)**.**

Element	Stable isotopes	Natural abundance (%)		Relative mass difference (%)	International reference standard
		$^{heavy}E$	$^{light}E$		
Hydrogen	$^{2}H/^{1}H$	0.0155	99.98	100	VSMOW
Carbon	$^{13}C/^{12}C$	1.1060	98.89	8.3	VPDB
Chlorine	$^{37}Cl/^{35}Cl$	24.220	75.78	5.7	SMOC

2.4.1. Carbon

The original reference material for carbon isotope analysis, the marine fossil Pee Dee Belemnite, is exhausted. Nevertheless, carbon stable isotope composition is still reported relative to the Vienna-PeeDee Belemnite (VPDB) international standard, which was assigned a $\delta^{13}C$ of 0‰ (Coplen, et al., 2006), by using alternative reference material. Variation of carbon isotope composition on earth was reported to range from the lowest $\delta^{13}C$ values of -130.3 ‰ for a material of natural terrestrial origin and the most positive $\delta^{13}C$ values of +37.5 ‰ reported for deep-sea pore waters (Elvert et al. 2000, Coplen et al. 2002). The three main carbon reservoirs on earth are sedimentary organic material, the biosphere including atmospheric carbon, and sedimentary carbonates. The three reservoirs may differ from each other according to the isotope fractionation they undergo during formation. Subsequently, isotope composition incorporated in plants, food and animal tissue is characteristic according to their carbon source and the metabolic pathway used for hydrocarbon formation (Coplen et al. 2002).

2.4.2. Chlorine

Chlorine isotope composition ($\delta^{37}Cl$) is usually expressed relative to standard mean ocean chlorine (SMOC), which is assumed to be isotopically homogeneous (Kaufmann et al. 1988). The use of chlorine stable isotopes to evaluate hydrological processes and sources of chloride in aqueous environments is, to some extent, limited by the small range of observed isotopic ratios. Due to the small relative mass difference between ^{35}Cl and ^{37}Cl isotopes only minor isotope fractionation was observed for chlorine. In groundwater, surface and pore waters $\delta^{37}Cl$ range generally from -8.00 to +3.00 ‰ (Coplen et al. 2002). The lowest $\delta^{37}Cl$ values for naturally occurring pore water samples were measured to be -7.7 ‰ and the highest +7.5 ‰ for Cl in smectite from a Costa Rica Rift ocean drill hole (Magenheim et al. 1995, Ransom et al. 1995). Chlorine isotope composition of organic solvents was reported to range from -6.0 to +4.4 ‰ (Tanaka and Rye 1991). However, due to the lack of routine available techniques (chapter 2.5), isotope fractionation of chlorine in chlorinated compounds is poorly investigated yet.

2.4.3. Hydrogen

The natural abundance of deuterium (^2H or D) is relatively low (0.0155%) and δ^2H values are usually reported relative to IAEA reference water VSMOW (Vienna Standard Mean Ocean Water), which is assigned a δ^2H of 0‰ (Coplen et al. 2002). Due to the highest relative mass difference between both isotopes (^1H and ^2H) undergoes hydrogen the strongest kinetic isotope effects, which results in isotope fractionation and is reflected in the largest range of isotope composition in comparison to other elements. Variation of hydrogen isotope composition is primarily caused by evaporation and condensation processes of meteoric water (water precipitation). Therefore, δ^2H values of water from different origin on earth can range from -495 ‰ in Atlantic ice shields to +129 ‰ evaporated lakes (Jouzel et al. 1987, Coplen et al. 2002). Isotope composition of plants usually reflects the isotope composition of regional water precipitates and is further incorporated into processed food and animal tissue.

2.5.Isotope analysis techniques

Though isotopes can generally be measured in one way or another by all types of mass spectrometers, the determination of accurate isotope composition is mainly dominated by isotope ratio mass spectrometry (IRMS) for more than five decades. The distinguishing characteristic of IRMS is the high-precision ($1\sigma < 0.1$ ‰) of measurements (Brenna et al. 1997). Measurements usually realized in a combination as dual-inlet isotope ratio mass spectrometry (DI-IRMS) or as continuous-flow isotope ratio mass spectrometry (CF-IRMS). High precision, however, is accomplished at an expense of flexibility, since for analysis of isotope composition the samples have to be converted to an appropriate analyte gas to which the Faraday cups of the IRMS is adjusted. Alternatively to the IRMS several other mass spectrometers are usually applied, as for instance the quadrupole mass spectrometer (qMS), the thermal ionization mass spectrometer (TIMS), and the inductively coupled plasma mass spectrometry (ICP-MS). Though, qMS is a more flexible device, precision of isotope measurement is usually about one order of magnitude lower in comparison to the IRMS. The precision of TIMS and ICP-MS, on the other hand, is in comparison to the IRMS similar or better (Brenna et al. 1997, Holmstrand et al. 2004, Elsner et al. 2012). However, TIMS cannot be applied for compound-specific analysis and the application of ICP-MS is currently limited by the high investment costs. Therefore, IRMS remains the system of choice for routine application of stable isotope analysis.

For analysis of stable isotope composition via IRMS the sample has to be converted quantitatively into a suitable purified gas that can be analyzed by a mass spectrometer, as for instance schematically shown for carbon or for hydrogen isotope analysis in Figure 1 (Elsner and Hofstetter 2011). The conversion of the organic sample into a gas can be done by an ’off-line’ method, where conversion of pure or bulk sample is usually done in a sealed tube, or an ‘on-line’ method, where a sample preparation line is connected directly to the mass spectrometer. ‘Off-line’ conversion techniques, however, are time-consuming and only a small number of samples can be analyzed per day. Moreover, ‘off-line’ techniques are not suitable for compound mixtures, as for instance in biological or environmental samples. Today continuous-flow isotope ratio mass spectrometry is frequently used as an ‘on-line’ method for stable isotope analysis. Isotope composition can be measured on-line for bulk or pure samples using elemental analyzed in combination with isotope-ratio mass spectrometry (EA-

IRMS or TC/EA-IRMS) (Figure 1). This type of system is appropriate for most organic tissue samples, sediment and soil samples containing sufficient organic matter. To make this technique appropriate for sample mixtures, gas chromatography in combination with isotope ratio mass spectrometry (GC-IRMS) analysis was introduced, where separation was applied prior to the conversion of a sample and the subsequent measurements of isotopes in the analyte gas (Figure 2) (Barrie et al. 1984, Meier-Augenstein 1999, Sessions 2006). Different conversion techniques are applied prior to MS analysis, as for instance combustion for carbon or pyrolysis for hydrogen, which will be discussed in more detail below. Additionally, liquid chromatography in combination with isotope ratio mass spectrometry (LC-IRMS) was introduced for polar compounds that are not GC compatible (Brenna et al. 1997, Krummen et al. 2004). The LC approaches may therefore extend the access to further substance classes, such as pharmaceuticals and polar pesticides.

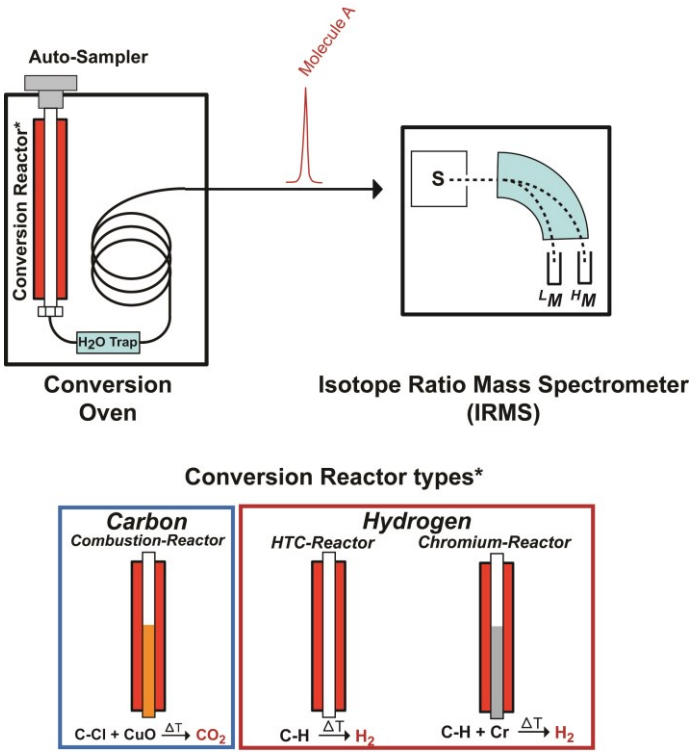

Figure 1: Continuous-flow methods for isotope analysis of bulk samples via EA-IRMS, combining a conversion oven/reactor (EA or TC/EA systems) and isotope ratio mass spectrometer (IRMS) for carbon and hydrogen. The conversion technique is indicated in respect to the analyzed element.

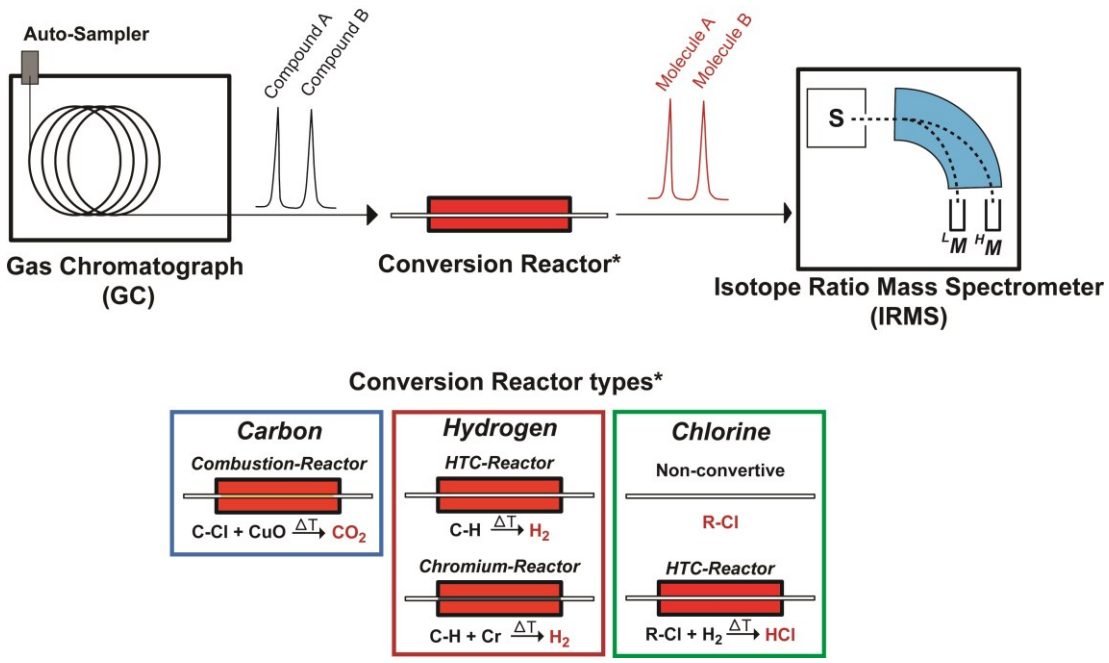

Figure 2: Compound-specific stable isotope analysis, combining gas chromatography (GC), conversion reactor (combustion or HTC) and isotope ratio mass spectrometry for carbon, chlorine and hydrogen. The conversion technique is indicated in respect to the analyzed element.

2.5.1. Carbon stable isotope analysis

Carbon isotope analysis is nowadays the most frequent application in CSIA. Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) is commonly used for the compound-specific measurements of carbon isotopes. In GC-C-IRMS target compounds are first chromatographically separated and subsequently converted to CO_2 as analyte gas in the combustion unit at 980-1050 °C. The combustion unit consists of a ceramic tube with CuO/NiO/Pt as oxygen donor and catalyst for a quantitative conversion of carbon to CO_2 as analysis gas for isotope measurements (Brenna et al. 1997, Elsner et al. 2012). After transfer of the analyte gas to the IRMS CO_2 becomes ionized and the different isotopes are separated in a magnetic field according to their mass to charge ratio (m/z). Faraday cups, adjusted to atomic mass units (amu) 44 [$^{12}C^{16}O_2$] and 45 [$^{13}C^{16}O_2$], are measuring the abundance of the different isotopes.

2.5.2. Chlorine isotope analysis

Although essential for numerous chlorinated compounds, as for instance a broad range of chlorinated pollutants (Sakaguchi-Soder et al. 2007, Cincinelli et al. 2012, Zhang and Qi 2012, Lebedev 2013),

compound-specific stable isotope analysis of chlorine was limited thus far by the difficulty of conversion of the sample into a simple chlorine-containing gas for isotope measurements by CF-IRMS (Brenna et al. 1997, Sessions 2006, Elsner et al. 2012). Nowadays chlorine isotope analysis is mainly restricted to traditional off-line methods, including time consuming conversion to either chloromethane (Kaufmann et al. 1984, Holt et al. 1997) or cesium chloride (Holmstrand et al. 2004) in combination with measurements via dual inlet isotope ratio mass spectrometry (DI-IRMS) or thermal ionization mass spectrometry (TIMS) (Figure 3). Further, pure compounds and bulk samples can be analyzed 'off-line' exclusively. Several techniques were applied during the last decade for on-line analysis of chlorine isotopes, which do not require previous conversion of organochlorides, but direct measurement of molecular or fragment ions (Figure 1) (Bernstein et al. 2011). These methods use a combination of GC-qMS (Aeppli et al. 2010, Jin et al. 2011) or GC-IRMS (Shouakar-Stash et al. 2006), which have already been successfully applied for chlorine isotope analysis of chlorinated ethenes and some chlorinated ethanes (Aeppli et al. 2010, Hunkeler et al. 2011, Wiegert et al. 2012, Audi-Miro et al. 2013, Cretnik et al. 2013, Wiegert et al. 2013, Cretnik et al. 2014, Palau et al. 2014, Renpenning et al. 2014). However, due to specific cup configurations required for IRMS or moderate precision of qMS ($1\sigma > 0.5‰$) both methods can, thus far, only be applied for a limited range of compound classes (Elsner et al. 2012). Moreover, at least two reference standards are required for calibration of the measurements to SMOC-scale for each compound class. Thus far, however, reference material for chlorine isotopes is limited or not available for many compound classes. In recent years application of inductively coupled plasma in combination with multi-collector mass spectrometry (MC-ICP-MS) for chlorine isotope measurements was demonstrated in several studies (Van Acker et al. 2006, Zakon et al. 2014). Though application of MC-ICP-MS is a promising universal method with a high precision ($1\sigma \approx 0.1‰$), it is inappropriate for routine application, due to the low ionization efficiency of chlorine, large sample size (several hundred nmol of chlorine) required for analysis, interference with $^{36}Ar^{1}H$ ions and high instrumental costs. A novel application introduced by Hitzfeld et al. (2011) involves a high-temperature conversion (HTC) of chlorinated hydrocarbons at 1300°C-1400°C to hydrochloric acid (HCl) as target compound for chlorine isotope analysis, may

overcome the limitation of chlorine isotope analysis and extend the range of compound classes available for chlorine isotope analysis in future.

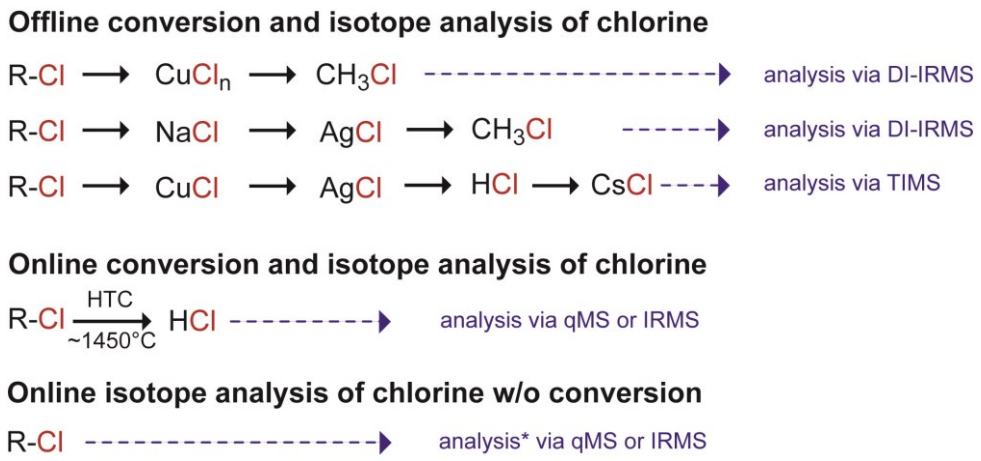

Figure 3: Overview of off-line and on-line conversion and spectrometric analysis techniques for chlorine isotopes. *Analysis of non-converted sample as molecular or fragment ion

2.5.3. Hydrogen isotope analysis

Traditionally, measurements of hydrogen isotopes were done via conversion of water or hydrogen containing substances to elementary hydrogen (H_2). 'Off-line' conversion of the samples was performed via oxidation into water (for samples other than water) and subsequent reduction by zinc, uranium, chromium, but also other metals (i.e. magnesium or tungsten) to elementary H_2 analyte gas and measurement via DI-IRMS (Figure 4) (Friedman 1953, Nief and Botter 1959, Kokubu et al. 1961, Schiegl and Vogel 1970, Coleman et al. 1982, Wong and Klein 1986, Gehre et al. 1996). Direct conversion of organic hydrogen to molecular hydrogen was achieved via high-temperature conversion at > 900 °C and later combined to CF-IRMS an 'on-line' stable isotope analysis of hydrogen (Sofer and Schiefelbein 1986, Wong and Klein 1986). Nowadays compound-specific stable isotope analysis of hydrogen is performed with a combination of GC-IRMS via pyrolysis of the target compound at 1400-1450 °C and the subsequent measurement of hydrogen isotopes as H_2 analyte gas (Brenna et al. 1997, Gehre and Strauch 2003). Though this is an excellent routine application for hydrocarbons, analysis of hetero-atoms containing compounds (i.e. N, S, P) and especially halogens (X = F, Cl, Br, I) remains challenging, since pyrolysis of those compounds results in formation of hydrogen containing byproducts (i.e. HCN, HX) (Chartrand et al. 2007, Brand et al. 2009, Hitzfeld et al. 2011). Thus far

only few techniques are available for hydrogen isotope analysis of chlorinated compounds. Application of chromium at 1050 °C in an elemental analysis reactor was demonstrated to be a promising technique (Gehre et al. 1996, Morrison et al. 2001, Armbruster et al. 2006). Hydrogen isotope analysis of chlorinated ethenes using chromium-based Quartz reactors for conversion at 850-1000 °C was applied by Kuder and Philp (2013) and Shouakar-Stash and Drimmie (2013) in combination with GC-IRMS for CSIA. Nevertheless, standard application of hydrogen isotope analysis of chlorinated compounds is thus far not routine.

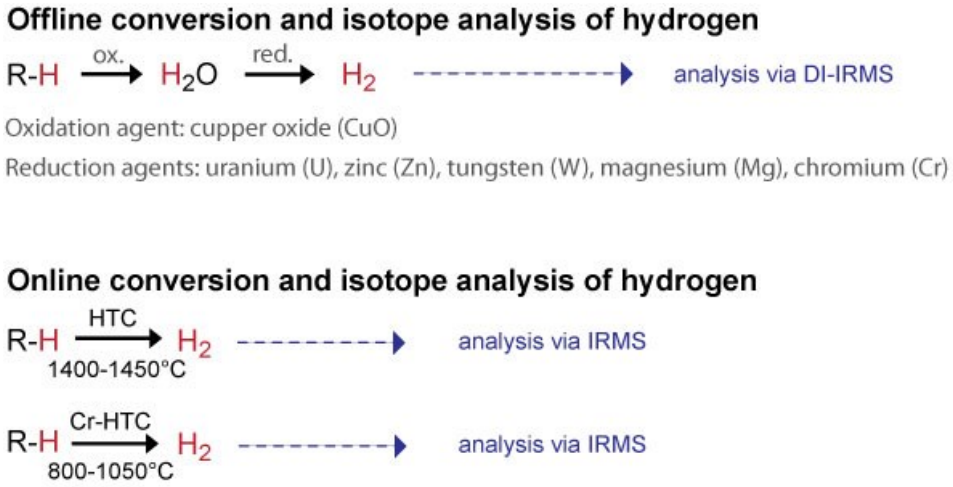

Figure 4: Overview of off-line and on-line conversion and spectrometric analysis techniques for hydrogen isotopes.

2.6.Isotope effects and isotope fractionation

2.6.1. Kinetic isotope effect (KIE)

Stable isotope analysis can be used to obtain information on sources, transport routes, degradation pathways and sinks of organic chemicals in the environment. (Bio)chemical degradation of contaminants leads to kinetic isotope fractionation, a result of the kinetic isotope effect (KIE), which may alter the isotope composition of organic chemicals. The isotope fractionation can be applied for characterization of (bio)chemical reaction mechanism by observing the transition state of a chemical bond cleavage (Northrop 1981, Paneth 2003, Dybala-Defratyka et al. 2007).

Chemical behavior of two isotopes of the same element is qualitatively similar, since the number of electrons harbored in outer shell of an atom is identical in both isotopes. However, both isotopes still differ in their reaction-rate and bond strength. Their chemical kinetics is frequently rationalized by

using the transition state theory (TST). The difference in reaction kinetics between the heavy and light isotopes in the transition state is called kinetic isotope effect (KIE) [equation 3].

$$KIE_E = \frac{^{L}k}{^{H}k} \qquad [3]$$

The kinetic isotope effect results from differences in the zero-point energy (ZPE) and the corresponding difference in the vibrational energy between heavy and light isotopes. Heavy atoms vibrate more slowly than lighter ones and therefore, have more stable and stronger bonds, since the zero point energy of the molecule with the heavy isotope is lower. The corresponding reaction-rate and bond strength differences among isotopes and isotopomers lead to isotopic differences between the source and product compounds of a chemical transformation and results in isotope fractionation (Northrop 1975).

Kinetic isotope effects arise in unidirectional reactions. Kinetic fractionation reactions are usually associated with processes such as evaporation, diffusion, dissociation reactions, and enzymatic effects. A simple example is the comparison of non-deuterated water ($^{1}H_2O$) and deuterated water ($^{2}H_2O$), where vapor pressure of deuterated water is nearly 40 torr lower than that of $^{1}H_2O$ (Hoefs 1987). Thus, evaporation will lead to observable fractionation, yielding in a depletion of ^{2}H in vapor and enrichment of ^{2}H in remaining water. Generally, stronger kinetic isotope effects can be expected for isotopes with a higher relative mass difference, for example, a deuterium atom has twice the mass of a hydrogen atom ($^{2}H/^{1}H = 2$), whereas the mass of carbon-13 is only about eight percent increase over the mass carbon-12 ($^{13}C/^{12}C = 1.0833$). Therefore, KIE for hydrogen are much stronger than for carbon. KIEs can be calculated when the mass of atoms in the bond and the vibrational frequency (i.e., its wave number) are given. The obtained values are known as Streitwieser semiclassical limits (Table 2) (Huskey 1991). Detailed discussions of isotope fractionation are found elsewhere (O'Neil 1986).

Table 2: Streitwieser semiclassical limits for KIE during bond cleavage at 25°C for halogenated hydrocarbons (Cook 1991).

Bond	Frequency (cm^{-1})	Isotope	KIE
C-H	2900	$^{12}C/^{13}C$	1.021
C-H		$^{1}H/^{2}H$	6.44
C-C	1000	$^{12}C/^{13}C$	1.049
C-Cl	750	$^{12}C/^{13}C$	1.057
C-Cl		$^{35}Cl/^{37}Cl$	1.013

2.6.2. Single-element isotope analysis

The changes in isotope composition during a reaction can be described using the Rayleigh model relating changes in isotope composition to changes in concentration under closed system conditions (Mariotti et al. 1981, Hoefs 1987). The extent of isotope fractionation is usually expressed as the enrichment factor ε or the isotope fractionation factor α where ε (‰) = (α-1)*1000. In the next sections, however, only the enrichment factor ε is used. The isotope enrichment factor (ε) can be determined from the linearized logarithmic form of the Rayleigh equation, as presented for carbon below (equation 4) (Mariotti et al. 1981). Since isotope enrichment factors are typically small, ε-values are reported in part per thousand (‰).The determined enrichment factors can then be used to characterize reaction mechanisms and degradation pathways.

$$\ln \frac{R_t}{R_0} = ln\left(\frac{\delta_t - {}^{13}C + 1}{\delta_0 - {}^{13}C + 1}\right) \times 1000 = \epsilon \times ln\left(\frac{C_t}{C_0}\right) \times 1000 \qquad [4]$$

2.6.3. Dual-element isotope analysis

Currently, stable isotope analysis of a single-element is frequently applied for evaluation of reaction mechanisms. Examples from recent studies, however, showed that stable isotope fractionation results for a single-element must be interpreted very carefully in natural multi-step processes. The additional analysis of two or more elements, however, enables a better characterization of a reaction mechanism (Zwank et al. 2005, Fischer et al. 2008). Usually, stable isotope analysis is done for elements involved in the reaction step, i.e. dual-element isotope analysis of carbon and chlorine during reductive dehalogenation. The results are usually presented as a dual-element isotope slope m (or Λ), as shown in equation 5 below for carbon and chlorine (Elsner 2010):

$$m = \frac{\Delta^{13}C}{\Delta^{37}Cl} \approx \frac{\epsilon_C}{\epsilon_{Cl}} \qquad [5]$$

Dual-element isotope slopes can elucidate variations for the same mechanism that were previously masked in a single-element isotope analysis. Such variations may bear enormous potential to learn something about the underlying processes (Elsner 2010). In addition to dual-element, multi-element isotope analysis of chemically complex substances can be used to analyze transformation pathways, making use of isotope fractionation processes altering the reactive position, as well as to analyze the isotope composition of reactive and non-reactive entities of an organic molecule to track sources. Though, thus far analysis of hydrogen and chlorine isotope of chlorinated hydrocarbons is limited by available analytical methods, first steps were done to establish multi-element analysis.

2.7.Reductive dehalogenation

2.7.1. Chlorinated solvents as groundwater and soil contaminants

Chlorinated aliphatic hydrocarbons (CAH) have seen a broad historical usage for a wide variety of applications, from cleaning of machinery and electronic parts, dry cleaning of clothes, but also for synthesis in chemical industry (Doherty 2000). Due to improper storage, handling and disposal chlorinated solvents were released into environment. Halogenated C_1 and C_2 solvents became especially a major concern in the 1970s and since then the production and application is in a steady decline. However, extensive use of chlorinated hydrocarbons left its footprint in the environment (Doherty 2000, Field and Sierra-Alvarez 2004, Moran et al. 2007, McCarty 2010). CAHs pollution is threatening to human health due to their toxicity, potential carcinogenicity and persistence in groundwater and soil. Common methods for remediation of groundwater and soil are for instance the *ex situ* treatment of contaminated (i.e. pump-and-treat, air striping) or other physical-chemical technology. However, these methods are costly to operate, resulted in the transfer of contaminants to other environmental compartments, or they require surface treatment. Therefore, the focus was put on alternative and innovative treatment methods that may prove to be more effective and less costly, as for instance bio-remediation strategies (Semprini 1995, McCarty 2010).

2.7.2. Chlorinated Ethenes

Tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE), vinyl chloride (VC) are some of the most common pollutants worldwide. They are found in surface water, in groundwater and in the atmosphere. TCE and PCE were the most commonly found contaminants in water supply wells, detected in maximum concentrations of several to several hundred micrograms per liter (Fetzner 1998, Moran et al. 2007).

The properties of chlorinated ethenes (higher density than water and modest solubility) contribute to the occurrence and persistence of these solvents in groundwater (Table 3). Sorption was demonstrated to be an important process not only causing retardation in the movement of the trace organics, but also resulting in transfer of a significant fraction of the organics to aquifer solid material. Subsequently, this was found to be one of the factors that increased the difficulty and time span for groundwater cleanup by i.e. pump-and-treat processes. A surprising observation was the disappearance of some chlorinated hydrocarbons by a unknown degradation process (Bouwer and McCarty 1983). Subsequent studies indicated that the chlorinated solvents could indeed be transformed by abiotic or biotic processes, leading to the production of many intermediate chlorinated compounds in groundwater that were also of health concern (Vogel et al. 1987).

Table 3: Properties of chlorinated ethenes, including M – molar weight, ρ – density, Mvol – molar volume, S – solubility, PC – partition coefficient. Property data according to: a - Horvath 1982.

Substance	M [g mol^{-1}]	ρ [g cm^{-3}]	M_{vol} [ml mol^{-1}]	S [mg L^{-1}]	PC log K_{OW}
PCE	165.83	1.62[a]	102.00	206	3.40
TCE	131.39	1.46[a]	89.02	1280	2.61
cis-DCE	96.94	1.28[a]	75.52	6410	1.86
trans-DCE	96.94	1.26[a]	77.20	4520	2.09
VC	62.50	0.91	68.50	2763	2.79

2.7.3. Microbial reductive dehalogenation of chlorinated hydrocarbons

Chlorinated hydrocarbons can be transformed through a range of biotic and abiotic reactions. Most abiotic transformations are slow. In contrast, biotic reactions are typically much faster provided that

appropriate conditions are present such as sufficient substrate, nutrients and suitable microbial populations (Semprini 1995). Degradation of chlorinated ethenes was mainly observed under anoxic conditions in field studies, in soil, in sediment, and aquifers microcosms.

Generally, transformation of chlorinated ethenes under anoxic conditions proceeds by sequential reductive dehalogenation of i.e. PCE, over TCE, DCE and VC to ethene as final product (Figure 5), while each chlorine is replaced by hydrogen (Freedman and Gossett 1989, Fetzner 1998, Janssen et al. 2001, Smidt and de Vos 2004).

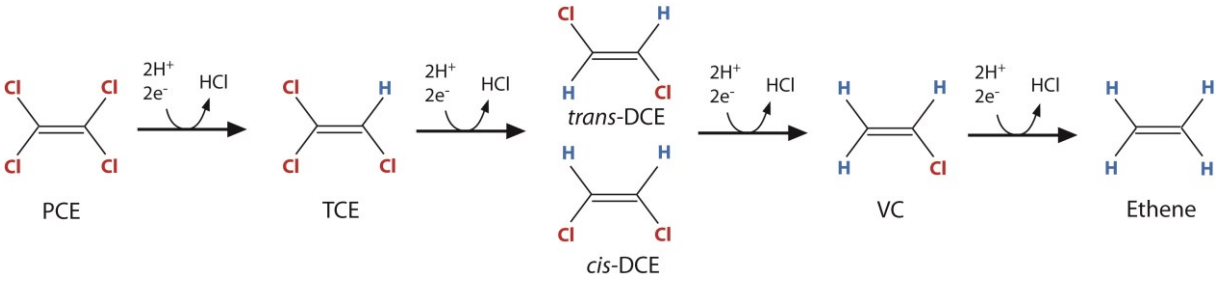

Figure 5: Chlorinated ethenes, including PCE, TCE, *cis*- DCE, *trans*-DCE, and vinyl chloride (VC) in the pathway of dechlorination and ethene as the non-chlorinated final product.

Organohalide-respiring bacteria are capable of conserving energy by coupling the anaerobic reductive dehalogenation of halogenated compounds to the generation of a proton motive force (Holliger et al. 1998). Such reactions are catalyzed by corrinoid-containing reductive dehalogenase enzymes (RDase). Organohalide-respiring microorganisms were identified as key players in the detoxification of halogenated groundwater contaminants such as chlorinated ethenes; therefore, strong efforts have been made during the last decades to understand the dehalogenation process and the underlying reaction mechanism. So far several organohalide-respiring bacteria from different phyla were isolated, e.g. *Sulfurospirillum*, *Desulfitobacterium*, *Dehalococcoides*, *Dehalobacter* and *Geobacter*, and the responsible genes encoding RDase enzymes were identified (Neumann et al. 1998, Seshadri et al. 2005, Nonaka et al. 2006, Wagner et al. 2012, Rupakula et al. 2013). However, only few RDases have been characterized biochemically. Among these RDases different substrate spectra have been observed; dehalogenation of e.g. chlorinated ethenes or chlorinated benzenes, as well as complete or partial dehalogenation. Remarkably, all RDases were found to be highly similar in structure, containing two iron-sulfur clusters and a corrinoid cofactor (Neumann et al. 1996, Miller et al. 1998,

Maillard et al. 2003, Hug and Edwards 2013, Hug et al. 2013). The evolutionary origin of organohalide-respiring bacteria or other microbial types that appear to be well adapted to growth on chlorinated solvents is uncertain. The existence of such organisms is intriguing when one considers the relatively recent and localized dispersal of halogenated solvents into the environment. However, from a global perspective, a number of naturally produced organochloride compounds have been in the environment over geologic time (McCarty 1997, Lee et al. 1998).

2.7.4. Evaluation of bioremediation processes *in situ*

Isotopic fractionation of (bio)chemical reaction processes may vary in magnitude depending on mechanism, reaction rates, concentrations of products and reactants, environmental conditions, and in the case of metabolic transformations species of the organism (Northrop 1981, Sherwood Lollar et al. 1999, Mancini et al. 2006). Evaluation of (bio)transformation *in situ* can be achieved by monitoring changes in isotope composition of the degraded compound (Meckenstock et al. 2004). Over the last decades, stable isotope fractionation concepts have been developed which allow qualitative and quantitative assessment of the *in situ* biodegradation, source identification and analysis of the reaction mechanism of organic contaminants. Concepts are available for the common groundwater contaminants such as BTEX, MTBE and chlorinated ethenes. To develop this approach, laboratory and field studies were performed and evaluated (Hunkeler et al. 1999, Sherwood Lollar et al. 1999, Bloom et al. 2000, Sherwood Lollar et al. 2001, Meckenstock et al. 2004, Elsner et al. 2005, Bombach et al. 2010). These initial field investigations confirmed that a shift in carbon stable isotope composition could be used as an indicator for biodegradation.

Along with the development of the *in situ* approaches, enrichment of ^{13}C was observed in laboratory experiments during microbial dehalogenation of the chlorinated ethenes (Bloom et al. 2000, Slater et al. 2001). However, although the reactions are thought to be the same during microbial dehalogenation by different strains, laboratory carbon isotope fractionation was highly variable as discussed in the next sections, providing a challenge for quantification of biodegradation using CSIA.

2.7.5. Investigation of degradation pathways via CSIA

Isotope fractionation often reflects the reaction mechanism involved in its degradation. Therefore, enrichment factors can be used for identification of degradation pathways, as it was already used for MTBE (Kuder et al. 2005, Zwank et al. 2005, Elsner et al. 2007). Halogenated hydrocarbons can be transformed *in situ* by different pathways, including abiotic and biotic (enzymatic) dehalogenation. Abiotic transformation can be mediated during reductive dehalogenation by zero-valent iron (Arnold and Roberts 2000, Elsner et al. 2008), dehalogenation mediated by corrinoids (Krone et al. 1989, Glod et al. 1997) or oxidation using for instance permanganates or persulfates (Hrapovic et al. 2005, Tsitonaki et al. 2010). Biotic transformation occurs under oxic and anoxic conditions. Aerobic degradation (metabolic), however, was observed only for lower chlorinated compounds, such as vinyl chloride (VC) and dichloroethene (DCE). Carbon isotope fractionation during aerobic degradation is usually small and can be explained by the catalytic reaction pathways, which do not involve a direct cleavage of the C-Cl bond (Chartrand et al. 2005, Tiehm et al. 2008, Abe et al. 2009, Mattes et al. 2010, Tiehm and Schmidt 2011, Clingenpeel et al. 2012). In contrast, dehalogenation of higher chlorinated compounds was observed to happen via microbial reductive dehalogenation under anaerobic conditions, where strict anaerobic bacteria use chlorinated hydrocarbons as terminal electron acceptors. During reductive dehalogenation C–Cl bonds are sequentially cleaved leading to formation of lower chlorinated hydrocarbons (Scholz-Muramatsu et al. 1995, Maymo-Gatell et al. 1997). The corresponding isotope fractionation is usually larger for VC, cis-DCE and trans-DCE, while for 1,1-DCE, TCE and especially PCE lower and more variable isotope fractionation was measured (Table 4).

Table 4: Experimental carbon isotope enrichment factors (ε_C) determined for abiotic and enzymatic catalysis.

	Abiotic		**Biotic**	
Compound	**Fe(0)**	**Corrinoids** (red. dehalogenation)	**Anaerobic** (red. dehalogenation)	**Aerobic** (Degradation)
PCE	-5.7 to -25.3	-13.0 to -25.3	-0.4 to -16.4	
TCE	-7.5 to -13.5	-15.0 to -21.3	-3.3 to -26.0	

cis-DCE	-6.9 to -16.0		-14.9 to -29.7	-0.9 to -9.8
trans-DCE			-20.8 to -30.3	
1,1-DCE			-5.1 to -23.9	
VC	-6.9 to -19.3		-23.2 to -31.1	-3.2 to -8.2

2.7.6. Assessment of the reaction mechanisms

To elucidate the reaction mechanisms of microbial strains capable of reductive dehalogenation, carbon stable isotope analysis was performed to investigate the involved (bio)catalytic step. Thus far isotope fractionation patterns were investigated for several microorganisms, including members of δ-Proteobacteria, ε-Proteobacteria, Firmicutes and Chloroflexi. Despite similarities of the reductive dehalogenase enzyme in all microorganisms, however, isotope analysis of carbon revealed highly variable isotope fractionation for different strains during dehalogenation of tetrachloroethene (PCE) and trichloroethene (TCE) (Table 5), making characterization of the reaction mechanism difficult (Nijenhuis et al. 2005, Lee et al. 2007, Cichocka et al. 2008). Observed variability in carbon isotope fractionation was thought to be related to the specific corrinoids incorporated into the RDase enzymes as well as the microbial cell envelope properties or growth conditions (Nijenhuis et al. 2005, Mancini et al. 2006, Cichocka et al. 2008). Furthermore, effects of substrate properties, i.e. hydrophobicity, were suggested to be responsible for variability in isotope fractionation (Cichocka et al. 2007, Thullner et al. 2013).

Therefore, the observed variability of isotope fractionation during reductive dehalogenation was suspected to be a result of isotope masking. Isotope masking in microbial systems is a result of rate limiting events prior to the actual catalytic reaction, as for instance extracellular and intracellular mass transfer (Elsner 2010). The effect of extracellular mass transfer was demonstrated to affect observable isotope fractionation in substrate availability studies (Aeppli et al. 2009, Kampara et al. 2009, Thullner et al. 2013). Similarly, intracellular mass transfer was suspected to affect isotope fractionation (Nijenhuis et al. 2005).

Table 5: Enzymatic and abiotic carbon isotope enrichment factors for dehalogenation of PCE and TCE catalyzed by corresponding microbial strain or corrinoids.

Enzymatic dehalogenation mediated by RDase				
Phyla	**Organism**	**$\varepsilon_{C\text{-}PCE}$**	**$\varepsilon_{C\text{-}TCE}$**	**Ref.**
Firmicutes	*Desulfitobacterium PCE-S*	-5.2 to -8.9	-10.9 to -12.9	Nijenhuis 2005, Cichocka 2007
	D. restrictus PER-K23	-4.0 to -6.3	-3.3 to -8.3	Lee 2007, Renpenning 2015
δ-Proteobacteria	*G. lovleyi SZ*	ns to -2.3	-8.5 to -12.2	Cichocka 2007, Cretnik 2013, Renpenning 2015
	D. michiganensis	-1.7 to -2.6	-3.5 to -7.1	Cichocka 2007, Renpenning 2015
ε-Proteobacteria	*S. halorespirans*	-0.5 to -3.2	-18.7 to -22.9	Cichocka 2007
	S. multivorans	-0.4 to -2.2	-16.2 to -26.0	Nijenhuis 2005, Cichocka 2007, Lee 2007, Renpenning 2014
Abiotic dehalogenation mediated by corrinoids				
Corrinoid type	Cyanocobalamin	-16.2 to -22.4	-15.0 to -16.5	Slater 2003, Nijenhuis 2005, Cichocka 2007, Cretnik 2013, Renpenning 2014
	Cobaloxime		-21.5	Cretnik 2013

*ns: not significant

3. Research Objectives

The aim of the PhD thesis is the characterization of microbial reductive dehalogenation using compound-specific stable isotope analysis. Stable isotope analysis was frequently applied during the last decades for evaluation of *in situ* (bio)remediation processes, as well as for characterization of degradation pathways and involved reaction mechanisms. Isotope analysis of carbon was mainly applied, since not routine methods are thus far available for analysis of chlorine and hydrogen. Characterization of microbial reaction mechanism by carbon as single-element, however, was observed to be challenging, since isotope effects were masked in microbial systems to an unknown extend. The extension of isotope analysis to two or more elements in the molecule was shown to be a promising solution in order to overcome those limitations and to elucidate the real magnitude of isotope effects in microbial systems. Therefore, development of novel methods for routine isotope analysis of chlorine and hydrogen in chlorinated hydrocarbons are mandatory to overcome the currently existing bottlenecks of compound-specific stable isotope analysis.

The objectives of this PhD study were therefore, (1) the development of analytical methods for chlorine and hydrogen stable isotope analysis and (2) the characterization of microbial reductive dehalogenation reaction using stable isotope analysis of carbon, as well as chlorine and hydrogen.

The PhD thesis is divided in two topics:

(1) Method development for compound-specific stable isotope analysis (Chapter 4.1)
    a. Development of a novel method and setup for compound-specific analysis of chlorine isotopes, including the characterization of the high-temperature conversion process and validation of chlorine isotope analysis via GC-HTC-MS/IRMS
    b. Development of a compound-specific isotope analysis method for hydrogen in presence of halogenated heteroatoms

(2) Characterisation of microbial reductive dehalogenation reaction using compound-specific stable isotope analysis (Chapter 4.2)

   a. Determination of factors affecting variability of microbial isotope fractionation for carbon isotope analysis

   b. Application of single- and dual-element stable isotope analysis (carbon and chlorine) in order to elucidate the dehalogenation mechanism in abiotic (mediated by corrinoids) and enzymatic (reductive dehalogenases) systems.

4. Summary of Results & Discussion

4.1. Method development for compound-specific stable isotope analysis of chlorinated hydrocarbons (Publication II and IV)

Isotope analysis of carbon and hydrogen evolved to a standard application for forensics, tracer studies, *in situ* remediation strategies and characterization of reaction mechanisms. This approach was successfully applied for a wide range of pollutants. However, it remains challenging for halogenated hydrocarbons. Though carbon isotope analysis can be applied as a standard procedure for a wide range of compounds, isotope analysis of hydrogen is simply not possible and isotope analysis of chlorine is limited to few compound classes (chlorinated methanes, ethanes and ethenes). On the one hand, hydrogen isotopes cannot be analyzed, if a halogen is present as heteroatom in the molecule. On the other hand, chlorine isotope analysis is problematic, since conversion of chlorine to an appropriate analyte gas is difficult with common techniques. This chapter is therefore focused on the method development on-line isotope analysis of chlorine and hydrogen in combination with continuous-flow IRMS systems.

4.1.1. Development and validation of an universal interface for compound-specific stable isotope analysis of chlorine ($^{37}Cl/^{35}Cl$) by GC-HTC-MS/IRMS (Publication II)

The feasibility of compound-specific chlorine isotope analysis via high-temperature conversion of chlorine containing compounds to HCl was already demonstrated by Hitzfeld et al. (2011). However, analysis of chlorine isotopes revealed serious instabilities during measurements ($1\sigma > 1.0$ ‰). Therefore, the conversion of chlorinated and non-chlorinated hydrocarbons was investigated in detail, in order to understand and optimize the high-temperature conversion process. Characterization of the conversion and simultaneous measurements of chlorine isotopes were established using a dual-detection system combining ion trap MS and IRMS. The conversion process was monitored, evaluated and optimized using the ion trap MS, while the isotopes [$H^{35}Cl$ and $H^{37}Cl$] were measured via IRMS.

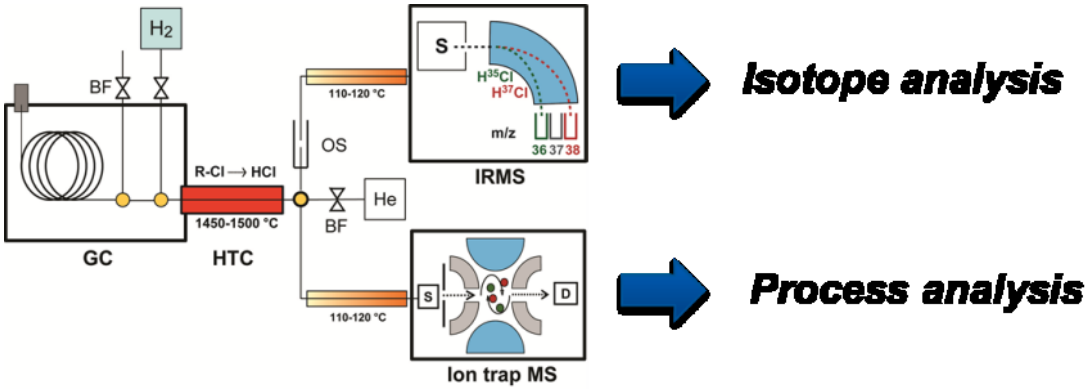

Figure 6: Set-up of the gas chromatography – high-temperature conversion in combination with an ion trap mass spectrometer and isotope-ratio mass spectrometer (GC-HTC-MS/IRMS).

4.1.1.1. Identification of interfering by-products

The analysis of chlorinated and non-chlorinated ethenes at previously applied standard HTC conditions of 1300°C revealed severe by-product formation (Figure 7). For the non-chlorinated ethene by-products could be assigned to acetylene (C_2H_2, m/z 26) and benzene (C_6H_6, m/z 78). Additionally, by-products in the mass range of m/z 37 − 43 and 49 − 54 were detected and associated with fragment ions of benzene, though C_3H_x or C_4H_x hydrocarbon by-products cannot be excluded at this point. The production of longer hydrocarbon chains could be explained by the thermal-coupling, which is

enhanced by additional availability of hydrogen during pyrolysis, as reported elsewhere (Gueret and Billaud 1994). Similar by-product formation was detected for the chlorinated ethenes during high-temperature conversion in presence of hydrogen. Moreover, some molecular or fragment ions of the by-product were interfering with the target ion $H^{37}Cl$ (m/z 38), which is required for chlorine isotope analysis. These interfering ions corrupted the chlorine isotope measurements, whereby previous instabilities of chorine isotope analysis could be assigned to overlapping ion signals of $H^{37}Cl$ and by-products at m/z 38. In conclusion, previously applied HTC conditions were inappropriate for a quantitative and clean conversion of chlorine containing compounds to HCl and had to be optimized.

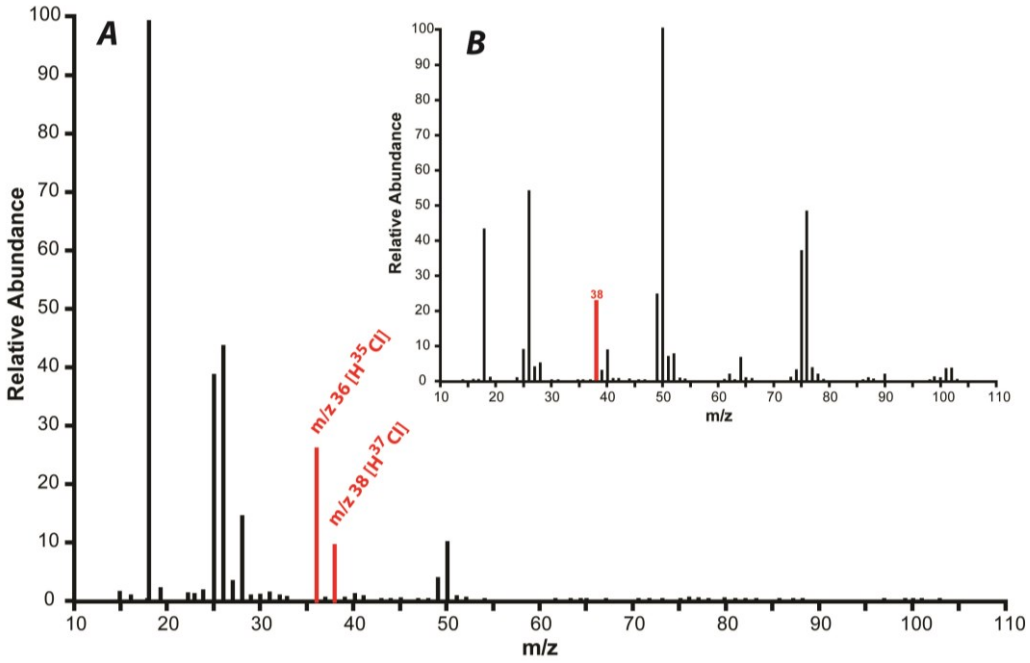

Figure 7: Conversion quality of trichloroethene (A) and ethene (B) at HTC of 1300°C. The target ions (HCl) are labeled in red (A). Interfering by-product from the non-chlorinated ethene at m/z 38 is labeled red (B). The conversion background was monitored via ion trap MS in combination to GC-HTC.

4.1.1.2. Characterization of the high-temperature conversion process

In order to characterize the conversion and optimize the HTC process, the effect of reactor load (9 − 45 nmol of ethene on column), reactant gas (H_2) concentration (0 - 0.5 mL/min), and conversion temperature (1100 − 1500 °C) (Figure 8) were analyzed. Furthermore, the compound residence time in the reactor was modified to improve conversion quality (data not shown).

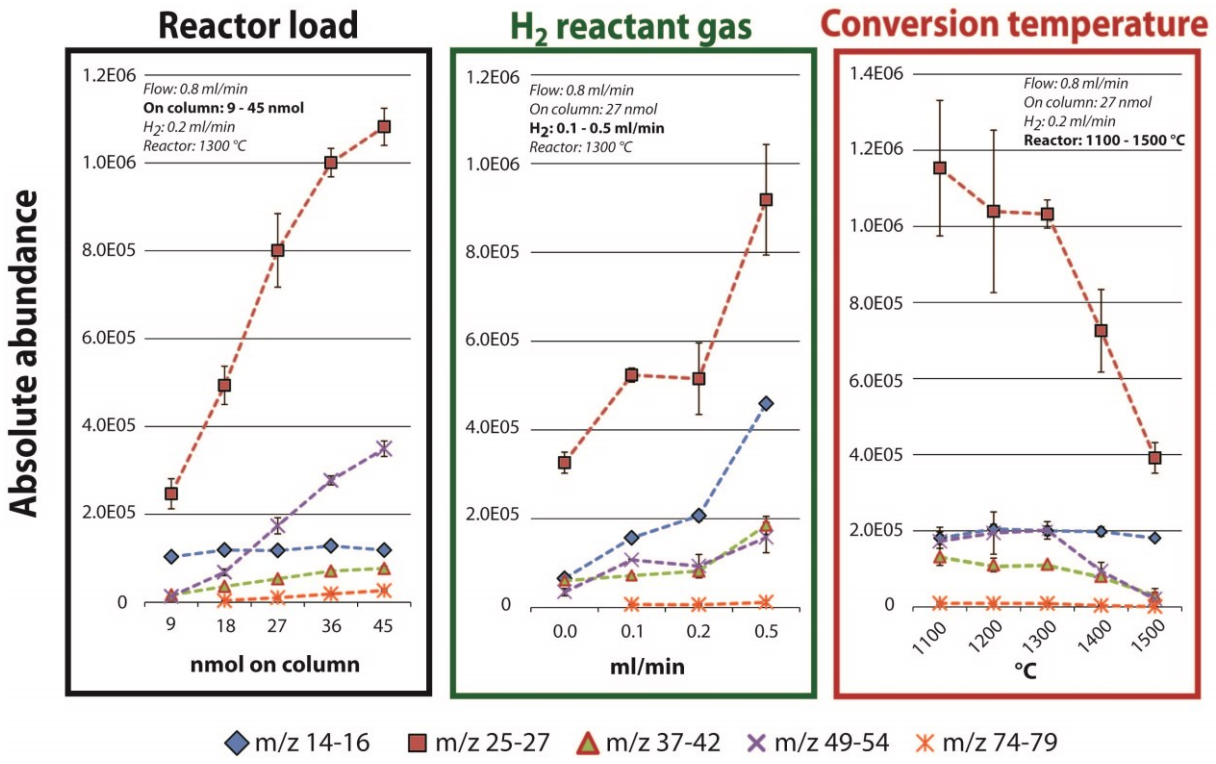

Figure 8: Characterization of by-product formation during HTC of ethene by considering reactor load (left), reactant gas (H_2) concentration (middle), and conversion temperature (right). Molecular and fragment ions were monitored via ion trap MS and given as absolute abundances.

For the characterization of the by-product formation non-chlorinated ethene was used as model compound (Figure 8). Interestingly, all investigated parameters were observed to affect by-product formation to a certain extent. Increase of reactor load (concentration on column) and reactant gas (H_2) concentration were observed to enhance by-products formation. The increase of reactor load resulted in formation of hydrocarbons with higher molecular masses (C_4H_x and C_6H_x), while increased H_2 concentration enhanced the formation of short hydrocarbon chains (C_1H_x and C_2H_x). The observed trends are in agreement with previous studies on thermal-coupling of methane (Billaud et al. 1992, Gueret and Billaud 1994). Higher conversion temperature, however, resulted in a significant reduction of byproducts produced during conversion.

Conclusively, formation of interfering byproducts could be significantly reduced at conversion temperature of 1500°C. Alternatively, extended residence time in the reactor and conversion temperature of 1450°C was also observed to be sufficient. The subsequent evaluation of trichloroethene conversion at 1300°C and 1500°C could confirm the previously observed trends. While hydrocarbon by-product formation could be significantly suppressed (Figure 9A), especially

C_3H_x (m/z 38 – 42) that interfere with the HCl target ions (m/z 38), conversion of chlorine to HCl remained unaffected (Figure 9B).

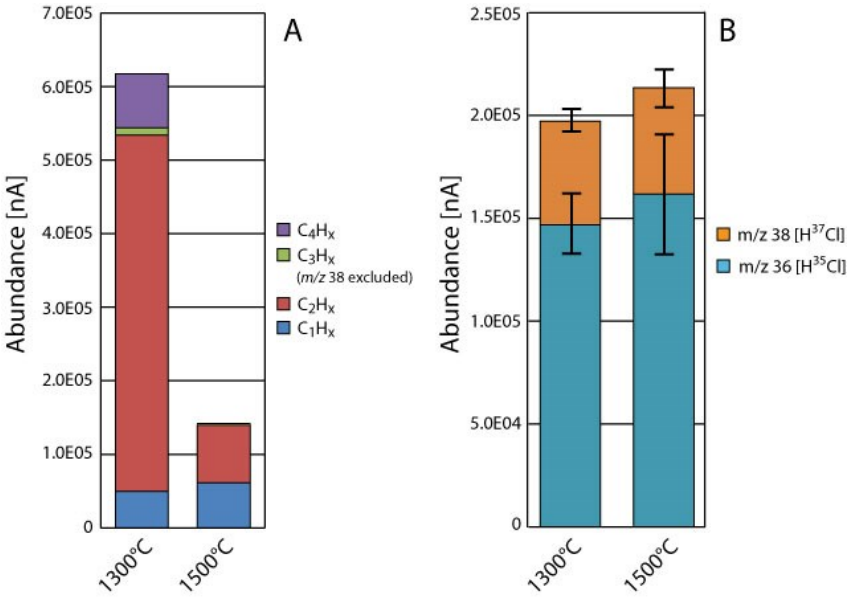

Figure 9: Evaluation of the conversion quality of trichloroethene (TCE) at 1300°C vs 1500°C by taking in account the hydrocarbon by-product formation (A) and the HCl formation (B). The abundance of products was monitored via ion trap MS.

4.1.1.3. Chlorine isotope analysis at optimized conversion conditions

All subsequent chlorine isotope measurements were done at optimized conversion conditions (GC flow 0.4 mL/min, H_2 flow 0.1 mL/min, HTC temperature 1450 − 1500 °C). The on column concentration of the measured compound was limited to about 30-40 nmol Cl. As shown in Figure 10 the optimized conditions clearly improved the background by suppressing the by-products.

At optimized HTC conditions isotope analysis of reference materials (TCE ref. 2 and 6) was measured to remain very stable over a period of 5 days with a standard deviation of ± 0.3 ‰. Therefore, all available reference material was used for validation of the on-line chlorine isotope analysis by the GC-HTC-IRMS. The following reference material was analyzed: trichloroethene (TCE refs 2 and 6), tetrachloroethene (PCE refs 1 and 5), methyl chloride (MeCl ref.), hexachlorocyclohexane (HCH refs 1 and 2), and trichloroacetic acid methyl ester (TCAA ref.). After two-point calibration with TCE reference 2 and 6, chlorine isotope composition of all reference compounds determined on-line via GC-HTC-IRMS was in very good agreement with chlorine reference values determined off-line via

DI-IRMS with a maximum deviation of ≤0.8‰ (Table 6). Also the precision was very satisfying with standard deviations below ±0.5‰ for all compounds.

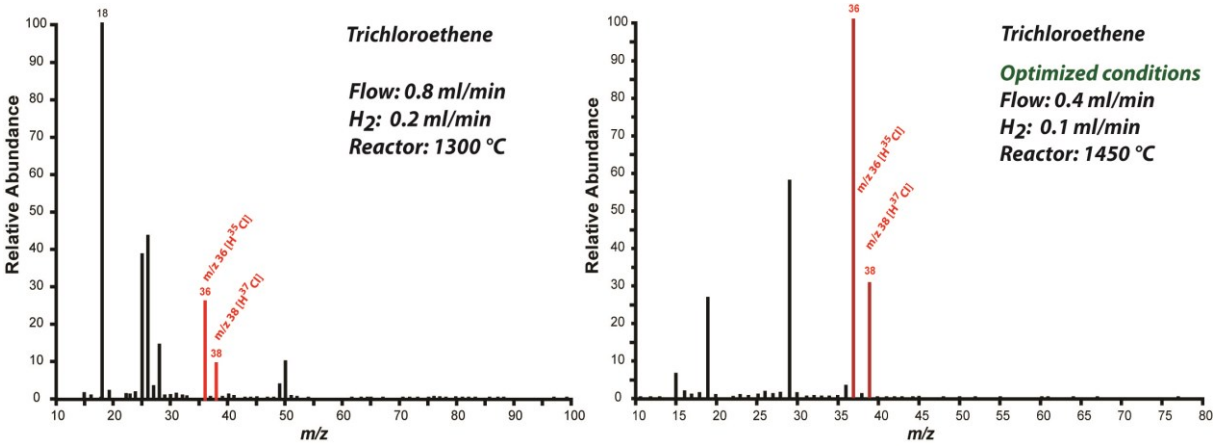

Figure 10: Conversion quality of trichloroethene (TCE) at insufficient conversion conditions (flow 0.8 mL/min, H_2 flow 0.2 mL/min, HTC at 1300 °C) vs optimized conditions (flow 0.4 mL/min, H_2 flow 0.1 mL/min, HTC at 1450 °C). The HTC background was monitored via ion trap MS.

Table 6: Chlorine isotope composition of reference compounds measured off-line via DI-IRMS ($\delta^{37}Cl$ [‰ vs. SMOC]) and is assumed to be the true value. Chlorine isotope composition of several reference compounds (blue) was analyzed and converted to the international SMOC-scale ($\delta^{37}Cl_{SMOC}$).

Reference material	DI-IRMS $\delta^{37}Cl$ [‰ vs. SMOC]	GC-HTC-IRMS $\delta^{37}Cl$ [‰ vs. SMOC]	n
TCE ref. 2	**-1.19 ± 0.01**	**anchor**	
TCE ref. 6	**2.17 ± 0.2**	**anchor**	
PCE ref. 1	**-0.49 ± 0.12**	**-0.49 ± 0.17**	10
PCE ref. 5	**1.03 ± 0.08**	**0.64 ± 0.15**	9
MeCl ref. (Linde)	**6.03 ± 0.02**	**6.85 ± 0.51**	10
HCH ref. 1 (HiMedia)	**-0.18 ± 0.03**	**0.27 ± 0.32**	9
HCH ref. 2 (Greyhound)	**-5.49 ± 0.12**	**-5.20 ± 0.27**	10
TCAA ref. (Sigma)	**-5.11 ± 0.17**	**-4.60 ± 0.28**	10

4.1.2. On-line hydrogen-isotope measurements of organic samples using elemental chromium - an extension for high temperature elemental-analyser techniques (Publication IV)

On-line hydrogen isotope analysis was routinely applied during the last decades by combining elemental analyser (EA) or gas chromatography (GC) to high-temperature conversion (HTC) and subsequent detection of the produced H_2 via isotope-ratio mass spectroscopy (IRMS) (Tobias and Brenna 1997). Though this technique is working well for hydrocarbons, many other heteroatom-containing compound classes cannot be measured accurately, due to the frequently occurring by-product formation during HTC, as for instance the formation of HCl from chlorinated hydrocarbons or HCN form nitrogen containing compounds (Chartrand et al. 2007, Hunsinger et al. 2013). Due to the formation of by-products the yield of H_2 for isotope analysis can be reduced. Moreover, by-product formation results often in isotope fractionation and inaccurate analysis of isotope composition. In order to understand the limitation of the HTC a set-up was designed combining EA with a dual-detection system consisting of IRMS and MS (Figure 12). Additionally to the standard approach (HTC), a chromium reactor based elemental analyser (Cr-EA) was tested in detail. The major focus of this study was the hydrogen isotope analysis of the nitrogen containing compounds.

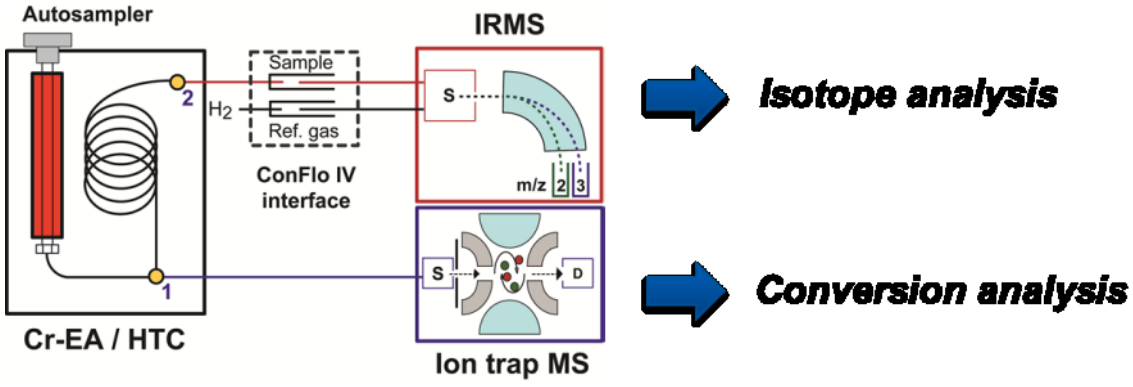

Figure 11: Set-up of the elemental analyser – high-temperature conversion (HTC) or chromium reactor based elemental (Cr-EA) – in combination with an ion trap mass spectrometer and isotope-ratio mass spectrometer (MS/IRMS).

4.1.2.1. By-product formation in presence of heteroatoms and hydrogen

The performance of both, HTC and chromium reactor, was analyzed via ion trap MS (Polaris*Q*, Thermo Fisher, Germany) for by-product formation and via IRMS (MAT 253, Thermo Fisher, Germany) for isotope composition. The exhaust of the reactors was sampled immediately at the post-reactor position 1 or 2 (Figure 11). First analysis revealed that undesirable by-products are partly trapped in the GC-column located between both positions. Therefore, only sampling position 2 could be used for the analysis of by-product formation.

Our results confirmed severe formation HCN (m/z 27 and partly m/z 28 due to auto-protonation in the ion source) as the main hydrogen bearing by-product during HTC of nitrogen-containing compounds, as for instance caffeine (Figure 12 A and C). Corresponding quantification of the hydrogen yield confirmed that for caffeine ($C_8H_{10}N_4O_2$) about 30-40% of the hydrogen is lost during HTC (Gehre et al. 2015, Nair et al. 2015). This trend was observed to occur in all nitrogen-bearing compounds (Nair et al. 2015). The conversion of several chlorinated hydrocarbons confirmed severe formation of HCl as by-product during HTC, as it was already reported elsewhere (Figure 12) (Chartrand et al. 2007).

The application of chromium-based reactors could significantly reduce by-products formation for all tested compounds (Figure 12 B and D). By-products were measured to be about one to two orders of magnitude lower and in the range of air-water background or below. Corresponding hydrogen yields exhausted from the chromium-based reactor were ≥ 96% for all tested compounds. The isotope measurements of heteroatom containing reference material approved that reduction at chromium significantly improves hydrogen isotope analysis.

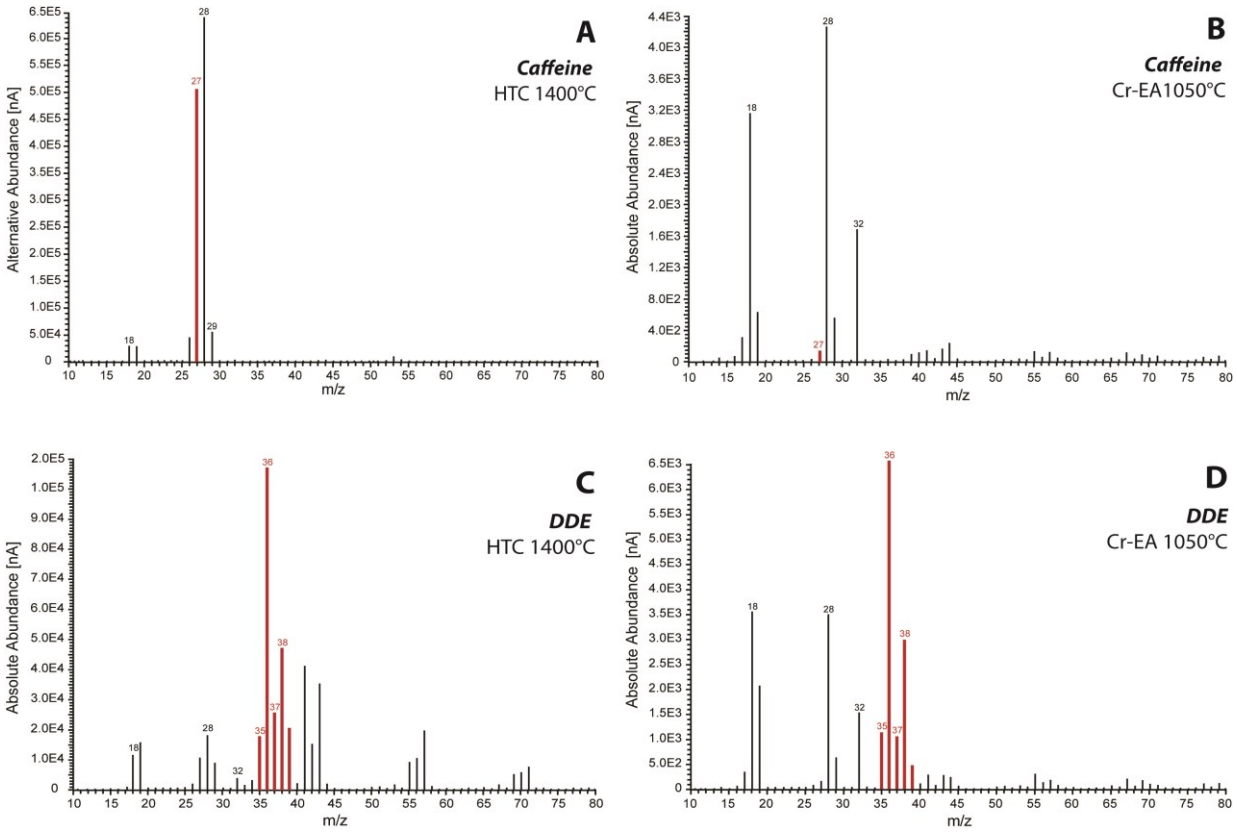

Figure 12: By-product formation caffeine (A and B) and dichlordiphenyldichlorethen (DDE; C and D) after conversion with HTC (A and C) or hot chromium reduction (B and D).

4.1.2.2. Inter-laboratory hydrogen isotope analysis

High-temperature conversion and the chromium-based reactor technique were compared in an inter-laboratory analysis (Table 7). Different caffeine references (caffeine IAEA 600, #1, #2 and #3 reference) with known isotope composition (determined off-line as described elsewhere (Kendall and Coplen 1985) were analyzed via on-line measurements with EA using HTC or chromium reduction. Analysis was performed by the Helmholtz Centre for Environmental Research, Stable Isotopes Laboratories (UFZ, Leipzig, Germany); Reston Stable Isotope Laboratory (RSIL, Reston, USA); Max-Planck Institution, Stable Isotopes Laboratories (MPI, Jena, Germany); and University of Groningen (Gröningen, Netherlands). Off-line analyses were performed at Indiana University (Bloomington, USA).

Conversion and hydrogen isotope analysis via HTC resulted in an average shift of δ^2H about 20 to 30 per mil for all caffeines. Though precision of the measurements was satisfying ($1\sigma \leq 3.0$ ‰), accuracy clearly differed from the reference δ^2H value (off-line value). In contrast, accuracy could be clearly

improved by the chromium reactor technique with a shift of $\delta^2H \leq 5$ ‰. Remarkably, each laboratory was using a different setup of the chromium reactor with a different chromium particle size, package length, particle mixture (chromium, glassy carbon or quartz chips). Still, all results were in a similar range and could demonstrate that chromium approach is a powerful tool for the measurement of hydrogen isotopes.

Table 7: The $\delta^2H_{VSMOW\text{-}SLAP}$ results for caffeine references obtained with three methods by four laboratories: UFZ Leipzig, RSIL Reston, MPI Jena and University of Groningen. *Off-line analyses were performed at Indiana University Bloomingten.

Laboratory	**Sample**	**On-line HTC (TC/EA)** $\delta^2H_{VSMOW\text{-}SLAP}$	**On-line Cr** $\delta^2H_{VSMOW\text{-}SLAP}$	**Off-line *** $\delta^2H_{VSMOW\text{-}SLAP}$
Leipzig UFZ	IAEA-600	-181.5 ± 1.2, n=10	-157.8 ± 1.2, n=15	
RSIL	IAEA-600	-177.9 ± 0.8, n=4	-158 ± 1.0, n=4	
Jena MPI	IAEA-600	-175.52 ± 2.4, n=24	-155.8 ± 1.2, n=8	
Groningen	IAEA-600	-	-158.3 ± 2, n=5	
		-178.3 ± 3	**-157.5 ± 1.1**	**-152.4 ± 1.3, n=8**
Leipzig UFZ	Caffeine #1	74.0 ± 1, n=10	96.9 ± 1.0, n=15	
RSIL	Caffeine #1	72.0 ± 0.9, n=6	95.8 ± 0.7, n=13	
Jena MPI	Caffeine #1	71.6 ± 1.1, n=24	97.6 ± 0.9, n=4	
Groningen	Caffeine #1	73.5 ± 0.7 n=6	98.6 ± 2.0 n=10	
		72.8 ± 2.1	**97.2 ± 1.2**	**98.0 ± 1.5, n=8**
Leipzig UFZ	Caffeine #2	-180 ± 1.4, n=10	–157.3 ± 1.1, n=15	
RSIL	Caffeine #2	–177.5 ± 2.4, n=17	–155.3 ± 0.3, n=14	
Jena MPI	Caffeine #2	–174.0 ± 1.6, n=24	−154.0 ± 0.8, n=4	
Groningen	Caffeine #2	-	-161.1 ± 2.0 n=10	
		–177.2 ± 3	**–156.9 ± 3.1**	**–152.4 ± 1.5, n=17**
Leipzig UFZ	Caffeine #3	151.2 ± 1.6, n=10	175.3 ± 1.4, n=15	
RSIL	Caffeine #3	148.9 ± 1.6, n=24	173.9 ± 0.6, n=14	
Jena MPI	Caffeine #3	148.1 ± 1.4, n=24	175.6 ± 1.2, n=4	
Groningen	Caffeine #3	152.4 ± 0.3 n=6	170.9 ± 2, n=10	
		150.2 ± 3	**173.9 ± 2.1**	**171.2 ± 4.2, n=9**

4.1.3. Discussion and outlook - Method development for on-line analysis of stable isotopes

4.1.3.1. Chlorine isotope analysis via high-temperature conversion

Analysis of chlorine isotopes via high-temperature conversion to HCl is a promising tool for routine analysis of chlorinated hydrocarbons, as well as a wide range of other GC compatible compounds. On-line analysis via GC-HTC-IRMS reduced the sample preparation time in comparison to previous off-line techniques significantly. This, a range of GC amendable chlorinated compound classes are now accessible for chlorine isotope analysis via IRMS. The accurate chorine isotope analysis was achieved for chloromethanes, chloroethenes, chlorocyclohexanes and chloroacetic acid methyl ester. In comparison to available techniques, as GC-qMS, GC-IRMS, MC-ICPMS and TIMS, chlorine isotope analysis via GC-HTC-IRMS showed a similar or lower detection limits (~ 10-15 nmol Cl on column). Lower detection limit was thus far only reported for GC-qMS (< 10 pmol), including considerable loss of precision ($1\sigma > 0.5‰$). Achieved precision with our setup ($1\sigma \sim 0.3$ ‰) is superior to that of GC-IRMS ($1\sigma \sim 0.1$ ‰), but not as good as by MC-ICPMS ($1\sigma \sim 0.06$ ‰) (Shouakar-Stash et al. 2006, Van Acker et al. 2006, Aeppli et al. 2010). Still, achieved precision is adequate for a compound-specific stable isotope analysis of chlorine.

Though on-line analysis of chlorine isotopes ($^{37}Cl/^{35}Cl$) via GC-HTC-IRMS is a promising universal and compound-specific approach, several limitations and challenges are remaining. Those are the memory effects, stability of the aluminum oxide HTC reactor, water formation, and effect of hydrochloric acid on instrumental set-up. Memory effects are a serious issue of the current set-up and were thus far assigned to the reactor material. The routinely applied reactors material for isotope analysis is aluminum oxide ceramic. Aluminum oxide ceramics are highly thermo-stable. However, it remains unclear if the material is appropriate for HTC of chlorinated compounds and the corresponding HCl formation as analyte gas. Recent results point to a potentially damaging effect of high HTC temperatures (1450-1500°C) in combination with hydrochloric acid, since reactor ceramics that usually last for months became leaky after less than one week. This indicates that HCl somehow reacts with

aluminum oxide ceramic. The observed memory effects and the short life time of the reactor could be the consequence of this side effect during HTC. Therefore, an appropriate reactor material is required that resists the extreme conditions of high temperature and HCl. Damaging effect on the IRMS source, however, was not observed after one year of application.

Formation of water in the background was generally higher than in routine HTC applications (i.e. hydrogen isotope analysis). This was shown to be a result of high hydrogen concentrations in the carrier gas required as reactant gas for HCl formation. However, in parallel hydrogen can also react to H_2O with background oxygen in the system. Though formation of H_2O was not observed to interfere with chlorine isotope measurements at moderate levels, water background was observed to rise with progressive decay of the aluminum oxide ceramic and had to be continuously monitored. Alternative reactor material may solve this issue. Therefore, new reactor designs have to be investigated in future.

4.1.3.2. Hydrogen isotope analysis in combination with reduction at hot chromium

In comparison to HTC as the current routine technique, conversion of hydrogen bearing compounds to H_2 at hot chromium could benefit from significant reduction of by-product formation. The high yield of H_2 from converted analyte resulted in an excellent precision ($1\sigma \leq 2$ ‰) and accuracy of isotope measurements. Similar results were confirmed in all laboratories. Reproducibility of δ^2H analyses on caffeine references from different laboratories was measured as precise as ±1.5 ‰. In contrast, δ^2H from caffeine samples measured with HTC was shifted vs off-line values by about −20 to −26 ‰, albeit with reasonably good reproducibility (< 2.6 ‰). Consequently, laboratory might not become aware of incorrect hydrogen isotope analysis of nitrogen-bearing compounds measured with routine HTC application.

The chromium-elemental analyser (Cr-EA) technique for conversion of organic hydrogen to molecular hydrogen is a powerful tool for the measurement of hydrogen isotopic compositions of heteroatom

(e.g., N, Cl, F, Br, S, P) containing organic matter. The method is far less susceptible to matrix effects and has the potential to replace the currently applied HTC as routine method.

4.1.3.3. Future application of chromium reactor in combination with GC-IRMS

The chromium-based approach could be recently extended from bulk isotope analysis (CrEA-IRMS) to the compound-specific stable isotope analysis (GC-CrHTC-IRMS) (Figure 13). Though compound-specific hydrogen isotope analysis in combination with chromium-based reactor systems will not be a matter of this dissertation, some preliminary results will be presented and discussed below, as an outlook for upcoming application and publication.

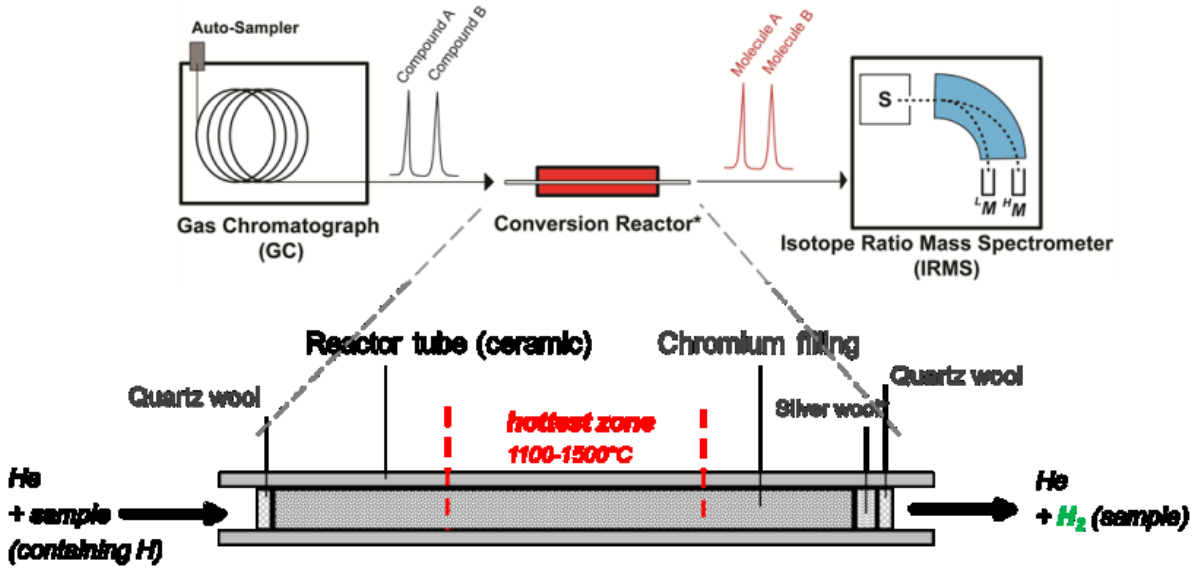

Figure 13: GC-CrHTC-IRMS setup. Chromium filled rector operating in a temperature range of 1100-1500 °C.

In contrast to the high flow Cr-EA system, a low flow chromium-based reactor was constructed for the GC-IRMS application. Previously applied quartz reactor tubular reactors were replaced by a more thermo-stable aluminum oxide ceramics. Application of aluminum oxide ceramic extended the temperature range of hot chromium from previously applied maximal temperature of ~1050 °C to technically possible 1500 °C or theoretically up to ~1800°C (limited by chromium melting point). The

most notable advantage, however, is the compatibility and simple incorporation of the constructed chromium reactor into standard analytical devices (GC IsoLink, Thermo Fisher, Germany).

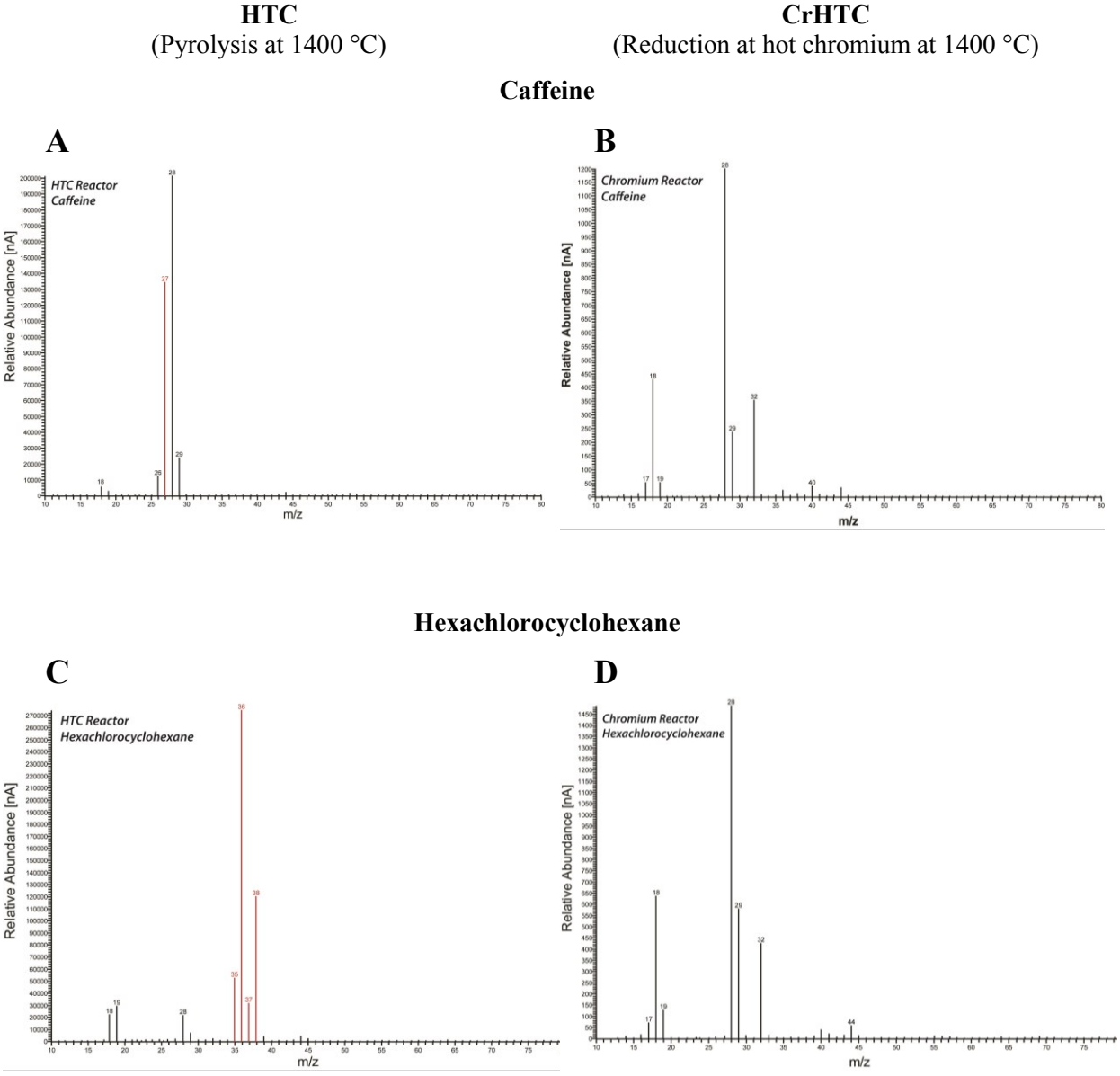

Figure 14: By-product formation after conversion of caffeine (A and B) and hexachlorocyclohexane (HCH; C and D) - high-temperature conversion (HTC) at 1400 °C (A and C) or hot chromium (CrHTC) at 1400 °C (B and D). Main by-products ions (HCN – *m/z* 27 or HCl – *m/z* 35-38) are indicated in red.

As already observed in previous experiments with the elemental analyser, heteroatom containing compounds produced severe byproducts during HTC, as for instance HCN and HCl byproducts of caffeine and hexachlorocyclohexane, respectively (Figure 14 A and B). Therefore, GC-HTC-IRMS cannot be applied for accurate hydrogen isotope analysis of heteroatom containing compounds for the

same reasons as discussed above. In comparison to the routinely applied GC-HTC-IRMS (operating at 1400°C), combination of GC-CrHTC-IRMS (chromium filled reactor tube operating at 1400°C) could immediately demonstrate the advantages of conversion via chromium reduction. The conversion at hot chromium resulted in a complete absence of by-products and a considerably low background (Figure 14 B and C).

Preliminary hydrogen isotope analysis of several types of compound classes, including nitrogen contains caffeine's and chlorine containing ethenes (DCE and TCE) resulted in a good to excellent agreement of measured hydrogen isotope composition (via GC-CrHTC-IRMS) in comparison to reference values (via off-line method) (Figure 15). Currently further development is in progress. In any case, reduction at hot chromium is already now a promising approach for routine hydrogen isotope analysis and has the potential to replace the routine high-temperature conversion as the standard method.

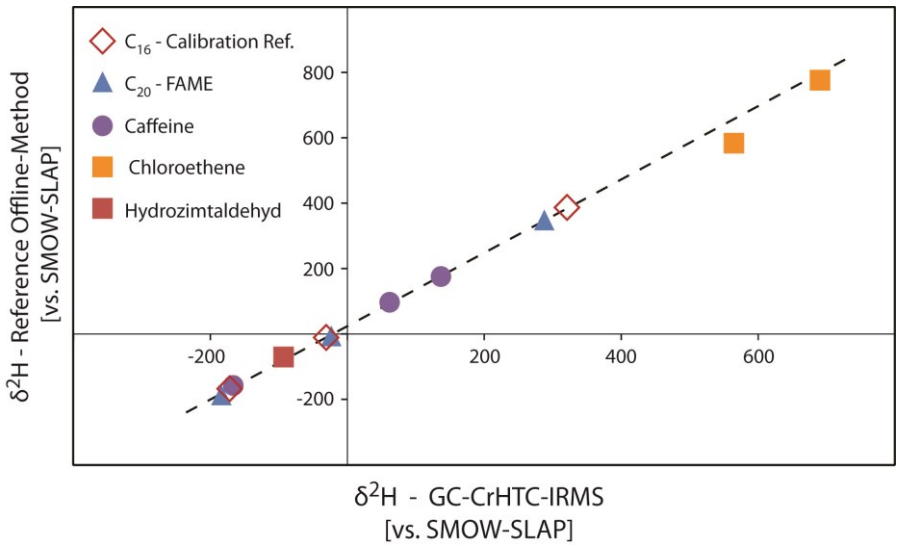

Figure 15: Hydrogen isotope composition of several reference compounds measured with GC-CrHTC-IRMS and compared to known isotope values. Off-line isotope composition δ^2H (‰ vs. SMOW) is given on the y-axis (determined via DI-IRMS after off-line conversion). On-line (via GC-CrHTC-IRMS) measured hydrogen isotope composition is plotted at the x-axis.

4.2. Characterization of microbial reductive dehalogenation using compound-specific stable isotope analysis (Publication I and III)

Stable isotope analysis of carbon was performed during the last decade as standard procedure in order to investigate the involved (bio)catalytic step of the microbial reductive dehalogenation. Thus far isotope fractionation patterns were investigated for several microorganisms, including members of δ- and ε-Proteobacteria, Firmicutes, as well as Chloroflexi. Despite similarities of the reductive dehalogenase enzyme in all microorganisms isotope analysis of carbon revealed highly variable isotope fractionation for different strains during dehalogenation of PCE and TCE, making characterization of the reaction mechanism difficult (Nijenhuis et al. 2005, Lee et al. 2007, Cichocka et al. 2008). Observed variability in carbon isotope fractionation was thought to be related to specific corrinoids incorporated into the RDase enzymes, or microbial cell envelope properties, or growth conditions (Nijenhuis et al. 2005, Mancini et al. 2006, Cichocka et al. 2008). Further, effects of substrate properties, i.e. hydrophobicity, were suggested to be responsible for variability in isotope fractionation (Cichocka et al. 2007, Thullner et al. 2013). The introduction of the dual-element approach (carbon and chlorine) offered a major step forward to overcome the bottlenecks of single-element isotope analysis. Simultaneous analysis of two (or more) elements involved in the catalytic step may potentially elucidates the real magnitude isotope fractionation (Zwank et al. 2005).

This chapter is focusing on the isotope effects and variability of isotope fractionation in microbial systems during single-element isotope analysis. Furthermore, dual-element isotope analysis was applied for characterization of the microbial reductive dehalogenation mechanism, as well as the role of the corrinoid co-factor in the catalytic dehalogenation step.

4.2.1. Substrate hydrophobicity and cell composition determine the extent of rate limitation and resulting isotope masking during microbial reductive dehalogenation of chlorinated ethenes (Publication III)

In order to understand the variability of single-element isotope analysis in microbial systems, effects of different cell envelopes in combination with properties of the degraded compound were investigated in detail. The role of the cell envelope was investigated using data from previous studies, as well as from additional experiments comparing growing cultures (membrane barriers) and corresponding crude extracts (no membrane barriers). Properties of gram-negative and gram-positive cell envelopes (sorption capacity, hydrophobicity and membrane composition) were investigated using two model organisms (*Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* strain PCE-S). The effect of cell envelope on carbon isotope fractionation was subsequently determined using PCE and TCE as model compounds.

4.2.1.1. Microbial isotope fractionation patterns of growing cells vs. crude extracts

The evaluation of microbial carbon isotope fractionation of PCE and TCE from previous studies and recent experiments revealed significant differences in isotope fractionation (Table 8). However, enrichment factors could be separated in distinct patterns. In contrast to abiotic dehalogenation, isotope fractionation was observed to be significantly smaller for PCE in all microbial systems, indicating the effect of compound properties on masking of the isotope effects. Furthermore, for gram-positive bacteria isotope fractionation of PCE was generally stronger as for gram-negative bacteria, indicating an additional effect of the cell envelope properties.

The results indicate that the cell envelope has a major effect on the observed variabilities of microbial isotope fractionation for single-element isotope analysis. Furthermore, properties of the degraded substrate may significantly affect the intracellular micro-scale mass transfer. Therefore, magnitude of

the isotope fractionation may be masked in general in microbial systems, as observed for the highly hydrophobic PCE.

Table 8: Stable isotope enrichment factors for growing cells vs crude extracts of *Geobacter lovleyi*, *Desulfuromonas michiganensis*, *Sulfurospirillum multivorans*, *Dehalobacter restrictus*, and *Desulfitobacterium hafniense* strain PCE-S, as well as representative corrinoids. *ns: not significant

		PCE				TCE			
		ε_C	CI (95%)	R^2	*Ref*	ε_C	CI (95%)	R^2	*Ref*
ε-Proteobacteria gram-negative	***S. multivorans***								
	Crude extract	-1.4	± 0.1 ‰	0.986	*(Renpenning et al. 2014)*	-20.0	± 0.5 ‰	0.999	*(Renpenning et al. 2014)*
	Crude extract	-1.4	± 0.1 ‰	0.924	*(Cichocka et al. 2007)*	-16.2	± 3.7 ‰	0.920	*(Cichocka et al. 2007)*
	Crude extract	-1.0	± 0.2 ‰	0.949	*(Nijenhuis et al. 2005)*	-13.2	± 1.8 ‰	0.990	*(Nijenhuis et al. 2005)*
	Growing culture	-0.4	± 0.2 ‰	0.668	*(Nijenhuis et al. 2005)*	-18.7	± 4.2 ‰	0.840	*(Cichocka et al. 2007)*
	Growing culture					-16.4	± 1.5 ‰	0.970	*(Lee et al. 2007)*
δ-Proteobacteria gram-negative	***G. lovleyi* SZ**								
	Crude extract	-2.3	± 0.4 ‰	0.854	*(Renpenning et al. 2015)*	-9.3	± 0.6 ‰	0.970	*(Renpenning et al. 2015)*
	Growing culture		ns		*(Cichocka et al. 2008)*	-8.5	± 0.6 ‰	0.980	*(Cichocka et al. 2008)*
	Growing culture					-12.2	± 0.5 ‰		(Cretnik et al. 2013)
	D. michiganensis								
	Crude extract	-2.6	± 0.4 ‰	0.878	*(Renpenning et al. 2015)*	-7.1	± 0.4 ‰	0.980	*(Renpenning et al. 2015)*
	Growing culture	-1.7	± 0.1 ‰	0.993	*(Renpenning et al. 2015)*	-4.4	± 0.3 ‰	0.999	*(Renpenning et al. 2015)*
	Growing culture		ns		*(Cichocka et al. 2008)*	-3.5	± 0.3 ‰	0.990	*(Cichocka et al. 2008)*
Firmicutes gram-positive	***D. restrictus***								
	Crude extract	-6.3	± 0.2 ‰	0.993	*(Renpenning et al. 2015)*	-7.7	± 0.4 ‰	0.980	*(Renpenning et al. 2015)*
	Growing culture	-4.0	± 0.2 ‰	0.996	*(Renpenning et al. 2015)*	-8.3	± 0.9 ‰	0.990	*(Renpenning et al. 2015)*
	Growing culture					-3.3	± 0.3 ‰	0.980	*(Lee et al. 2007)*
	***D. hafniense* strain PCE-S**								
	Crude extract	-7.6	± 0.6 ‰	0.991	*(Renpenning et al. 2015)*	-12.9	±0.4 ‰	0.999	*(Renpenning et al. 2015)*
	Crude extract	-8.9	± 1.8 ‰	0.933	*(Nijenhuis et al. 2005)*	-10.9	±1.1 ‰	0.990	*(Cichocka et al. 2007)*
	Growing culture	-5.2	± 1.5 ‰	0.931	*(Nijenhuis et al. 2005)*	-12.2	± 2.3 ‰	0.880	*(Cichocka et al. 2007)*
Corrinoids (abiotic dehalogenation)	Cyanocobalamin	-16.2		0.990	*(Slater et al. 2003)*	-16.5		0.990	*(Slater et al. 2003)*
	Cyanocobalamin	-13.2	± 1.5 ‰	0.983	*(Nijenhuis et al. 2005)*	-15.4	± 2.1	0.961	*(Cichocka et al. 2007)*
	Cyanocobalamin	-22.4	± 0.8 ‰	0.998	*(Renpenning et al. 2014)*	-15.0	± 2.0	0.979	*(Renpenning et al. 2014)*
	Cyanocobalamin					-16.1	± 0.9		*(Cretnik et al. 2013)*
	Norpseudo-B12	-25.3	± 0.8 ‰	0.998	*(Renpenning et al. 2014)*	-18.5	± 2.8	0.961	*(Renpenning et al. 2014)*
	Dicyanocobinamide	-25.2	± 0.5 ‰	0.999	*(Renpenning et al. 2014)*	-16.5	± 0.7	0.997	*(Renpenning et al. 2014)*
	Cobaloxime					-21.3	± 0.5		*(Cretnik et al. 2013)*

4.2.1.2. Effect of cultivation conditions and properties of cell envelope

In order to investigate effects of cultivation conditions on cell envelope properties, the gram-negative *S. multivorans* and gram-positive *D. hafniense* strain PCE-S were used as model organisms. Different cultivation conditions were tested using either fumarate or chlorinated ethene (PCE or TCE) as electron acceptors. Remarkably, both bacteria were observed to modify the fatty acid composition of the membrane during cultivation with chlorinated ethenes. Fatty acids ware observed to alter from cis- to trans-isomers or saturated fatty acids. This adaptation of the membrane can be assumed to be a reaction to solvent stress conditions during cultivation and is in agreement with previous reports (Ramos et al. 2002, Vodovnik et al. 2012). However, the effect of the cell envelope properties was observed to be more pronounced in the gram-negative *S. multivorans* where a strong alteration of cell envelope hydrophobicity was measured, while for *D. hafniense* no or minor changes of cell envelope hydrophobicity were detected (Table 9). Further, cellular sorption of PCE was observed to be about three to four times higher for *S. multivorans* in comparison to *D. hafniense*. In addition, cultivation conditions were observed to affect RDase localization, as already reported by John et al. (2006). However, contrary to that PceA of *S. multivorans* was found at both sites of the membrane, periplasmic and cytoplasmic, if cultivated with fumarate for about ten sub-cultivations, while PceA was localized only at the periplasmic site if cultivated with chlorinated ethenes.

Table 9: Sorption properties and cell envelope hydrophobicity of *S. multivorans* and *D. hafniense* strain PCE-S. Sorption of chlorinated ethenes at the microbial membrane is given as sorption coefficient K_d. The hydrophobicity of the cell envelope was determined via contact angle [°] measurement of a water drop on a cell surface layer; conclusively, higher contact angle represents higher hydrophobicity. *ns: not significant, - : not determined.

	Cultivation ED/EA	K_d for PCE [L g^{-1}]	K_d for TCE [L g^{-1}]	Contact angle [°]
Sulfurospirillum multivorans	Pyr/Fum	0.42 ± 0.09	0.05 ± 0.02	72.2 ± 2.0
	Pyr/PCE	0.51 ± 0.27	ns	33.0 ± 3.4
Desulfitobacterium hafniense strain PCE-S	Pyr/Fum	0.13 ± 0.09	ns	59.1 ± 3.7
	Pyr/PCE	-	-	60.8 ± 3.0

Our results indicate that cultivation with chlorinated ethenes as electron acceptors may result in modification of the cell envelope composition, which apparently affects mass transfer across microbial membranes (Figure 16). The localization of the RDase enzyme may additionally enhance the masking of isotopic effects, if both membranes have to be passed. Though thus far only confirmed for *S. multivorans*, this may occur generally in organohalide respiring microorganisms. In conclusion, with about 10-fold higher hydrophobicity, rate limitation prior to the catalytic dehalogenation step and corresponding masking of the real magnitude of isotope effects is more likely for PCE.

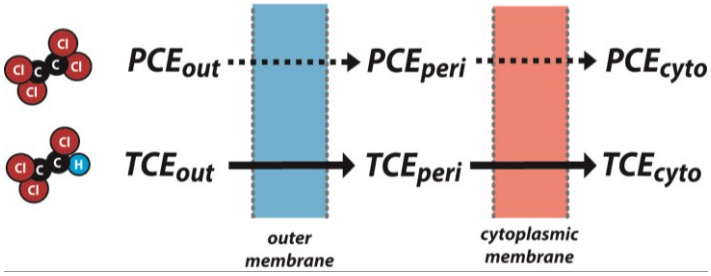

Figure 16: Micro-scale mass transfer of PCE and TCE at microbial membranes. Due to higher hydrophobicity of PCE in comparison to TCE the overall mass transfer of TCE is faster, while PCE transfer is potentially rate-limited.

4.2.1.3. Effect of cell envelope properties and RDase lokalization on isotope fractionation

In order to investigate the effect of the cell envelope on isotope fractionation resting cells of *S. multivorans* were used for dehalogenation experiments. Different electron acceptors (fumarate vs PCE) and electron donor (methyl viologen vs hydrogen) for subsequent dehalogenation experiments were applied in order to isolate the effects of the outer- and cytoplasmic membrane (Figure 17). Carbon isotope fractionation was measured for all scenarios.

While for TCE no significant difference in isotope fractionation could be observed for all scenarios, significant masking of isotope fractionation could be confirmed for PCE. On the one hand, isotope fractionation of PCE was generally much lower for the microbial dehalogenation reaction in comparison to the abiotic dehalogenation reaction (Table 8). On the other hand, isotope fractionation was shown to be affected by cell envelope properties (Figure 17 A and 18 A) and membranes that had

to be passed during transport to the enzyme (Figure 17 B and 18 B). Therefore, masking of isotope effects was proven to be affected by differences in micro-scale mass transfer of the degraded compound, the corresponding rate-limiting transport trough the cell membranes, as well as the RDase enzyme localization in the cell.

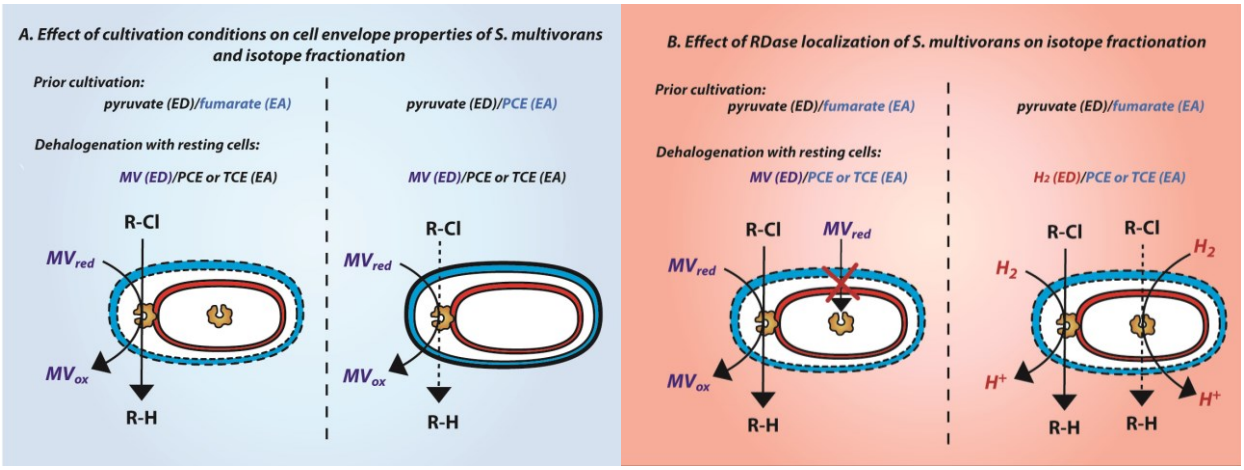

Figure 17: Concept figure for dehalogenation experiments with resting cells. (A) The effect of *S. multivorans* outer-membrane on isotope fractionation was investigated with cells previously cultivated with fumarate or PCE as electron acceptor. Subsequent dehalogenation experiments were conducted with reduced methyl viologen as artificial electron acceptor, addressing only the periplasmic located PceA. (B) The effect of transport across both membranes (outer and cytoplasmic-membrane) and corresponding isotope fractionation was investigated with cells previously cultivated with fumarate only. In order to assess the periplasmic or the cytoplasmic and periplasmic RDase, methyl viologen or H_2 was used as electron donor, respectively.

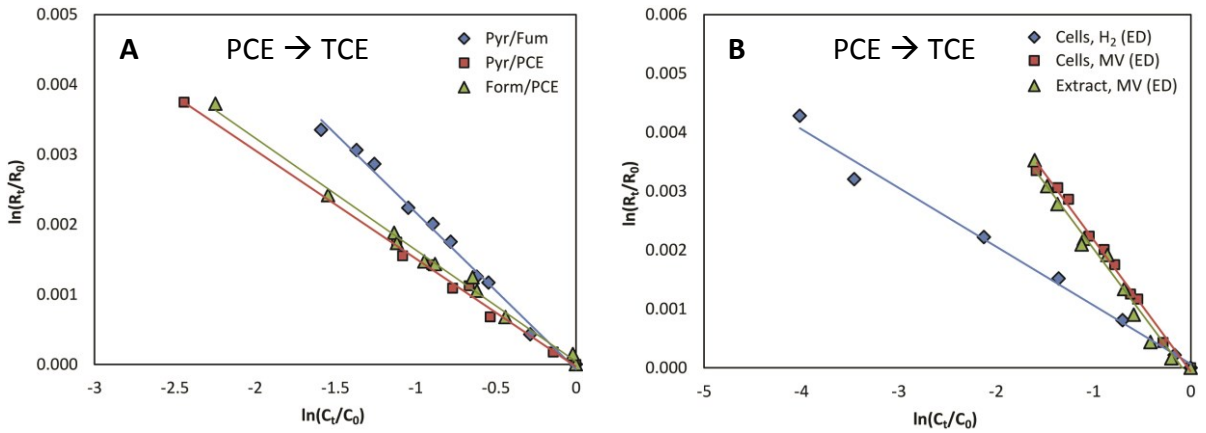

Figure 18: Carbon isotope fractionation of PCE during dehalogenation by *S. multivorans*. The effect of the cellular membranes was investigated using resting cells in combination with methyl viologen as electron donor (outer membrane as only barrier) (A) or resting cell in combination with hydrogen as physiological electron donor (outer + cytoplasmic membrane barriers) (B). Effect of cytoplasmic-membrane on masking of isotope fractionation is linked to the substrate dependent localization of the PceA RDase enzyme.

4.2.2. Combined C and Cl isotope effects indicate differences between corrinoids and enzyme (*Sulfurospirillum multivorans* PceA) in reductive dehalogenation of tetrachloroethene, but not trichloroethene (Publication I)

In order to overcome the limitation of the single-element isotope analysis, dual-element isotope analysis was applied, combining carbon and chlorine isotopes. Due to the simultaneous analysis of both elements during the bond cleavage, masking of isotope effects becomes negligible, since both elements in the molecule are similarly affected by rate limiting events prior to the catalytic step. Thus, dual-element analysis allows to elucidate the isotope effect during the bond cleavage solely.

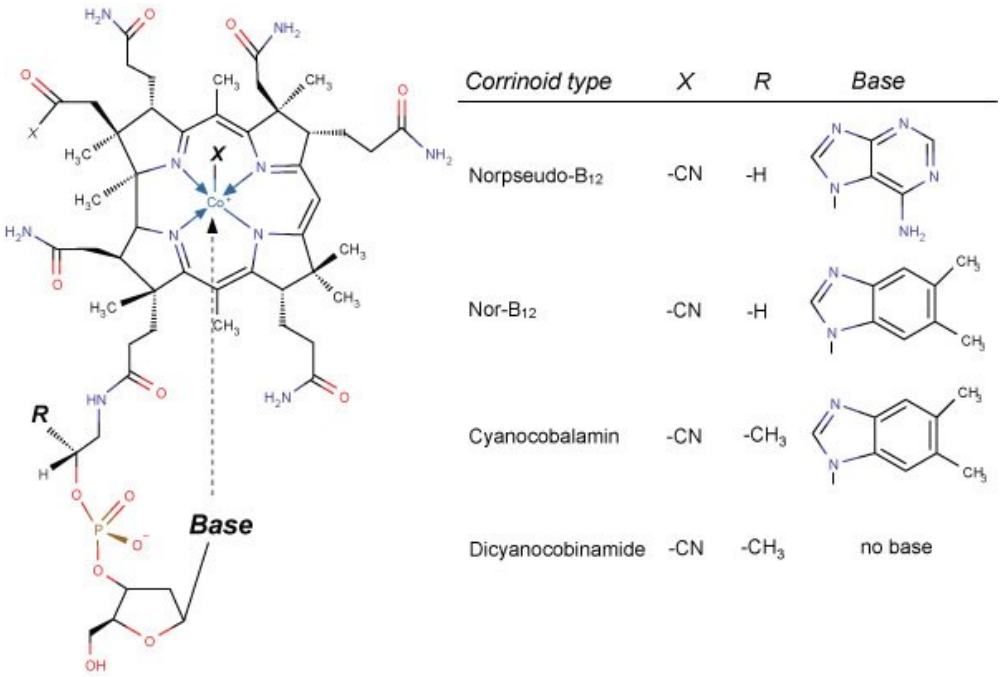

Figure 19: Structure of used corrinoids in this study. The different corrinoids are modified in their structure in the upper ligand (X), lower ligand (Base) or the substituent at carbon-176 (R).

Using dual-element analysis, the effect of corrinoids was investigated in abiotic and enzymatic reactions with *S. multivorans* as model organism. Corrinoids are the key co-factors for the dehalogenation reaction and present in almost all know RDase enzymes (Figure 19) (Hug et al. 2013). However, different corrinoids were found to be present in different organohalide respiring bacteria and assumed to affect the dehalogenation reaction to a certain extend (Yan et al. 2012, Yan et al. 2013,

Keller et al. 2014). Therefore, dual-element isotope analysis was applied in order to elucidate the reaction specific isotope fractionation for abiotic and enzymatic dehalogenation reaction.

4.2.2.1. Comparison of abiotic and enzymatic dehalogenation reaction

Abiotic dehalogenation experiments were done with commercially available corrinoids (cyanocobalamin – vitamin B_{12}, dicyanocobinamide) and purified corrinoids (*norpseudo*-B_{12}, *nor*-B_{12}) from *S. multivorans*. Abiotic dehalogenation was conducted at low redox potential (E_h ~ -0.6 V) and reaction rates were generally significantly slower in comparison to enzymatic dehalogenation reaction. Rapid PCE dehalogenation (pseudo first order reaction rate = 0.30 ± 0.06 min^{-1}) and accumulation of TCE as intermediate was observed, while the dehalogenation rate of TCE was one order of magnitude lower (first order reaction rate = 0.029 ± 0.004 min^{-1}). During abiotic dehalogenation of chloroethenes, cis-DCE, traces of trans-DCE, and VC, as well as ethene were detected. Enzymatic dehalogenation reaction was mediated with crude extracts of *S. multivorans* containing either the PceA-*norpseudo*-B_{12} or PceA-*nor*-B_{12} form of the RDase enzyme. In both cases PCE or TCE were dehalogenated to cis-DCE as final product. In contrast to abiotic experiments, dehalogenation reaction already occurred at lower redox potential (E_h ~ -0.2 V) and was generally much faster. Moreover, enzymatic reaction rates were similar for both, PCE and TCE.

4.2.2.2. Dual-element isotope analysis of reductive dehalogenation reaction

Despite prior differences in single-element isotope analysis, dual-element isotope slopes during TCE dehalogenation reaction were remarkably consistent between abiotic dehalogenation mediated by different corrinoids (cyanocobalamin, dicyanocobinamide, *norpseudo*-B_{12}, *nor*-B_{12}) and enzymatic dehalogenation mediated by PceA-*norpseudo*-B_{12} and PceA-*nor*-B_{12} (Figure 20). Enzymatic dehalogenation of TCE resulted in similar dual-element isotope slopes of 5.3 ± 0.3 (ε_C = -20.0 ± 0.5 ‰, ε_{Cl} = -3.7 ± 0.2 ‰) for PceA-*norpseudo*-B_{12} and 5.0 ± 0.8 (ε_C = -20.2 ± 1.1 ‰, ε_{Cl} = -3.9 ± 0.6 ‰) for PceA-*nor*-B_{12}. For abiotic dehalogenation with corresponding purified corrinoids isotope fractionation was not significantly different. Here the dual-element slopes were 4.5 ± 0.8 (ε_C = -18.5 ±

2.8 ‰ and ε_{Cl} = -4.2 ± 0.8 ‰) for *norpseudo*-B_{12} and 3.7 ± 0.3 (ε_C = -15.0 ± 2.7 ‰ and ε_{Cl} = -3.9 ± 0.8 ‰) for *nor*-B_{12}. Also commercially available cyanocobalamin and dicyanocobinamide gave dual-element slopes of 4.4 ± 0.7 (ε_C = -15.0 ± 2.0 ‰ and ε_{Cl} = -3.2 ± 1.0 ‰) and 4.2 ± 0.6 (ε_C = -16.5‰ ± 0.7 ‰ and ε_{Cl} = -3.9‰ ± 0.5 ‰), which were not significantly different. Therefore, dual-element isotope results in this study suggest a similar reaction for reductive dehalogenation via enzymes as well as corrinoids.

In the case of PCE, however, dual-element isotope slopes partly differed (Figure 20). The enzymatic dehalogenation of PCE with PceA-*norpseudo*-B_{12} resulted in a dual-element isotope slope of 2.2 ± 0.7 (ε_C = -1.4 ± 0.1 ‰ and ε_{Cl} = -0.6 ± 0.2 ‰). For the alternative PceA-*nor*-B_{12} a dual-element isotope slope of 2.8 ± 0.5 (ε_C = -1.3 ± 0.1 ‰, and ε_{Cl} = -0.4 ± 0.1 ‰) was determined. Therefore, dual-element isotope analysis suggests that reductive dehalogenation reaction is similar for both enzymes, despite the different corrinoid in the active center. In contrast, abiotic dehalogenation of PCE with the corresponding purified corrinoids resulted in distinct dual-element isotope slopes compared to the enzymatic dehalogenation. For *norpseudo*-B_{12} a dual-element isotope slope of 6.9 ± 0.7 (ε_C = -25.3 ± 0.8 ‰ and ε_{Cl} = -3.6 ± 0.4 ‰) and for *nor*-B_{12} of 5.0 ± 0.8 (ε_C = -23.7 ± 1.2 ‰ and ε_{Cl} =-4.8 ± 0.9 ‰) was measured. Further, dual-element analysis of cyanocobalamin resulted in a slope of 4.6 ± 0.2 (εC = -22.4 ± 0.8 ‰ and εCl = -4.8 ± 0.2 ‰), similar to extracted *nor*-B_{12}. In contrast, for dicyanocobinamide the observed dual-element isotope fractionation was different with a significantly higher slope of 7.0 ± 0.8 (εC = -25.2‰ ± 0.5 ‰ and εCl = -3.4‰ ± 0.4 ‰), similar to extracted *norpseudo*-B_{12}. Therefore, the significant difference in dual element isotope slopes between *nor*-B_{12} and *norpseudo*-B_{12} could also be reproduced in chemical reactions with commercially available cobalamins.

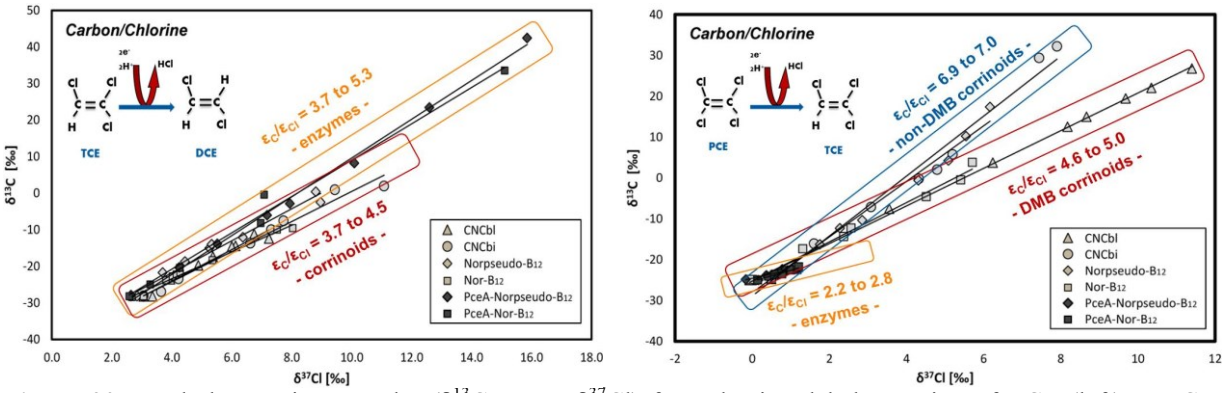

Figure 20: Dual-element isotope plot ($\delta^{13}C$ versus $\delta^{37}Cl$) for reductive dehalogenation of TCE (left) or PCE (right). Dehalogenation was performed using pure corrinoids (cyanocobalamin - CNCbl, dicyanocobinamide - CNCbi, *norpseudo*-B_{12} and *nor*-B_{12}), as well as crude extract harboring PceA-*norpseudo*-B_{12} or PceA-*nor*-B_{12} of *S. multivorans*.

4.3.1. Discussion and outlook - Characterization of microbial reductive dehalogenation via CSIA

4.3.1.1. Intracellular limitation of micro-scale mass transfer and variability of isotope fractionation in microbial systems

Generally variability in microbial isotope fractionation is considered to be a result of rate-limiting steps during mass transport prior to C-Cl bond cleavage (Nijenhuis et al. 2005, Cichocka et al. 2007, Thullner et al. 2013). For example, mass transfer of the substrate to the enzyme may be affected by extracellular (dissolution, solubility, transport to the cell) and intracellular (membrane barriers, sorption at the membranes or enzymes) rate-limitation, resulting in a reduction of the magnitude of isotope fractionation (Aeppli et al. 2009, Thullner et al. 2013). The effects of extracellular mass transfer of masking of isotope effects were already investigated and confirmed by several studies (Kampara et al. 2008, Thullner et al. 2008, Thullner et al. 2013). The effect of intracellular mass transfer, however, was thus far poorly investigated.

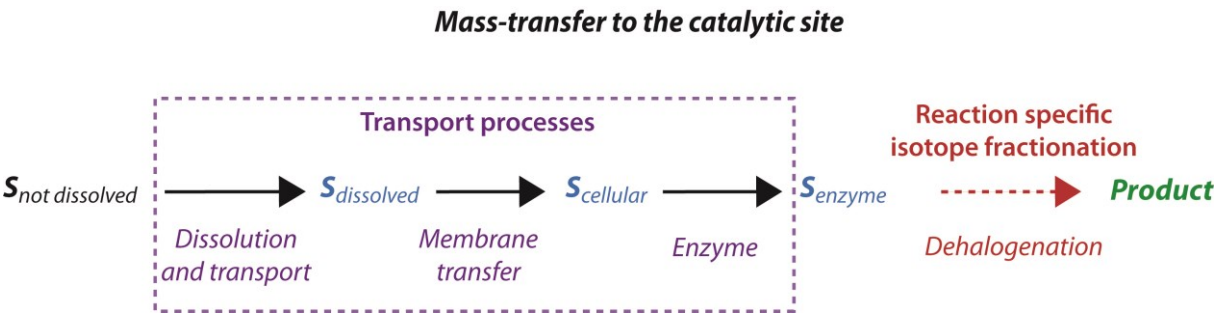

Figure 21: Theoretical mass transfer of substrate from dissolution, extracellular transfer, intracellular transfer to substrate enzyme association and the final catalytic step (dehalogenation).

According to our results limitation in micro-scale mass transfer of chlorinated ethenes during microbial dehalogenation is determined by the properties of the cell envelope and the properties of the degraded compound. This hypothesis is supported by several observations during single-element isotope analysis of carbon:

- Enzymatic dehalogenation of PCE could be divided in two groups: the gram-negative Proteobacteria with εC -0.4 to -1.7 ‰ and gram-positive Firmicutes with εC -4.0 to -5.2 ‰, suggesting a general effect of the cell envelope composition.
- Isotope fractionation for TCE, in comparison to the more hydrophobic PCE, was overall higher in all experiments. Moreover, enzymatic isotope fractionation of TCE was more similar to abiotic isotope fractionation catalyzed by pure corrinoids, while enzymatic isotope fractionation of PCE was much weaker and diverse, suggesting an effect of compound hydrophobicity.
- Stronger isotope fractionation was measured in experiments with *S. multivorans* cells cultivated with fumarate (higher cell surface hydrophobicity and lower relative content of saturated fatty acids), compared to cells cultivated on chloroethenes (lower cell surface hydrophobicity and higher relative content of unsaturated *trans*-isomeric fatty acids).
- For *S. multivorans* the cytoplasmic membrane was observed to be an additional mass transfer barrier for PCE, in case the RDase enzyme is localized in the cytoplasm.

Masking of isotope fractionation for PCE indicates serious micro-scale mass transfer limitation across the membrane. Differences between PCE and TCE, therefore, may be partly explained by different hydrophobicity of both compounds. Since TCE is a less hydrophobic compound, transport across the cell envelope might be less affected. Therefore, hydrophobicity of the degraded compound is probably an important factor that determines micro-scale mass transfer and the corresponding masking of the isotope fractionation.

During transport three main, potentially rate- or mass transfer limiting, barriers can be considered for the chlorinated substrate prior to the catalytic cleavage of the C-Cl bond: 1) the outer membrane or cell wall, 2) the cytoplasmic membrane in case of a cytoplasmic location of the enzyme as well as, 3) the reductive dehalogenase enzyme (Figure 22).

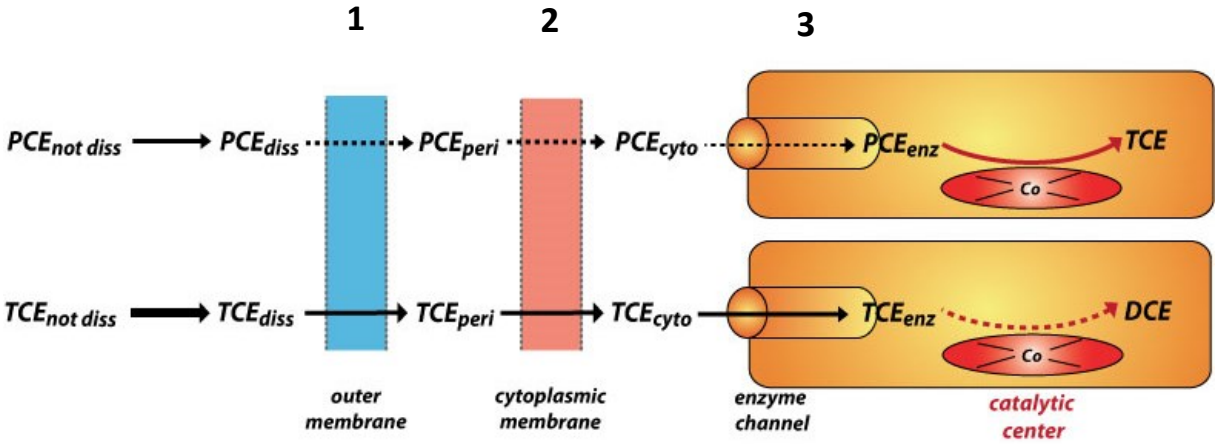

Figure 22: Potentially rate-limiting steps in the micro-scale mass transfer of PCE and TCE during reductive dehalogenation of *S. multivorans* cultivated with fumarate as electron acceptor. During transport compound are extracellularly dissolved (diss), transported through the outer-membrane into the periplasm (peri), transported through the cytoplasmic-membrane into cytoplasm (cyto), and transported through the enzyme channel to the corrinoid in the enzyme (catalytic center). Since mass transfer of PCE is rate-limited (at the membranes and enzyme channel) prior to the catalytic step, measured isotope fractionation is masked. In contrast, mass transfer of TCE is not limited since the catalytic step is generally slower. Therefore, isotope fractionation of TCE is not masked at these conditions.

1) Outer-membrane

In contrast to the reports of Harding et al. (2013), different growth conditions were observed to affect cell envelope properties of *S. multivorans*, as well as the extent of isotope fractionation of PCE. Isotope fractionation of PCE was significantly reduced from ε_C = -2.2 ± 0.2 ‰ (only periplasmic PceA-RDase active) down to ε_C = -1.5 ± 0.1 ‰ (cytoplasmic and periplasmic PceA-RDase active). Micro-scale mass transfer induced masking of isotope fractionation was already observed in bioavailability studies at low substrate concentration and concentration gradients (Kampara et al. 2008, Thullner et al. 2008). Furthermore, the effect of rate-limiting mass transfer on isotope fractionation was demonstrated in several studies using high biomass concentration (Templeton et al. 2006, Staal et al. 2007, Kampara et al. 2009). Therefore, our results suggest a partial rate-limiting mass transfer at the outer-membrane of *S. multivorans*. However, since no outer-membrane is present in gram-positive Firmicutes, as well as Chloroflexi, rate-limiting effect on isotope fractionation supposed to be negligible for bacteria from both phyla. Still, isotope fractionation was measured to be significantly different between growing cells and crude extracts for gram-positive *D. hafniense* und *D. restrictus* (chapter 4.2.1, publication III). This outcome cannot be explained by the rate limiting effect

of the outer-membrane alone, but could be explained by the cytoplasmic RDase activity and rate limitation at the cytoplasmic-membrane, as discussed below.

2) Cytoplasmic-membrane

For cell with cytoplasmic PceA activity, the cytoplasmic-membrane was shown to result in a significant rate-determining step prior to dehalogenation. Isotope fractionation of PCE was significantly reduced from ε_C = -2.2 ± 0.2 ‰ (periplasmic dehalogenation only) down to ε_C = -1.0 ± 0.1 ‰ (periplasmic and cytoplasmic dehalogenation) (chapter 4.2.1, publication III). Though thus far investigated for *S. multivorans* solely (John et al. 2006), active cytoplasmic dehalogenase may occur frequently in organohalide respiring bacteria during the initial growth, and therefore, affect the observed isotope fractionation. Cytoplasmic dehalogenation may explain the variability of isotope enrichment factors observed in different studies for growing cells in comparison to crude extracts or purified enzyme, as for instance isotope enrichment factors for PCE dehalogenated by mixed culture KB-1 (-2.6 to -5.5 ‰), *Desulfitobacterium* strain PCE-S (-5.2 to -8.9 ‰), and *Geobacter lovleyi* (not significant to -2.3 ‰) (Slater et al. 2001, Nijenhuis et al. 2005, Cichocka et al. 2008, Renpenning et al. 2014).

3) Reductive dehalogenase

Indication for extensive masking of isotope fractionation at the active site of the PceA enzyme was already demonstrated in our dual-element study (Renpenning et al. 2014). In addition, the first crystal structure of PceA of *S. multivorans* was recently published by Bommer et al. (2014) and revealed an enzyme structure with an active site deeply embedded in the core of the protein. To get access to the active site chlorinated hydrocarbons have to pass a 12 Å long and 5.5 Å wide hydrophobic channel. The channel forms a restriction filter and is thought to disfavor access for molecules much larger than halogenated ethenes. Therefore, the enzymes itself potentially restricts the mass-transfer for highly hydrophobic compounds and enhances masking of measured isotope fractionation. Evidence for rate-limitation at the active-site of PceA-RDase was provided by results of this thesis comparing abiotic reaction rates mediated by several corrinoids (i.e. cyanocobalamin, dicyanocobinamide, norpseudo-B_{12} and nor-B_{12}) to enzymatic reaction rates catalyzed by PceA of *S. multivorans* (Renpenning et al. 2014). Abiotic dehalogenation rates were observed to be about 10-times faster for PCE vs. TCE, while the

rates for dehalogenation catalyzed by *S. multivorans* PceA were similar for both chlorinated ethenes. Rate-limitation at the active-site of the enzyme would explain the overall low isotope fractionation of hydrophobic PCE by *S. multivorans,* but also by *G. lovleyi* and *D. michiganensis*, whereas the less hydrophobic TCE was not observed to be affected. Nonetheless, mass transfer of TCE may be affected. However, since the abiotic dehalogenation is reported to be about 10-times slower for TCE in comparison to PCE, the rate of transport apparently exceeds the overall rate of the reaction (Glod et al. 1997, Renpenning et al. 2014). Therefore, rate-limitation of the catalytic step (C-Cl cleavage) and corresponding masking of isotope effects cannot be observed for TCE (Figure 22).

In summary, our experiments revealed significant masking of isotope fractionation of PCE by the outer- and cytoplasmic-membrane, as well as the RDase of *S. multivorans*. The effects were mainly observed for PCE, but not for TCE. Nevertheless, as discussed above, TCE mass transfer may still be limited at the membranes, but does not limit the rate of the overall reaction due to the lower rate of abiotic reaction for TCE compared to PCE. Micro-scale mass transfer of chlorinated ethenes in this case will be limited by the outer-membrane, the cytoplasmic-membrane and the RDase enzyme. Determined single-element enrichment factors will not elucidate the real magnitude of isotope fractionation. Conclusively, isotope fractionation may be underestimated in microbial systems compared to the actual chemical reaction and not appropriate for evaluation of reaction mechanisms.

4.3.1.2. Effect of different corrinoids on dehalogenation reaction

Dual-element stable isotope analysis provides an alternative way to overcome masking effects in single-element isotope analysis. Thus far better evaluation and elucidation of degradation pathways could be achieved in lab and field studies, as already shown in other studies investigating degradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA) and the corresponding isotope effects on hydrogen and carbon (Zwank et al. 2005, Rosell et al. 2007). Therefore, dual-element isotope analysis was applied to compare the abiotic and enzymatic dehalogenation reaction by measuring carbon and chlorine isotope fractionation.

In the case of TCE, the dual-element isotope slopes observed in this study suggests that reductive dehalogenation proceeded via a similar reaction mechanism in all enzymes and corrinoids

investigated. In the case of PCE, however, dual-element isotope slopes differed between corrinoids harboring DMB as lower ligand base (cyanocobalamin and *nor*-B_{12}) on the one hand and non-DMB corrinoids (*norpseudo*-B_{12} and dicyanocobinamide) on the other hand (chapter 4.2.2, publication I). This raises the question for underlying reasons.

Different dual-element isotope slopes – when observed in elementary chemical reactions – reflect differences in reaction mechanisms (i.e., in the order and manner of bond cleavage within a molecule) (Elsner et al. 2012, Kummel et al. 2013). If the observed differences in dual element slopes (Figure 20, PCE) were indeed caused by *mechanistic* differences, the putative alternative reductive dehalogenation scenarios "nucleophilic addition", "nucleophilic substitution", or "electron transfer" could be invoked (or a simultaneous combination of them). However, it is not clear if only one mechanism is involved or a combination of more than one. Alternatively, as shown in Penning et al. (2008), different dual-element isotope plots may be caused by the same mechanism (i.e., the same manner of bond changes), but by different *kinetics* (i.e., bonds are cleaved in different steps of which the rates change relative to each other). If the observed differences were caused by such *kinetic* differences, that it is interesting to consider that upon reduction, the corrinoid may be transformed into its *base-off* conformation. The rate of the intramolecular ligand dissociation was shown to vary among structurally different corrinoids (Lexa and Saveant 1983, Butler et al. 2006). Structural differences in the lower ligand may affect a rate determining step prior to the bond cleavage and might cause the observed differences in dual-element stable isotope analysis for different corrinoid types. So far, however, it is not possible to disentangle the different isotope effects.

4.3.1.3. Similarities and differences of enzymatic reductive dehalogenation

Despite the different corrinoid cofactors, enzymatic isotope fractionation mediated by PceA-*norpseudo*-B_{12} or PceA-*nor*-$B_{12}$was not significantly affected. Consequently, stable isotope analysis indicated that the corrinoid types are most probably not responsible for previously observed variabilities in microbial isotope fractionation of carbon. Moreover, dehalogenation of TCE with both enzyme types, as well as all corrinoids was observed to be in a similar range with $\varepsilon_C/\varepsilon_{Cl}$ from 3.7 to 5.3

(Figure 20). This correlates with recently published observations of Cretnik et al. (Cretnik et al. 2013), with dual-element isotope slopes for *Geobacter lovleyi*, *Desulfitobacterium hafniense* of $\varepsilon_C/\varepsilon_{Cl}$ 3.4 to 3.8.

In the case of PCE, however, dual-element isotope fractionation clearly varied between enzymatic ($\varepsilon_C/\varepsilon_{Cl}$ of 2.2 to 2.8) and abiotic ($\varepsilon_C/\varepsilon_{Cl}$ of 4.6 to 7.0) dehalogenation. Therefore, it should be considered if dual-element analysis reflects differences in the reaction mechanism or if differences in dual-element isotope slopes are caused by the kinetics of subsequent reaction steps. Nonetheless, the small isotope fractionation of PCE observed for both carbon and chlorine with PceA makes it unlikely that any significant changes in C-Cl bond chemistry were involved in the rate-determining step. In this study PCE dehalogenation rates were one order of magnitude higher for abiotic reaction with pure corrinoids compared to TCE, but not for enzymes, were PCE and TCE dehalogenation rates were reported to be similar (Neumann et al. 1996). Therefore, it may be hypothesized that the actual *chemical* reaction at the corrinoid cofactor within the enzyme would proceed similarly ten-fold faster for PCE compared to TCE, but that difference is not observed in the *enzymatic* reaction, because preceding steps may significantly reduce the overall rate, as discussed above for single-element isotope analysis. This would affect PCE dehalogenation more strongly than TCE dehalogenation, because of the faster intrinsic rate for PCE. Consequently, dual-element analysis of PCE would reflect a preceding rate-limiting step (e.g., enzyme-substrate association), whereas in the case of TCE it would still reflect the underlying enzymatic reaction. In such a case PCE data would not be suited to elucidate the underlying reaction chemistry of C-Cl bond cleavage, but would give insight into the kinetics of enzyme catalysis instead.

4.3.1.4. Potential application of hydrogen isotope analysis for proton transfer studies

Thus far only one study about hydrogen isotope effects during the protonation step of the reductive dehalogenation reaction was published (Kuder et al. 2013). On the one hand, routine application for hydrogen isotope analysis is not available for chlorinated compounds, as discussed in chapter 4.1.2.

On the other hand, only minor isotope effects were expected for the protonation step. Furthermore, the source of the proton was not clarified.

Our preliminary results, however, confirmed water as a preferential proton source (data not published). Furthermore, strong hydrogen isotope fractionation was observed during the protonation reaction and could be significantly distinguished in enzymatic and abiotic dehalogenation reaction (Figure 23). All experiments were performed in deuterium enriched water (D_2O/H_2O = 1:1). Therefore, hydrogen isotope effects could be already monitored qualitatively via MS analysis. Nonetheless, all experiments have to be confirmed using water of a natural isotope composition.

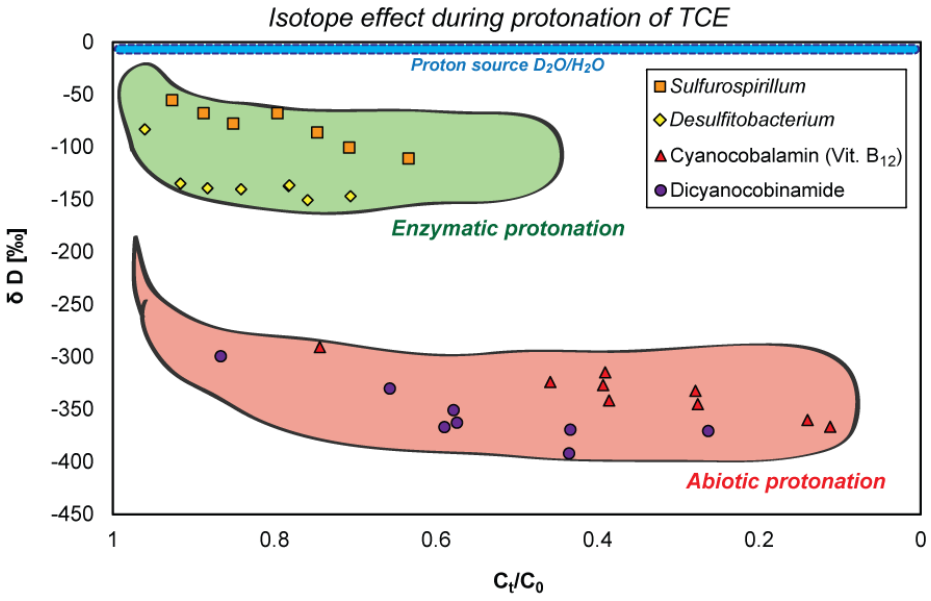

Figure 23: Hydrogen isotope analysis after dehalogenation of PCE and protonation of TCE. Dehalogenation reaction was mediated enzymatically by bacterial crude extracts of *S. multivorans* and *D. hafniense*, or abiotically by cyanocobalamin and dicyanocobinamide. The proton source was deuterium enriched water (D_2O/H_2O = 1:1) defined as $\delta^2H \sim 0$ ‰.

Our results unravel the potential of hydrogen isotope analysis. The development of on-line isotope analysis via chromium-based reactor technique (discussed in chapter 4.1.3) will make hydrogen isotope analysis accessible for halogenated compounds as routine application. The combination of carbon, chlorine and hydrogen could therefore reveal the whole potential of CSIA for a full characterization of the microbial reductive dehalogenation by compound-specific stable isotope analysis of the microbial reductive dehalogenation.

5. General Outlook

5.1. Method development for hydrogen and chlorine isotope analysis

Though major steps were undertaken to overcome the restriction of chlorine and hydrogen isotope analysis, the analysis of halogenated hydrocarbons remains a challenging task. First steps for a routine hydrogen isotope analysis of halogenated compound were already achieved with the Cr-EA approach, where the chromium-based reactor system was demonstrated to be applicable for precise and accurate isotope measurements of various hetero-atom containing compounds. However, this method can be only applied for pure or bulk samples, but not for mixtures. Therefore, a compound-specific method will be required. Remarkably, first attempts to transfer the chromium-based reactor to a compound-specific GC-IRMS application were successful. Moreover, only standard equipment was applied, which can be easily incorporated to available analytical devices. The combination of aluminum oxide ceramic and chromium-based reactor system was able to extend the range of application of hot chromium up to 1500 °C. The elevated temperature was observed to improve the conversion properties and may give access to a broader range of thermo-stable compounds, and cover not only halogenated compounds, but also a wide range of heteroatom containing organics in compound-specific stable isotope analysis. Preliminary results of chromium-based reactor systems in combination with GC-IRMS are highly promising (Chapter 4.1.3.3) and may establish the chromium-based reactor systems as a routine application for hydrogen isotope analysis.

Compound-specific isotope analysis of chlorine via high-temperature conversion to HCl was for the first time able to demonstrate the potential of this technique for several types of chlorinated hydrocarbons. Conversion of chlorinated organics to HCl as analyte gas for isotope analysis offers in contrast to non-convertive methods (GC-qMS or GC-IRMS) a universally applicable approach. Moreover, the basic principal could be further extended to TC/EA systems for organics, as well as inorganics. In addition, similar approach could be applied for isotope analysis of other elements, as for instance bromine or sulfur isotopes, if converted to HBr or H_2S as analyte gas. However, for GC-HTC-IRMS stability of the reactor material and memory effects were thus far a bottleneck for a routine application. Alternative thermo-stable reactor material and new reactor designs are currently in

development and may provide a solution to overcome the limitations. Therefore, routine isotope analysis of chlorine may be achieved in near future.

5.2.Application of CSIA of carbon and chlorine for evaluation of microbial reaction systems

Application of carbon and chlorine isotope analysis in dehalogenation studies could already profit from the extension of single-element to a dual-element approach. Analysis of both elements was able to provide valuable information about the reductive dehalogenation reaction. Simultaneous isotope analysis of carbon and chlorine isotopes during dehalogenation of chloroethenes provided strong evidence for a similar reaction mechanism during abiotic (mediated by the corrinoids) and enzymatic (mediated by microbial enzymes) dehalogenation reaction (Cretnik et al. 2013, Cretnik et al. 2014, Renpenning et al. 2014). Further, first successful attempts to connect laboratory experiment and field site application were demonstrated by Wiegert et al. (2013) and Badin et al. (2014), which may offer an significant improvement of monitoring and evaluation of *in situ* remediation processes. Moreover, a similar approach is currently applied for chlorinated methanes and ethanes (Audi-Miro et al. 2013, Palau et al. 2014, Palau et al. 2014) and may evolve to a routine method in lab and field side application.

Though chlorine isotope analysis via non-convertive GC-IRMS or GC-qMS was thus far particularly limited to chlorinated methane, ethene, and ethane, it already placed chlorine isotope analysis a major step forward. However, isotope analysis of frequent pollutants as for instance HCHs, chlorinated benzenes, chloroacetic acids, as well as chlorinated pharmaceuticals is a field of interest, which was so far hardly accessible for the non-convertive techniques. The extension of chlorine isotope analysis to those pollutants using the high-temperature conversion approach could help to access a novel group of compound classes for CSIA, as well as the opportunity to evaluate and characterize abiotic and biotic degradation/conversion mechanisms in lab, as well as in field site experiments.

5.3.Extension of hydrogen isotope analysis to chlorinated hydrocarbons

Hydrogen isotope fractionation during dehalogenation of chlorinated hydrocarbons was thus far hardly investigated, due to the fact that no routine methods were available for compound-specific hydrogen isotope analysis. Though first attempts were undertaken by Shouakar-Stash and Drimmie (2013) and Kuder and Philp (2013) to provide a method for hydrogen isotope analysis of chlorinated ethenes, only one application study was thus far published targeting hydrogen isotope effects during reductive dehalogenation (Kuder et al. 2013). Remarkably, strong shifts in isotope composition were observed after the dehalogenation step mediated by a *Dehalococcoides* mixed culture. Similarly, our preliminary results of hydrogen isotope analysis have shown pronounced hydrogen isotope fractionation after dehalogenation reaction mediated by corrinoids or RDase of *S. multivoarans* and *D. hafniense*. Moreover, hydrogen isotope effects could be clearly distinguished between abiotic and enzymatic reaction with a difference of about 200 to 300 ‰. The observed effects are assumed to be the result of events prior to the protonation step, since protonation supposed to result in a minor isotope fractionation. These results have to be confirmed. However, hydrogen isotope analysis of halogenated compounds may provide additional information related to the involved reaction mechanism and extend the application of CSIA in lab and field site studies. Furthermore, the field of isotope forensics would highly benefit from hydrogen isotope analysis of halogenated compounds, since variations of isotope composition is the most pronounced for hydrogen in comparison to any other element. Therefore, hydrogen is ideal for fingerprint analysis and source identification. In conclusion, the extension of CSIA to a multi-element stable isotope analysis, including carbon, chlorine and hydrogen, may reveal a more detailed insight into the (bio)chemical process, as well as the field of isotope forensics.

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7. Appendix

Publication I

Renpenning, J.; Keller, S.; Cretnik, S.; Shouakar-Stash, O.; Elsner, M.; Schubert, T.; Nijenhuis, I., Combined C and Cl isotope effects indicate differences between corrinoids and enzyme (*Sulfurospirillum multivorans* PceA) in reductive dehalogenation of tetrachloroethene, but not trichloroethene. *Environ Sci Technol* **2014**, 48, (20), 11837-45.

Publication II

Renpenning, J.; Hitzfeld, K. L.; Gilevska, T.; Nijenhuis, I.; Gehre, M.; Richnow, H. H., Development and Validation of an Universal Interface for Compound-Specific Stable Isotope Analysis of Chlorine ($^{37}Cl/^{35}Cl$) by GC-High-Temperature Conversion (HTC)-MS/IRMS. *Anal Chem* **2015**, 87, (5), 2832-9.

Publication III

Renpenning, J.; Rapp, I.; Nijenhuis, I., Substrate Hydrophobicity and Cell Composition Influence the Extent of Rate Limitation and Masking of Isotope Fractionation during Microbial Reductive Dehalogenation of Chlorinated Ethenes. *Environ Sci Technol* **2015**, 49 (7), pp 4293–4301

Publication IV

Gehre, M.; **Renpenning, J**.; Gilevska, T.; Qi, H.; Coplen, T. B.; Meijer, H. A. J.; Brand, W. A.; Schimmelmann, A., On-line hydrogen-isotope measurements of organic samples using elemental chromium—an extension for high temperature elemental-analyser techniques. *Anal Chem* **2015**.

Publication I

Environmental Science & Technology

Article

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Combined C and Cl Isotope Effects Indicate Differences between Corrinoids and Enzyme (*Sulfurospirillum multivorans* PceA) in Reductive Dehalogenation of Tetrachloroethene, But Not Trichloroethene

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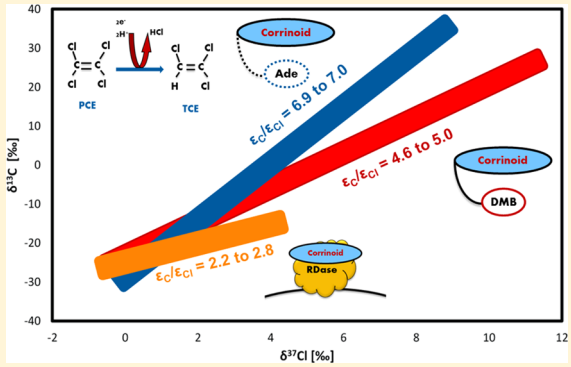
Supporting Information

ABSTRACT: The role of the corrinoid cofactor in reductive dehalogenation catalysis by tetrachloroethene reductive dehalogenase (PceA) of *Sulfurospirillum multivorans* was investigated using isotope analysis of carbon and chlorine. Crude extracts containing PceA—harboring either a native *norpseudo*-B_{12} or the alternative *nor*-B_{12} cofactor—were applied for dehalogenation of tetrachloroethene (PCE) or trichloroethene (TCE), and compared to abiotic dehalogenation with the respective purified corrinoids (*norpseudo*vitamin B_{12} and *nor*vitamin B_{12}), as well as several commercially available cobalamins and cobinamide. Dehalogenation of TCE resulted in a similar extent of C and Cl isotope fractionation, and in similar dual-element isotope slopes ($\varepsilon_C/\varepsilon_{Cl}$) of 5.0−5.3 for PceA enzyme and 3.7−4.5 for the corrinoids. Both observations support an identical reaction mechanism. For PCE, in contrast, observed C and Cl isotope fractionation was smaller in enzymatic dehalogenation, and dual-element isotope slopes (2.2−2.8) were distinctly different compared to dehalogenation mediated by corrinoids (4.6−7.0). Remarkably, $\varepsilon_C/\varepsilon_{Cl}$ of PCE depended in addition on the corrinoid type: $\varepsilon_C/\varepsilon_{Cl}$ values of 4.6 and 5.0 for vitamin B_{12} and *nor*vitamin B_{12} were significantly different compared to values of 6.9 and 7.0 for *norpseudo*vitamin B_{12} and dicyanocobinamide. Our results therefore suggest mechanistic and/or kinetic differences in catalytic PCE dehalogenation by enzymes and different corrinoids, whereas such differences were not observed for TCE.



INTRODUCTION

Organohalide-respiring bacteria are capable of conserving energy by coupling the anaerobic reductive dehalogenation of halogenated compounds to the generation of a proton motive force.[1] Such reactions are catalyzed by corrinoid-containing reductive dehalogenase enzymes (RDase). Organohalide-respiring microorganisms were identified as key players in the detoxification of halogenated groundwater contaminants such as chlorinated ethenes; therefore, strong efforts have been made during the last decades to understand the dehalogenation process and the underlying reaction mechanism. So far several organohalide-respiring bacteria from different phyla were isolated, for example, *Sulfurospirillum*, *Desulfitobacterium*, *Dehalococcoides*, *Dehalobacter*, *Geobacter*, and the responsible genes encoding RDase enzymes were identified.[2−6] However, only few RDases have been characterized biochemically. Among these RDases different substrate spectra have been observed; dehalogenation of, for example, chlorinated ethenes or chlorinated benzenes, complete or partial dehalogenation. However, all RDases were found to be highly similar in structure, containing two iron−sulfur clusters and a corrinoid cofactor.[7−11]

A distinguishing feature by which corrinoid cofactors do differ is a structural diversity with respect to the lower ligand of the cobalt in the center of the corrin ring (Figure 1).[12,13]

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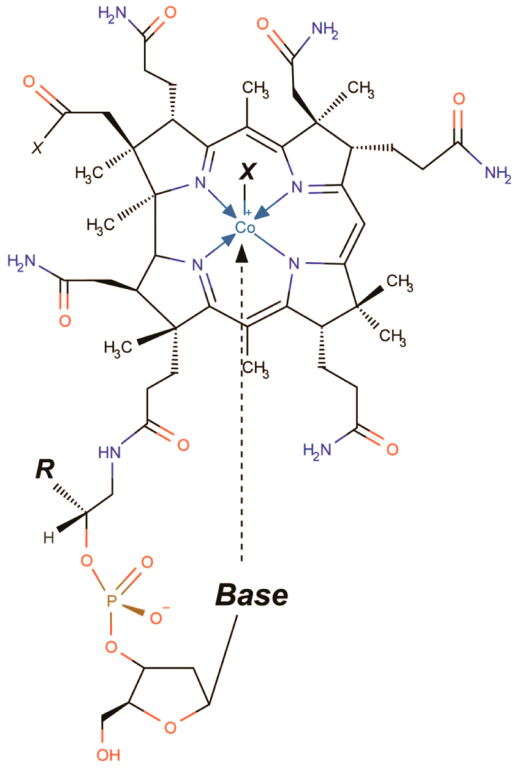


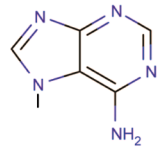
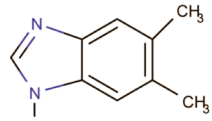
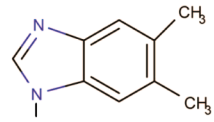
Corrinoid type	X	R	Base
Norpseudo-B_{12}	-CN	-H	
Nor-B_{12}	-CN	-H	
Cyanocobalamin	-CN	-CH_3	
Hydroxocobalamin	-OH		
Methylcobalamin	-CH_3		
Adenosylcobalamin	-Ado		
Dicyanocobinamide	-CN	-CH_3	no base

Figure 1. Structure of corrinoids used in this study. The different corrinoids differ in the structure of the upper ligand (X), lower ligand (Base) and the substituent at carbon-176 (R).

Benzimidazole analogues and purines (e.g., adenine) are frequently encountered as lower ligand bases. Recently, the importance of the lower ligand of the corrinoid cofactor for the *Dehalococcoides mccartyi* reductive dehalogenase was pointed out.[14,15] Specifically, the corrinoid cofactor type was assumed to be important for the overall activity, as well as the substrate spectrum of the reductive dehalogenase enzyme.

Sulfurospirillum multivorans, a strain which reductively dehalogenates tetrachloroethene (PCE) and trichloroethene (TCE), is capable of *de novo* biosynthesis of an unusual type of adenine-harboring corrinoid cofactor, the *norpseudo*-B_{12} (Co_{α}-adeninyl-176-norcobamide) (Figure 1).[16] Previous studies observed significantly higher dechlorination rates of trichloroacetic acid for *norpseudo*vitamin B_{12} in comparison to the common vitamin B_{12} (cyanocobalamin).[17] Cultivation of *Sulfurospirillum multivorans* in the presence of 4,6-dimethylbenzimidazole (DMB) resulted in formation of the DMB-harboring *nor*-B_{12} (Co_{α}-5,6-dimethylbenzimidazolyl-176-norcobamide)(Figure 1) cofactor. For *nor*-B_{12} much lower dechlorination rates were measured.[18] Overall, the dissociation rate of the lower ligand is thought to affect the reaction rates and potentially the reaction mechanism which are currently not elucidated.[19] Considered reactions are nucleophilic addition, nucleophilic substitution and single electron transfer for both corrinoids and enzymatic dehalogenation.[20−22]

In recent years, compound specific stable isotope analysis (CSIA) has increasingly been used for the characterization of reaction mechanisms. CSIA takes advantage of stable isotope fractionation during degradation of organic compounds, since light isotopes (e.g., ^{12}C, ^{35}Cl) typically react faster than the heavy isotopes (e.g., ^{13}C, ^{37}Cl), leading to an enrichment of heavier isotopes in the residual fraction which is not yet degraded. The corresponding isotope enrichment factors can be related to the respective (bio)chemical reactions; consequently, identification of similar enzymes or mechanisms may be accomplished with CSIA.[23] Frequently, isotope fractionation of a single element is used for evaluation of reaction mechanisms; meanwhile, an additional dual-element analysis enables a better characterization of a reaction mechanism.[24,25]

Interestingly, previous single-element isotope analysis of bacteria capable of chloroethene dehalogenation resulted in highly variable carbon isotope enrichment factors, even for similar dehalogenation reactions.[26−28] The observed differences were partially attributed to rate limitation during transport of the substrate to the enzyme, but also thought to be attributable to different corrinoid cofactors harbored by RDases. These differences may effectuate differences in intrinsic RDase reactivity and resultant putative differences in the mechanism (i.e., the manner and order of bond cleavage) and kinetics (i.e., the corresponding rates) affecting the individual elementary reaction steps involved in catalytic reductive dehalogenation.[27] Recently, Kuder et al.[29] and Wiegert et al.[30] reported on dual-element isotope fractionation of TCE using mixed cultures. The results, however, varied in both studies. In contrast, Cretnik et al.[31] observed similar dual-element isotope fractionation during TCE dehalogenation with commercial vitamin B_{12} (cyanocobalamin) compared to microbial dehalogenation and suggested similar reaction mechanisms in both systems. Thus far only one recent study was investigating dual-element isotope fractionation of PCE by a pure culture of *Desulfitobacterium* sp. strain Viet1.[22] However, the specificity of different enzymatic corrinoid cofactor and their effect on enzymatic reaction was not investigated.

In this study, for the first time, we report the dual element isotope analysis of carbon and chlorine of PCE and TCE during reductive dehalogenation by enzymes and corresponding

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corrinoids. We characterized the effect of different corrinoids (Figure 1) as well as the enzyme on isotope fractionation and the reaction. Therefore, microbial dehalogenation of PCE and TCE by *Sulfurospirillum multivorans* crude extracts, with PceA containing *norpseudo*-B_{12} or *nor*-B_{12} as corrinoid cofactors, was compared to abiotic dehalogenation by the respective purified corrinoids, as well as structurally different commercially available corrinoids.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased either from Merck (Darmstadt, Germany) or Sigma-Aldrich Chemie (Munich, Germany) in highest purity available. Gases were purchased either from Linde Gas AG (Pullach, Germany) or AirProducts (Hattingen, Germany).

Cultivation of Sulfurospirillum multivorans. *Sulfurospirillum multivorans* was cultivated in an anoxic mineral medium as previously described by Scholz-Muramatsu et al.[32] in the absence of vitamin B_{12} (cyanocobalamin) and yeast extract. Pyruvate (40 mM) was used as carbon source and electron donor. Fumarate (40 mM) and tetrachloroethene (10 mmol L^{-1}) dissolved in hexadecane were used as electron acceptors. The medium was inoculated with 10% of a fresh preculture and incubated at 28 °C, 120 rpm. When required, 5,6-dimethylbenzimidazole (DMB) was added to the culture medium from a sterile 1 mM stock solution in ultrapure water for cultivation of *Sulfurospirillum multivorans* containing *nor*-B_{12} as the corrinoid cofactor.

Purification and Analysis of Cobamides. The extraction, purification, and analysis of *norpseudo*vitamin B_{12} and *nor*vitamin B_{12} was done as described by Keller et al.[18]

Bacterial Crude Extract Preparation. The bacterial cells were harvested in the end of the log-phase. Bacterial crude extract was prepared as described by Nijenhuis et al.[26] under anoxic conditions by using a nitrogen stream or working in an anaerobic glovebox (Coy Lab Products, Grass Lake Charter Township, MI). For cell lysis 10 mg lysozyme and 1 mg DNase I was added and the mixture incubated for 10−15 min at room temperature. Subsequently, the cells were disrupted via French press (Thermo Scientific, Waltham, MA) at 20.000 psi. The produced crude extract was stored under anoxic conditions at −20 °C until further use.

Dehalogenation Experiments. *Enzymatic Reductive Dehalogenation Experiment.* The enzymatic dehalogenation reaction was conducted in 20 mL vials filled with 10 mL reaction buffer and sealed with Teflon coated rubber stopper. Methyl viologen was used as an artificial electron donor to the reaction buffer containing 100 mM Tris-HCl (pH 7.5), 0.2 mM $(NH_4)_2SO_4$ and 1.6 mM methyl viologen. As electron acceptor a final concentration of 500 μmol L^{-1} PCE or TCE in ethanol solution was used. Subsequently, the reaction buffer was reduced with titanium(III)citrate. The titanium(III)citrate solution was prepared by adding 5 mL 15% titanium(III)-chloride to 48 mL 0.2 M sodium citrate solution mixed with 6 mL saturated sodium carbonate solution.[33] The dehalogenation reaction was started by adding 25−50 μL of bacterial crude extracts to the reaction vial and was incubated at room temperature on a rotary shaker (160 rpm). The reaction was stopped at different reaction time points by the addition of a saturated Na_2SO_4 solution (adjusted to pH 1.0 with H_2SO_4). Concentrations of chlorinated ethenes were analyzed by taking 0.5 mL head space sample and the remaining sample volume was used for stable isotope analysis as described below. For the determination of isotope enrichment factors at least eight independent reaction samples taken at different time points were used.

Abiotic Reductive Dehalogenation Experiment. The abiotic dehalogenation reaction was conducted in 20 mL vials filled with 10 mL of reaction buffer (see above). As electron acceptor a final concentration of 500 μmol L^{-1} of PCE or 250 μmol L^{-1} of TCE were used. The reaction buffer was reduced with titanium(III)citrate to a redox potential of about −0.6 V. The respective corrinoid with a final concentration of 5 μM for PCE and 12.5 μM for TCE dehalogenation was added to start the reaction. Incubation was done at room temperature on a rotary shaker (160 rpm). The reaction was stopped at different time points and sacrificed for analysis by adding 1 mL saturated Na_2SO_4 solution (adjusted to pH 1.0 with H_2SO_4). Concentrations of chlorinated ethenes were analyzed by taking 0.5 mL head space sample as described below. The remaining sample volume was used for stable isotope analysis. For the determination of the isotope enrichment factor at least eight independent reaction samples taken at different time points were used.

Analytical Methods. *Chlorinated Ethene Concentration.* Analysis of chlorinated ethenes was performed as described elsewhere[26] and can additionally be found in the Supporting Information (SI).

Stable Carbon Isotope Analysis. Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) was applied to determine the carbon isotope composition of the chlorinated ethenes (Thermo GC Trace 1320 in combination with Thermo-Finnigan MAT 253 IRMS; Bremen, Germany). All samples were measured at least in triplicate and the standard deviation was generally below 0.5‰. Samples were injected automatically using 200 μL head space using an autosampler (Thermo TriPlus RSH Autosampler). All injections were done in split mode 1:5−1:10 using a split/splitless injector at 250 °C. For chromatographic separation a Zebron ZB-1 capillary column (60 m × 0.32 mm, 0.5 μm film; Phenomenex Inc., Torrance, CA) was used.

Stable Chlorine Isotope Analysis. Chlorine isotope analysis of TCE was performed according to a method adapted from Shouakar-Stash et al.[34] as described in detail in Cretnik et al.[31] In this new approach for GC/IRMS the TCE is directly transferred in the gas phase to the IRMS through the He carrier stream, where TCE is ionized and fragmented for isotope ratio measurements. TCE-EIL-1 (δ^{37}Cl = +3.05‰) and TCE-EIL-2 (δ^{37}Cl = −2.70‰) as well as PCE-EIL-1 (δ^{37}Cl = +0.29‰) and PCE-EIL-2 (δ^{37}Cl = −2.52‰), were used as external compound-specific isotope standards (determined according to Holt et al.[35] in the stable isotope laboratory of the University of Waterloo). The measurements were conducted on masses m/z = 95, 97, and 97 and m/z = 94, 96 for TCE and PCE, respectively. A GC-IRMS system (Thermo Scientific) consisting of a Trace GC connected via a heated transfer line to a MAT 253 IRMS with dual inlet system was used to carry out the CSIA measurements. The gas chromatograph was equipped with a 30 m VOCOL column (Supelco) with 0.25 mm inner diameter, a film thickness of 1.5 μm and operated with a He carrier gas at 1.4 mL/min. The GC program used started at 50 °C (7 min), increasing at 60 °C/min to 70 °C (2.70 min) and at 80 °C/min to 140 °C (0.10 min).

Calculation and Definitions. *Carbon and Chlorine Isotope Analysis.* The carbon isotope composition is reported in the δ-notation (‰) relative to the Vienna Pee Dee

Table 1. Enrichment Factors for Abiotic and Enzymatic Reductive Dehalogenation[a]

		PCE											
		carbon				chlorine				dual-element			
	S. multivorans	εC [‰]	CI (95%)	R^2	n	εCl [‰]	CI (95%)	R^2	n	εC/εCl	CI (95%)	R^2	n
enzymatic	norpseudo-B_{12} type	−1.4	±0.1	0.986	10	−0.6	±0.2	0.934	8	2.2	±0.7	0.902	8
	nor-B_{12} type	−1.3	±0.05	0.998	10	−0.4	±0.1	0.976	8	2.8	±0.5	0.971	8
	corrinoid type												
abiotic	norpseudo-B_{12}	−25.3	±0.8	0.998	11	−3.6	±0.4	0.986	9	6.9	±0.7	0.986	9
	nor-B_{12}	−23.7	±1.2	0.995	11	−4.8	±0.9	0.971	7	5.0	±0.8	0.98	7
	cyano-B_{12}	−22.4	±0.8	0.998	11	−4.8	±0.2	0.997	9	4.6	±0.2	0.998	9
	dicyanocobinamid	−25.2	±0.5	0.999	11	−3.4	±0.4	0.978	11	7.0	±0.8	0.975	11

		TCE											
		carbon				chlorine				dual-element			
	S. multivorans	εC [‰]	CI (95%)	R^2	n	εCl [‰]	CI (95%)	R^2	n	εC/εCl	CI (95%)	R^2	n
enzymatic	norpseudo-B_{12} type	−20.0	±0.5	0.999	9	−3.7	±0.2	0.997	8	5.3	±0.3	0.998	8
	nor-B_{12} type	−20.2	±1.1	0.998	7	−3.9	±0.6	0.99	6	5.0	±0.8	0.987	6
	corrinoid type												
abiotic	norpseudo-B_{12}	−18.5	±2.8	0.961	11	−4.2	±0.8	0.962	8	4.5	±0.8	0.972	8
	nor-B_{12}	−15.1	±2.7	0.955	10	−3.9	±1.0	0.930	9	3.7	±0.3	0.989	9
	cyano-B_{12}	−15.0	±2.0	0.979	10	−3.2	±1.0	0.892	9	4.4	±0.7	0.969	9
	dicyanocobinamid	−16.5	±0.7	0.997	10	−3.9	±0.5	0.981	8	4.2	±0.6	0.979	8

[a]Enzymatic isotope fractionation was measured with crude extracts of PceA from *Sulfurospirillum multivorans* harboring different cofactors; PceA-*norpseudo*-B_{12} or PceA-*nor*-B_{12}. In abiotic experiments the stable isotope fractionation of purified *norpseudo*-B_{12} and *nor*-B_{12} were measured, as well as cyanocobalamin and dicyanocobinamide. PCE and TCE were used as terminal electron acceptors. n = number of replicates.

Belemnite standard.[36] For chlorine isotope analysis, one of the bellows of the dual inlet system on the IRMS was utilized to introduce a reference gas of PCE for the PCE measurements and a TCE reference gas for the TCE measurements. The reference gas was introduced at the beginning and at the end of each measurement. The conversion to delta values relative to the international reference Standard Mean Ocean Chloride (SMOC) was performed by an external two-point calibration as described by Bernstein et al.[37] and Cretnik et al.[31] Each of these standards was added in triplicate before, during and at the end of each sequence, in order to calibrate the obtained values of the samples with respect to SMOC.

Evaluation of Enrichment Factors and the Dual-Element Stable Isotope Slope. The isotope fractionation during dehalogenation reaction was calculated by application of the Rayleigh-equation (eq 1) with R_0 and R_t as isotope values, and C_0 and C_t as concentrations at time 0 and time t, respectively.[38]

$$\ln(R_t/R_0) = \varepsilon \times \ln(C_t/C_0) = \varepsilon \times \ln(C_t/C_{total}) \quad (1)$$

In alternative to C_0, the total chloroethene concentration C_{total} in each sample was calculated from the mass balance to correct for losses or variations due to e.g. sampling, as well as the variability of amounts added per individual vial (eq 2). Determination of isotope enrichment factors via mass balance was observed to improve the precision of the results without affecting the magnitude of the obtained enrichment factor (SI Table S2).

$$C_0 = C_{total} = C_{PCE} + C_{TCE} + C_{DCE} + C_{VC} + C_{ethene} \quad (2)$$

The corresponding dual-element stable isotope slope was determined by plotting δ^{13}C versus δ^{37}Cl data and presented as slope of $\Delta\delta^{13}C/\Delta\delta^{37}Cl \approx \varepsilon_C/\varepsilon_{Cl}$. The slope and the 95% confidence intervals were determined via linear regression (not forced through zero) using Excel Analysis Toolpak (Microsoft). The statistical significance of the linear relationships was tested using t test statistics. The linear models were considered to significantly fit the data; with overall $P < 0.0003$ (SI).

RESULTS AND DISCUSSION

Dehalogenation Reaction. *Enzymatic Dehalogenation Reaction.* Crude extracts were used to investigate reductive dehalogenation with PceA dehalogenase enzymes from *Sulfurospirillum multivorans*. PceA dehalogenases harbored either *norpseudo*-B_{12} (PceA-*norpseudo*-B_{12}) as cofactor—when *Sulfurospirillum multivorans* had been cultivated in the absence - or *nor*-B_{12} (PceA-*nor*-B_{12})—when *Sulfurospirillum multivorans* had been cultivated in its presence of 4,6-dimethylbenzimidazole (DMB). In both cases PCE or TCE were dehalogenated to *cis*-DCE as final product. Dehalogenation rates with PceA-*norpseudo*-B_{12} reductive dehalogenase were faster than with PceA-*nor*-B_{12}, in agreement with observations by Keller et al.[18]

Abiotic Dehalogenation. Abiotic dehalogenation experiments were conducted using purified *norpseudo*vitamin B_{12} and *nor*vitamin B_{12},[18] as well as a range of commercially available corrinoids (cyanocobalamin, CNCbl; adenosylcobalamin, AdoCbl; methylcobalamin, MeCbl; Hydroxocobalamine; OHCbl; dicyanocobinamide, CNCbi). Dechlorination rates were different for various corrinoid types, and generally slower compared to enzymatic rates, in agreement with previous observations by Neumann et al.[17] In particular, *norpseudo*vitamin B_{12} and dicyanocobinamide gave rise to higher dehalogenation rates in comparison to the other corrinoids (results not shown) in agreement with the trend observed with PceA enzymes (see above). Interestingly, lowering the redox potential down to $E_h \sim -0.6$ V accelerated all reaction rates to a similar extent irrespective of the corrinoid type. Rapid PCE dehalogenation (pseudo first order reaction rate = 0.30 ± 0.06 min^{-1} determined according to Glod et al.[20]) and accumulation

of TCE as intermediate was observed. The dehalogenation rate of TCE was 1 order of magnitude lower (first order reaction rate = 0.029 ± 0.004 min^{-1}). Here formation of *cis*-DCE and traces of *trans*-DCE, and VC, as well as ethene were detected, while, in contrast to earlier studies,[20,21,39] significant formation of acetylene was not observed. According to the mass balance no losses occurred during dehalogenation of PCE to TCE. By compiling the mass balance for TCE dehalogenation, however, an overall loss of chloroethenes by up to 30−40% was observed at the end of the experiment, as it was also mentioned in earlier studies.[20,39] Since leakage and adsorption could be excluded and no other intermediates could be found in the head space, missing products are expected to be nonvolatiles trapped as dichlorovinyl-cobalamin complexes, as previously observed by Lesage et al.[40] and proposed by Kliegman and McNeill[21] for TCE dehalogenation.

Stable Isotope Analysis of Carbon and Chlorine. Highly variable single-element isotope fractionation was measured in all experiments for carbon, ranging from enrichment factors of −1.3‰ to −25.3‰, in agreement with previous isotope studies.[26,41] Isotope fractionation of chlorine was smaller, but similarly variable with values from −0.4 ‰ to −4.8 ‰ (Table 1 and SI S1 to S4). Highest fractionation for both elements was observed for corrinoid mediated dehalogenation, while the lowest fractionation was related to dechlorination by crude extracts of *S. multivorans*.

Isotope Fractionation of PCE during Enzymatic and Abiotic Dehalogenation. Enzymatic dehalogenation of PCE with PceA-*norpseudo*-B_{12} enzyme resulted in a dual-element isotope slope of 2.2 ± 0.7 (ε_C = −1.4 ± 0.1‰ and ε_{Cl} = −0.6 ± 0.2‰). For the alternative PceA-*nor*-B_{12} a dual-element isotope slope of 2.8 ± 0.5 (ε_C = −1.3 ± 0.1‰, and ε_{Cl} = −0.4 ± 0.1‰) was determined. Even though different corrinoid cofactors were harbored in both PceA enzymes, enzymatic carbon and chlorine isotope enrichment factors were indistinguishable.

In contrast, abiotic dehalogenation of PCE with the corresponding purified corrinoids resulted in much stronger single-element isotope fractionation as well as distinct dual-element isotope slopes compared to the enzymatic dehalogenation. For *norpseudo*vitamin B_{12} a dual-element isotope slope of 6.9 ± 0.7 (ε_C = −25.3 ± 0.8‰ and ε_{Cl} = −3.6 ± 0.4‰) and for *nor*vitamin B_{12} of 5.0 ± 0.8 (ε_C = −23.7 ± 1.2‰ and ε_{Cl} =-4.8 ± 0.9‰) was measured. Surprisingly, within this data set dual-element slopes of *norpseudo*vitamin B_{12} and *nor*vitamin B_{12} were therefore significantly different.

In addition to the experiments with extracted corrinoids, this difference was further investigated in experiments with commercially available cobalamins (CNCbl, AdoCbl, MeCbl, and OHCbl) and dicyanocobinamide (CNCbi). Consistent single-element isotope enrichment factors for carbon in PCE were measured for all commercial cobalamins, ranging from −22.4‰ to −23.2‰ (SI Table S1). Thus, only cyanocobalamin was utilized for additional dual-element analysis representing cobalamins. Here the dual-element analysis of cyanocobalamin resulted in a slope of 4.6 ± 0.2 (ε_C = −22.4 ± 0.8‰ and ε_{Cl} = −4.8 ± 0.2‰), similar to extracted *nor*vitamin B_{12}. In contrast, for dicyanocobinamide the observed dual-element isotope fractionation was different with a significantly higher slope of 7.0 ± 0.8 (ε_C = −25.2‰ ± 0.5‰ and ε_{Cl} = −3.4‰ ± 0.4‰), similar to extracted *norpseudo*vitamin B_{12}. Therefore, the significant difference in dual element isotope slopes between *nor*vitamin B_{12} and *norpseudo*vitamin B_{12} could also be reproduced in chemical reactions with commercially available cobalamins. A summary of all results is provided in Table 1 and Figure 1.

In conclusion, when comparing abiotic to enzymatic isotope effects during PCE dehalogenation two trends emerge. On the one hand, single-element isotopic enrichment factors were much larger in the abiotic reaction compared to enzyme catalysis. On the other hand also the dual-element isotope slope was significantly different compared to that obtained with PceA enzymes. For pure corrinoids, in addition a significant difference in dual element isotope slopes could be distinguished between DMB containing (*nor*vitamin B_{12}) and non-DMB containing structures (*norpseudo*vitamin B_{12}). These differences were not detected anymore when the corresponding corrinoids were incorporated in the enzyme. A tentative explanation for this observation is provided below.

Isotope Fractionation of TCE during Enzymatic and Abiotic Dehalogenation. Single-element isotope effects of both carbon and chlorine were stronger for TCE in comparison to PCE for enzymatic dehalogenation, similar to previous studies for only carbon.[26,41] When comparing isotope effects in TCE dehalogenation with enzymes and corrinoids, however, all values were all in a similar range; the same could be observed for chlorine isotope values. The pronounced difference between enzymes and corrinoids that could be observed for isotope values of PCE (both with respect to their magnitude, as well as dual element-isotope slopes) was therefore not observed for TCE. Specifically, enzymatic dehalogenation of TCE resulted in similar dual-element isotope slopes of 5.3 ± 0.3 (ε_C = −20.0 ± 0.5‰, ε_{Cl} = −3.7 ± 0.2‰) for PceA-*norpseudo*-B_{12} and 5.0 ± 0.8 (ε_C = −20.2 ± 1.1‰, ε_{Cl} = −3.9 ± 0.6‰) for PceA-*nor*-B_{12}. For abiotic dehalogenation with corresponding purified corrinoids isotope fractionation was not significantly different. Here the dual-element slopes were 4.5 ± 0.8 (ε_C = −18.5 ± 2.8‰ and ε_{Cl} = −4.2 ± 0.8‰) for *norpseudo*vitamin B_{12} and 3.7 ± 0.3 (ε_C = −15.0 ± 2.7‰ and ε_{Cl} = −3.9 ± 0.8‰) for *nor*vitamin B_{12} (SI Figure S4). Also commercially available cyanocobalamin (CNCbl) and dicyanocobinamide (CNCbi) gave dual-element slopes of 4.4 ± 0.7 (ε_C = −15.0 ± 2.0‰ and ε_{Cl} = −3.2 ± 1.0‰) for CNCbl and 4.2 ± 0.6 (ε_C = −16.5‰ ± 0.7‰ and ε_{Cl} = −3.9‰ ± 0.5‰) for CNCbi, which were not significantly different. A summary of all results is provided in Table 1 and Figure 2.

In conclusion, isotope effects in TCE dehalogenation reaction were remarkably consistent between different corrinoids and PceA enzymes. While this is consistent with previous findings of Cretnik et al.,[31] the observation is in stark contrast with the trend observed in PCE dehalogenation.

Dehalogenation Reaction Mediated by Different Corrinoids. In the case of TCE, the dual-element isotope slopes observed in this study suggest that reductive dehalogenation proceeded via a similar way with all enzymes and corrinoids investigated. In the case of PCE, in contrast, dual-element isotope slopes differed between corrinoids harboring DMB as lower ligand base (cyanocobalamin and *nor*vitamin B_{12}) on the one hand and non-DMB corrinoids (*norpseudo*vitamin B_{12} and dicyanocobinamide) on the other hand (Table 1, Figures 2 and 3). These differences are interesting in the context of the observed variability in abiotic reaction rates at more oxidized redox potentials. This raises the question for underlying reasons.

Different dual-element isotope slopes—when observed in elementary chemical reactions—reflect differences in reaction

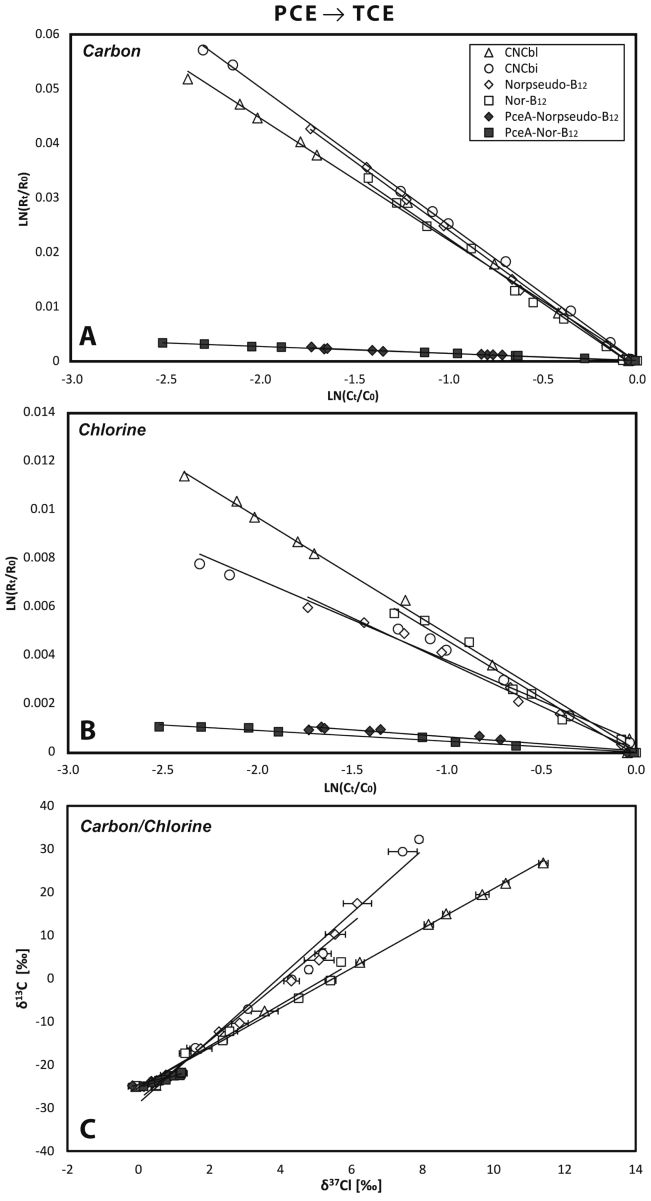

Figure 2. Rayleigh plots for carbon (A), chlorine (B) and the dual-element isotope plot (C) ($\delta^{13}C$ versus $\delta^{37}Cl$) for reductive dehalogenation of PCE. Dehalogenation was performed using pure corrinoids (CNCbl, CNCbi, norpseudo-B12 and nor-B12), as well as crude extract harboring PceA enzymes of *S. multivorans* equipped with different corrinoid cofactors.

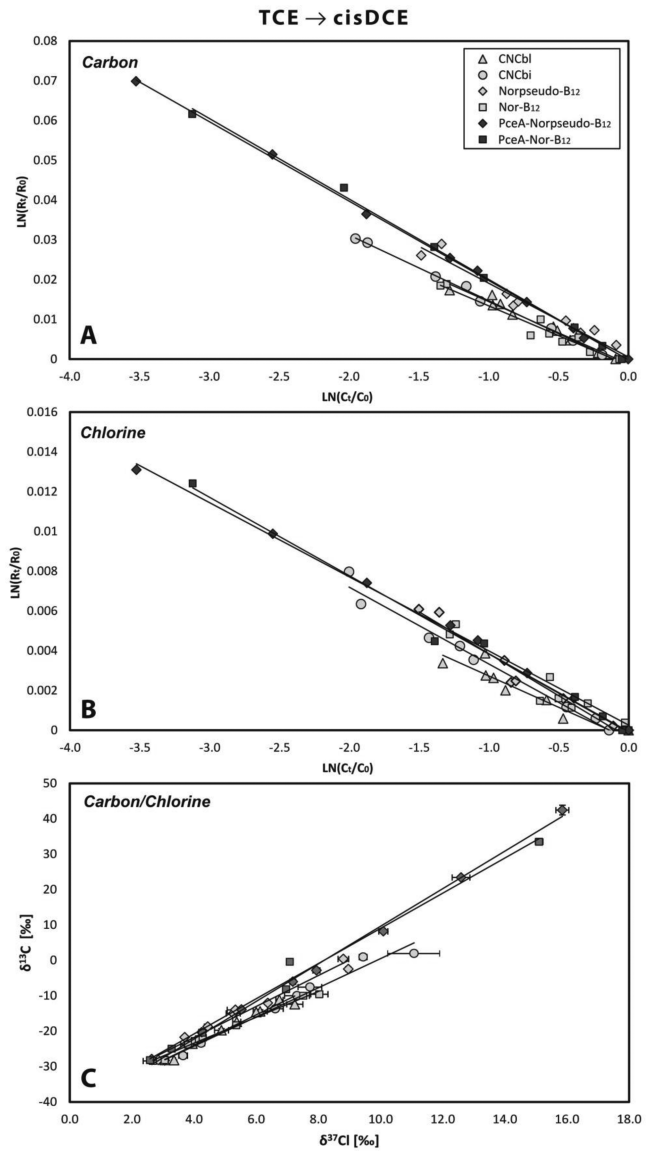

Figure 3. Rayleigh plots for carbon (A), chlorine (B) and the dual-element isotope plot (C) ($\delta^{13}C$ versus $\delta^{37}Cl$) for reductive dehalogenation of TCE. Dehalogenation was performed using pure corrinoid cofactors (CNCbl, CNCbi, norpseudo-B_{12} and nor-B_{12}), as well as crude extract harboring PceA enzymes of *S. multivorans* equipped with different corrinoid cofactors.

mechanisms (i.e., in the order and manner of bond cleavage within a molecule).[42] If the observed differences in dual element slopes (Figure 2C) were indeed caused by *mechanistic* differences, the putative alternative reductive dehalogenation scenarios "nucleophilic addition", "nucleophilic substitution", or "electron transfer" could be invoked (or a simultaneous combination of them). However, it is not clear if only one mechanism is involved or a combination of more than one. Considering the wide range of byproducts after abiotic dehalogenation of TCE simultaneous occurrence of different reaction mechanisms cannot be excluded. Alternatively, as shown in Penning et al.[43] different dual-element isotope plots may be caused by the same mechanism (i.e., the same manner of bond changes), but by different *kinetics* (i.e., bonds are cleaved in different steps of which the rates change relative to each other). If the observed differences in Figure 2C were caused by such *kinetic* differences, it is interesting to consider that upon reduction, the corrinoid may be transformed into its *base-off* conformation. The rate of the intramolecular ligand dissociation was shown to vary among structurally different corrinoids.[44,45] This step may affect also isotope effects in PCE if the substrate is associated in some form with the corrinoid when base dissociation occurs. In more general terms, structural differences in the lower ligand may affect a rate-determining step prior to the bond cleavage and might cause the observed differences in dual-element stable isotope analysis for different corrinoid types. So far, however, it is not possible to disentangle the different isotope effects.

Effect of the PceA Enzyme. Despite the different corrinoid cofactors, enzymatic isotope fractionation of PceA-*norpseudo*-B_{12} and PceA-*nor*-$B_{12}$was not affected for TCE and

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PCE, respectively. Consequently, compound-specific stable isotope analysis indicated that corrinoid cofactors are most probably not responsible for previously observed variabilities in microbial isotope fractionation of carbon. With TCE, the dehalogenation reaction with both enzyme types, as well as all corrinoids was observed to be in a similar range with $\varepsilon_C/\varepsilon_{Cl}$ from 3.7 to 5.3 (Table 1 and Figure 3). This correlates with recently published observations of Cretnik et al.,[31] with dual-element isotope slopes for *Geobacter lovleyi*, *Desulfitobacterium hafniense* of $\varepsilon_C/\varepsilon_{Cl}$ 3.4 to 3.8. Furthermore, Kuder et al.[29] and Wiegert et al.[30] reported for TCE dehalogenation dual-element isotope slopes of 4.8 (published as $\underline{\varepsilon_{Cl}}/\underline{\varepsilon_C}$ = 0.21 ± 0.2) for a *Dehalococcoides* mixed culture and 2.7 (published as $\underline{\varepsilon_{Cl}}/\underline{\varepsilon_C}$ = 0.35 ± 0.11) for a *Desulfitobacterium* mixed culture.

In contrast, in the case of PCE dual-element isotope fractionation clearly varied between enzymatic ($\varepsilon_C/\varepsilon_{Cl}$ of 2.2−2.8) and abiotic ($\varepsilon_C/\varepsilon_{Cl}$ of 4.6−7.0) dehalogenation in this study (Table 1 and Figure 2C). Likewise single-element isotope enrichment factors for PCE were also highly variable for enzymatic (ε_C −1.3 to −1.4 ‰ and ε_{Cl} −0.4 to −0.6‰) and abiotic (ε_C −22.4 to −25.3‰ and ε_{Cl} −3.4 to −4.8 ‰). Consequently, similar as for the reaction with the corrinoid types, it should be considered if dual-element analysis reflects differences in the reaction mechanism or if differences in dual-element isotope slopes are caused by the kinetics of subsequent reaction steps.

A priori it cannot be excluded that different (bio)chemical reaction mechanisms may be responsible for the observed differences in dual-element isotope fractionation, similar to recent reports of Kummel et al.,[46] where different subtypes of benzylsuccinate synthase were observed to produce distinct dual-element (C and H) patterns. However, the small isotope enrichment factors of PCE observed for both carbon and chlorine with PceA in this study (Table 1, Figure 2A+B) make it unlikely that any significant changes in C−Cl bond chemistry were involved in the rate-determining step. This indicates that the dual-element isotope slope did *not* reflect a chemical bond conversion, but instead a preceding step prior to the C−Cl cleavage. This step would on the one hand become rate-determining and, therefore, mask the isotope effects of the C−Cl bond cleavage. On the other hand, if this step has a small, but non-negligible isotope effect itself (e.g., during enzyme−substrate association), which may in addition give rise to a distinctly different dual-element isotope slope[47] such as observed in Figure 2C.

In this study PCE dehalogenation rates were 1 order of magnitude higher for abiotic reaction with pure corrinoids compared to TCE, but not for enzymes, were PCE and TCE dehalogenation rates were reported to be similar.[7] Therefore, it may be hypothesized that the actual *chemical* reaction at the corrinoid cofactor within the enzyme would proceed similarly 10-fold faster for PCE compared to TCE, but that this difference is not observed in the *enzymatic* reaction, because preceding steps may significantly reduce the overall rate. This would affect PCE dehalogenation more strongly than TCE dehalogenation, because of the faster intrinsic rate for PCE. Consequently, dual-element analysis of PCE would reflect a preceding rate-limiting step (e.g., enzyme−substrate association), whereas in the case of TCE it would still reflect the underlying enzymatic reaction. In such a case PCE data would not be suited to elucidate the underlying reaction chemistry of C−Cl bond cleavage, but would instead give insight into the kinetics of enzyme catalysis. So far Wiegert et al.[30] reported a similar dual-element isotope slope $\varepsilon_C/\varepsilon_{Cl}$ of 2.9 (published as $\underline{\varepsilon_{Cl}}/\underline{\varepsilon_C}$ = 0.37 ± 0.11) for the microbial dehalogenation of PCE using an enrichment culture. Furthermore, recent studies of Cretnik et al.[22] and Badin et al.[48] presented the first dual-element isotope analysis for microbial dehalogenation of PCE with *Desulfitobacterium* sp. strain Viet1 as pure culture ($\varepsilon_{Cl}/\varepsilon_C$ = 3.8 ± 0.2) and two bacterial enrichment cultures containing *Sulfurospirillum* spp. ($\varepsilon_{Cl}/\varepsilon_C$ = 2.7 ± 0.2 and 0.7 ± 0.2). Here also, different dual-element isotope slopes for a similar reaction were presented. Our study provides a first tentative explanation for this trend, which would need to be substantiated in further studies.

Environmental Significance. CSIA of carbon has proven a useful method for detecting in situ biotransformation of chlorinated ethenes. However, the elucidation of transformation mechanisms, as well as the quantification of degradation, is hampered by a remarkable variability in carbon stable isotope fractionation.[26−28,41] Therefore, dual-element stable isotope analysis provides an elegant way for characterization of reaction mechanisms and to improve the quantification using CSIA. Since a dual-element isotope analysis is less affected by masking of isotope effects or methodological differences, a more reliable evaluation and characterization of reaction mechanisms is possible. According to that, better evaluation and elucidation of degradation pathways may be achieved in lab and field studies, as already shown for hydrogen and carbon.[24,49]

In this study the analysis of carbon and chlorine isotopes fractionation during reductive dehalogenation revealed similar reaction for TCE dehalogenation by pure corrinoids, as well as PceA reductive dehalogenase of *Sulfurospirillum multivorans*. The dual-element slopes were observed to be remarkably robust and in similar range as reported recently by Cretnik et al.[31] Interestingly, dual-element isotope fractionation for PCE dehalogenation reaction showed differences between enzymes and corrinoids, and even between different corrinoid structures, contrary to the findings by Cretnik et al.[22] Our results suggest that during enzymatic dehalogenation of PCE isotope fractionation does not reflect the *chemical* reaction mechanism, but in addition preceding rate-limiting steps during enzyme−substrate association or enzymatic structure. Though these effects cannot be resolved so far, they may be used for characterization of different corrinoid structures or enzyme classes. In case that corrinoid or enzyme structures have a significant effect on preceding rate-limiting steps, the overlapping isotope effects may help to distinguish corrinoids or enzymes. Therefore, CSIA may allow identification of the prevailing microbial community responsible for biodegradation. In conclusion, future studies should attempt to reveal when microbial dual-element isotope analysis reflect a certain reaction mechanism and when differences in isotope fractionation result from isotope masking caused by interactions of enzyme, microbial membranes, or properties for the degraded compound as suggested by Nijenhuis et al.[26] Nonetheless, further studies have to be done in order to evaluate the scope of the dual-element approach for characterization of microbial reaction mechanisms as we are only starting to obtain an understanding.

■ ASSOCIATED CONTENT

Supporting Information

Isotope fractionation plots for carbon and chlorine and isotope enrichment factors of adenosylcobalamin, methylcobalamin and

hydroxocobalamin are presented. Concentration measurements via GC-FID, calculation of "isotope balance", and statistical data for regression of the dual-element isotope analysis. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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Publication II

analytical chemistry

Article

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Development and Validation of an Universal Interface for Compound-Specific Stable Isotope Analysis of Chlorine ($^{37}Cl/^{35}Cl$) by GC-High-Temperature Conversion (HTC)-MS/IRMS

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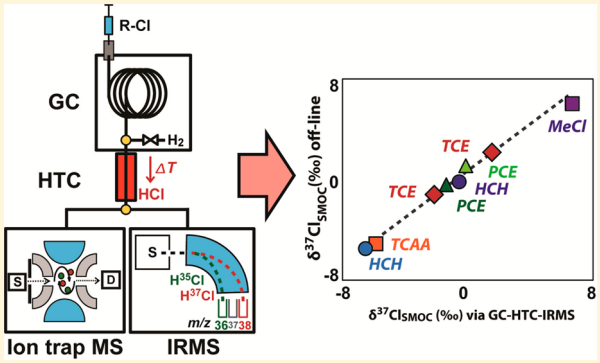
Supporting Information

ABSTRACT: A universal application of compound-specific isotope analysis of chlorine was thus far limited by the availability of suitable analysis techniques. In this study, gas chromatography in combination with a high-temperature conversion interface (GC-HTC), converting organic chlorine in the presence of H_2 to gaseous HCl, was coupled to a dual-detection system, combining an ion trap mass spectrometer (MS) and isotope-ratio mass spectrometer (IRMS). The combination of the MS/IRMS detection enabled a detailed characterization, optimization, and online monitoring of the high-temperature conversion process via ion trap MS as well as a simultaneous chlorine isotope analysis by the IRMS. Using GC-HTC-MS/IRMS, chlorine isotope analysis at optimized conversion conditions resulted in very accurate isotope values ($\delta^{37}Cl_{SMOC}$) for measured reference material with known isotope composition, including chlorinated ethylene, chloromethane, hexachlorocyclohexane, and trichloroacetic acids methyl ester. Respective detection limits were determined to be <15 nmol Cl on column with achieved precision of <0.3‰.



Identification and quantification of environmental pollutants is a field of major importance in modern science and analytical chemistry.[1−4] In addition to the identity, valuable information about source and fate of a chemical compound can be obtained from its isotopic composition. Although essential for source identification, assessment of biodegradation and characterization of (bio)catalytic reaction mechanisms for numerous chlorinated compounds, as for instance environmental pollutants, compound-specific stable isotope analysis of chlorine was thus far partly limited by the difficulty of conversion to a simple chlorine-containing gas for isotope measurements by continuous-flow isotope ratio mass spectrometry (CF-IRMS).[5−7] Currently, chlorine isotope analysis is mainly restricted to conventional off-line methods, including time-consuming conversion to either chloromethane[8,9] or cesium chloride[10] in combination with measurements via dual inlet isotope ratio mass spectrometry (DI-IRMS) or thermal ionization mass spectrometry (TIMS). Those off-line techniques, however, allow the determination of chlorine isotope composition of preoperatively isolated fractions or pure compounds only. In addition, online methods are available which do not require previous conversion of organochlorines[11] but involve the direct measurement of molecular and fragment ions via quadrupole MS (qMS)[12,13] or fragment ions via IRMS.[14] Though GC-qMS was shown to be highly sensitive (detection limit <10 pmol) and more flexible in terms of accessible compound classes, only moderate precision could be achieved (1σ > 0.5‰)[3,5,13] In contrast, GC-IRMS is less sensitive (detection limit ∼10 nmol on column), but able to achieve better precision (1σ = 0.1‰).[14] However, because of specific cup configurations required, GC-IRMS is limited to a narrow range of compound for analysis. In addition, both methods are restricted in availability of reference materials, since at least two isotope standards are required for calibration, chemically identical to the analyzed compound.[5] During the past decade, application of inductively coupled plasma in combination with multicollector mass spectrometry (MC-ICPMS) coupled to a gas chromatography for chlorine isotope measurements was demonstrated in several studies.[1,15,16] Though application of MC-ICPMS is a promising universal method with a precision of $1\sigma \approx 0.1-0.2$‰, low chlorine ionization efficiency, interference with $^{36}Ar^{1}H$ ions, and high instrumental costs limit the use in routine application. Moreover, combination of GC for separation with MC-ICPMS requires large sample size for analysis and, thus, may restrict the analysis of environmental samples.

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Hitzfeld et al.[17] introduced a novel approach for online chlorine isotope analysis via high-temperature conversion (HTC) of chlorinated compounds into gaseous hydrochloric acid and the analysis of $H^{37}Cl/H^{35}Cl$ ions by quadrupole mass spectrometry (GC-HTC-qMS). The fundamental principles of this conversion technique were first demonstrated by Hoering and Parker[18] but were not applied for online chlorine analysis before. Briefly, chlorinated hydrocarbons undergo a chromatographic separation, followed by a subsequent high-temperature conversion at 1300 °C to hydrochloric acid (HCl). Quantitative conversion to HCl was achieved by providing additional hydrogen as reactant gas during conversion. The conversion products $H^{35}Cl$ and $H^{37}Cl$ (m/z 36 and 38) were detected via qMS (gas analyzer).[17] The presented setup, however, revealed instrumental instability ($1\sigma \leq 1.4‰$ (n = 3), delta shifts of 3.0−4.0‰ over 3 days) and referencing against the international standard (SMOC, Standard Mean Ocean Chloride) was not established. Even so, chlorine isotope analysis via HTC holds a great promise because of the universal applicability for a wide range of chlorinated compounds as well as the cost efficient incorporation into standard instrumentation for compound-specific stable isotope analysis.

The objective of this study was a detailed investigation and characterization of the HTC process using nonchlorinated and chlorinated hydrocarbons as well as isotope analysis of chlorinated hydrocarbons via conversion of the measured compounds to HCl. Therefore, the instrumental setup previously presented by Hitzfeld et al.[17] was equipped with a dual-detection system combining ion trap mass spectrometry (MS) and isotope ratio mass spectrometry (IRMS) in order to analyze HTC products and chlorine isotope composition simultaneously. Thus, we were able to identify limitations of HTC and to improve the conversion procedure. Furthermore, we were able to demonstrate the precision that can be achieved by online chlorine isotope analysis of various chlorinated compounds via high-temperature conversion and detection of $H^{35}Cl$ and $H^{37}Cl$ by isotope ratio mass spectrometry.

EXPERIMENTAL SECTION

Chemicals. All solvents were purchased from Merck (Darmstadt, Germany) in the highest purity available. Gases were purchased either from Linde Gas AG (Pullach, Germany) or AirProducts (Hattingen, Germany).

Reference Material for Chlorine Isotope Analysis. Tetrachloroethene (PCE) and trichloroethene (TCE) were purchased from Merck, Germany (PCE reference 1 and TCE reference 2) and from PPG, USA (PCE reference 5 and TCE reference 6). Methyl chloride reference was obtained from Linde Gas AG, Germany. Hexachlorocyclohexane (Lindane) was obtained from HiMedia, India (HCH reference 1) and Greyhound, Global (HCH reference 2). Trichloroacetic acid was obtained from Sigma-Aldrich and derivatized as described elsewhere.[19]

Determination of $\delta^{37}Cl_{SMOC}$ of Reference Material. The $\delta^{37}Cl_{SMOC}$ values of reference standards were determined by off-line analysis via dual inlet isotope ratio mass spectrometry (DI-IRMS). Prior to analysis all reference compounds were converted to methyl chloride in order to obtain accurate $\delta^{37}Cl$ values.[9,20−22] Isotope composition determined by dual-inlet mass spectrometry (DI-IRMS) was directly related to the international SMOC scale (Standard Mean Ocean Chloride; $\delta^{37}Cl$ = 0.0‰, R_{SMOC} = 0.319 644). The determination of $\delta^{37}Cl_{SMOC}$ was done in a triple collector gas-source dual-inlet mass spectrometer (DI-IRMS VG Optima, Isoprime Inc., U.K.) as described elsewhere.[23,24] Determined chlorine isotope composition of all utilized reference material is presented in Table 1.

Instrumental Setup of GC-HTC-MS/IRMS. The previous GC-HTC-qMS instrumental setup presented by Hitzfeld et al.[17] was modified to GC-HTC-MS/IRMS (Figure 1). The

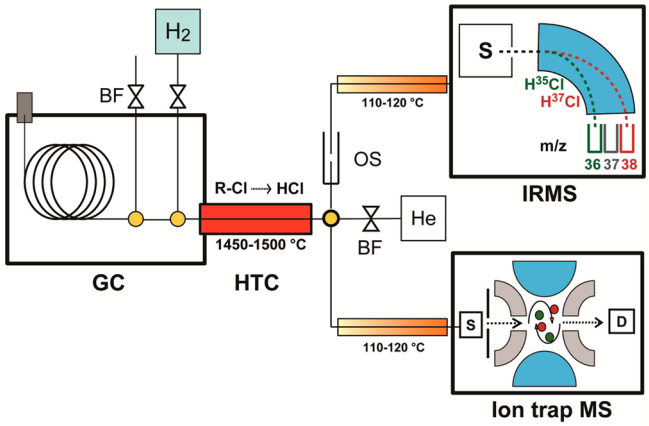

Figure 1. Schematic setup of the GC-HTC-MS/IRMS device, equipped with a gas chromatograph (GC), high-temperature conversion reactor (HTC), ion trap mass spectrometer (MS), and a multi collector isotope-ratio mass spectrometer (IRMS). OS: open split; BF: back flush.

device was equipped with an autosampler (A200S, CTC Analytics AG, Switzerland). The conversion unit consists of a standard gas chromatograph (HP6890N, Agilent Technologies, Germany) and a high-temperature conversion (HTC) tubular reactor (320 mm, 0.8 mm i.d., 1.6 mm o.d.; Degussit AL23 aluminum oxide ceramic, Friatec, Germany) mounted to a high-temperature combustion oven (ThermoFinnigan, Germany). The originally installed qMS (gas analyzer) was replaced by two simultaneously operating mass spectrometers: the ion trap mass spectrometer (PolarisQ, ThermoFinnigan, Germany) and isotope ratio mass spectrometer (DELTAplus XP, ThermoFinnigan, Germany). All transfer-lines were heated (110−120 °C) to avoid condensation of H_2O and HCl by regulated heating tubing (HORST GmbH, Germany).

Characterization of Conversion via MS. The high-temperature conversion was characterized and optimized via ion trap mass spectrometry. The ion trap MS was controlled by Xcalibur 1.4 software (Thermo Electron) and was able to operate within the mass range of 10−600 amu. All analytes were ionized via positive electron impact ionization (EI+ at 70 eV) at a source temperature of 225 °C and a vacuum pressure of 10^{-5} bar.

Ethylene and benzene were used as nonchlorinated model compounds for characterization of the HTC process and in particular the byproduct formation. Samples were injected manually via head space as pure compound in helium atmosphere using a split ratio of 1:10 to 1:40. The GC was equipped with a Zebron ZB-1 column (60 m, 0.32 mm i.d., 1 μm film thickness; Phenomenex Inc.) running at isothermal conditions.

For HTC characterization with ethylene as the model compound, standard conditions were applied: 27 nmol of ethylene on column, 0.8 mL/min flow rate, 0.2 mL/min hydrogen flow rate, and 1300 °C conversion temperature. The

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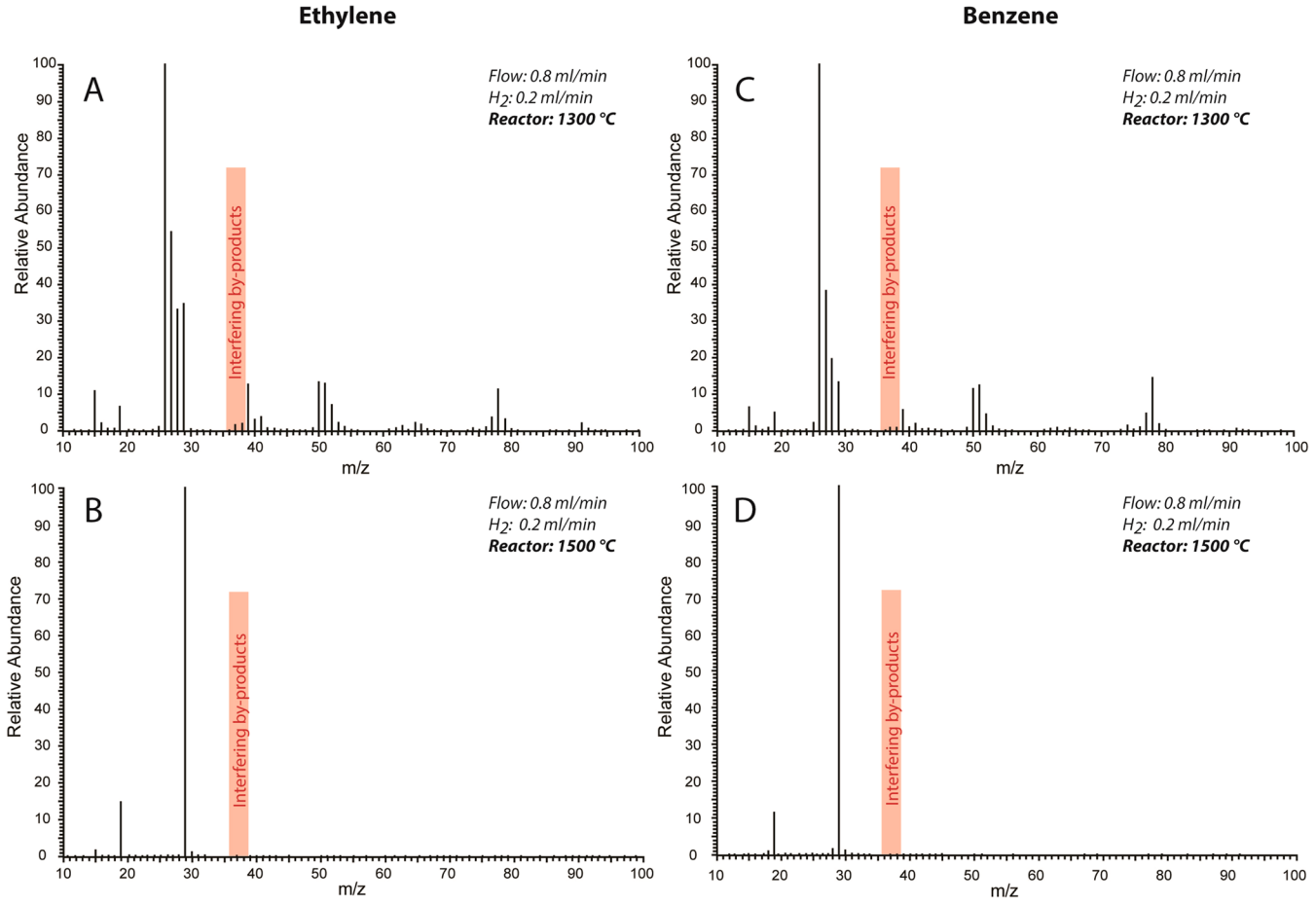

Figure 2. Byproduct formation (monitored by ion trap MS) during high-temperature conversion of the nonchlorinated ethylene ((A) 1300 °C and (B) 1500 °C) and benzene ((C) 1300 °C and (D) 1500 °C) at a GC flow of 0.8 mL/min and 0.2 mL/min reactant gas (H_2).

conversion was characterized by modifying one of the following parameters: ethylene on column (9−45 nmol on column), hydrogen gas flow (0.0−0.5 mL/min), and conversion temperatures (1000−1500 °C). All HTC products were transferred online to the ion trap MS. Conversion was characterized by quantitative analysis of molecular and fragment ions in a range of 10−200 amu produced simultaneously during HTC.

Chlorine Isotope Analysis via IRMS. Reference material was introduced into the GC via head space injection (chemicals in helium atmosphere) or liquid injection (chemicals diluted in acetone). The GC was equipped with a Zebron ZB-1 column (60 m, 0.32 mm i.d., 1 μm film thickness; Phenomenex Inc.) operating at a flow rate of 0.4 mL/min and reactant gas (H_2) flow of 0.1 mL/min. Chlorinated hydrocarbons were converted at 1450 °C. Positive electron impact ionization (EI+ at 85 eV) at a vacuum pressure of 10^{-6} bar was used and the ions m/z 36 ($H^{35}Cl$) and 38 ($H^{37}Cl$) were measured by IRMS with an amplifier gain of 1 (m/z 36), 100 (m/z 37; peak center), and 1 (m/z 38). The analyzed reference material was periodically injected in an amount of approximately 20−30 nmol of Cl on column until stabilization of the isotope values was reached. The measurements were considered stable when five repeat samples were within $1\sigma \leq 0.5$ per mil.

Calculations and Definitions. The abundance of $[H^{37}Cl]/[H^{35}Cl]$ was used to determine the relative chlorine isotope ratio R, measured by IRMS as molecular ions m/z 38 and 36 [eq 1]. For the correction from the measured mass 38/mass 36 (hydrogen chloride) to the chlorine isotopes (mass 37 and mass 35) this formula was used.

$$R(^{35}Cl/^{35}Cl)_{Sample} = \text{area}[H^{37}Cl]_{Sample}/\text{area}[H^{35}Cl]_{Sample} \quad (1)$$

Isotopic composition of chlorine isotopes was expressed as $\delta^{37}Cl_{Sample}$ in standard δ-notation in per mil (‰) relative to "Standard Mean Ocean Chloride" (SMOC) as the reference scale in delta notation [eq 2].

$$\delta^{37}Cl_{sample}[‰] = (R_{Sample}/R_{SMOC} - 1) \times 1000 \quad (2)$$

No reference gas was available in our system. Therefore, the chlorine isotope ratio of seawater (R_{SMOC} = 0.319 644 of the IAEA standard ISL354, Xiao et al.[25]) was used as an fixed value for the conversion of measured isotope ratios into delta-notation ($\delta^{37}Cl_{Sample}$).

Linear Normalization of Chlorine Isotope Composition vs SMOC Scale. In order to normalize the chlorine isotope composition to SMOC-scale, we applied a linear regression of measured (δ^M) and true (δ^T) δ-values as suggested by Paul et al.[26] The two-point normalization involved plotting the δ^M ($\delta^{37}Cl_{Sample}$ [‰] via GC-HTC-IRMS) of the reference standards on the x-axis and the δ^T ($\delta^{37}Cl$ [‰ vs SMOC] via DI-IRMS) on the y-axis. The "expansion factor" (m) and "additive correction factor" (b) were defined by linear regression ($\delta^T = m \times \delta^M + b$) as described by eq 3. For

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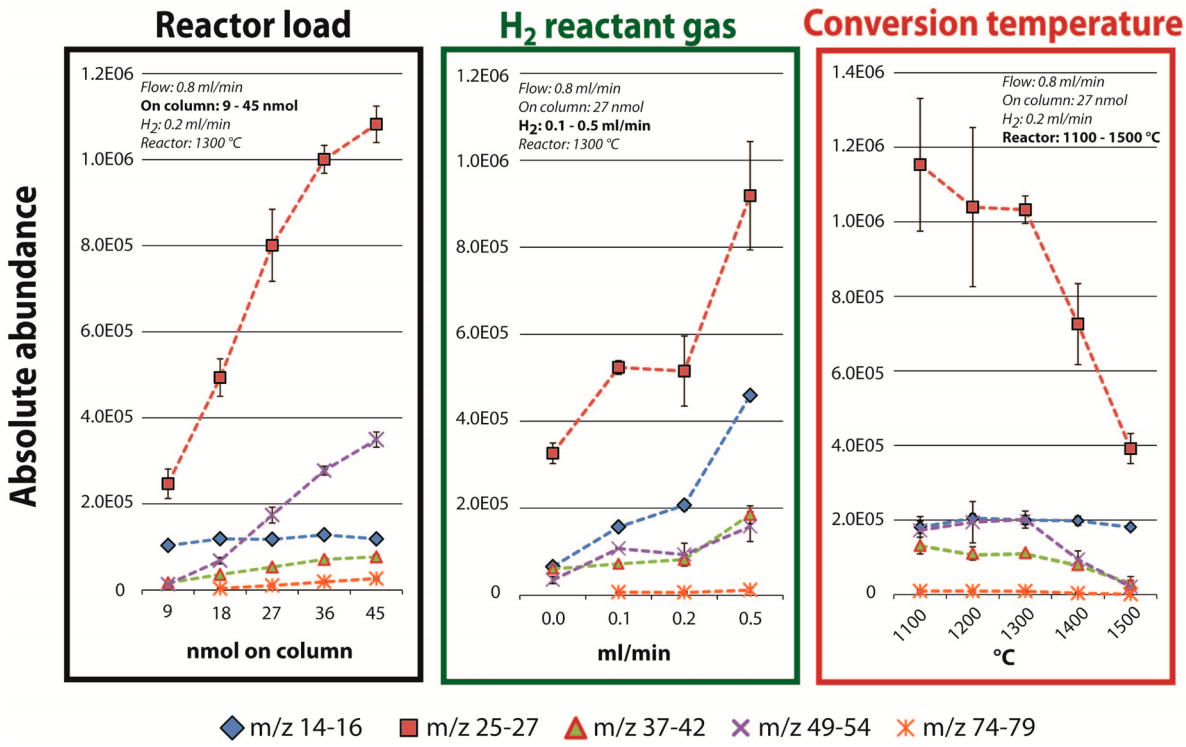

Figure 3. Characterization of byproduct formation during HTC of ethylene by considering reactor load (left), reactant gas (H_2) concentration (middle), and conversion temperature (right). Molecular and fragment ions were monitored via ion trap MS and given as absolute abundances.

normalization of sample values vs SMOC-scale, TCE references 2 and 6 were used as anchor points.

$$\delta^{37}\mathrm{Cl}[\text{‰ vs SMOC}] = m \times \delta^{37}\mathrm{Cl}_{\mathrm{Sample}} + b \qquad (3)$$

RESULTS AND DISCUSSION

Interfering Byproducts during High-Temperature Conversion. The feasibility of a compound-specific chlorine isotope analysis via HTC at 1300 to 1400 °C was already proposed by Hitzfeld et al.[17] However, the stability was insufficient for routine operation (standard deviation up to ±2.0 per mil) and efforts to establish a universal online application for chlorine isotopes analysis remained challenging. The originally applied qMS was replaced by an IRMS, as suggested by Hitzfeld et al.,[17] since instabilities of chlorine isotope measurement were related to qMS detection. Replacement of the qMS (gas analyzer) by an IRMS, however, confirmed instability of chlorine isotope ratios [$H^{37}Cl$] and [$H^{35}Cl$] (m/z 36 and 38). Therefore, we analyzed the HTC process in detail in order to understand and optimize the conversion.

For the investigation of the product spectrum, a combination of ion trap MS and IRMS was established (Figure 1) for a simultaneous analysis of HTC quality as well as the chlorine isotope composition. The analysis of high-temperature conversion at 1300 °C with nonchlorinated ethylene as model compound, however, revealed formation of several molecular and fragment ions of which some interfered with the mass of m/z 38 used for quantification of chlorine isotopes (Figure 2A and Supporting Information). Detected byproduct ions could be assigned to acetylene (C_2H_2, m/z 26), ethylene (C_2H_4, m/z 28), and benzene (C_6H_6, m/z 78). Additionally, ions in the mass range of m/z 37−43 and 49−54 were detected and associated with fragment ions of benzene, though further C_3 or C_4 hydrocarbon byproducts cannot be excluded at this point. Benzene formed during ethylene pyrolysis (Figure 2A and Supporting Information) is assumed to be a product of thermal-coupling during HTC in the presence of hydrogen, as it was previously reported for thermal-coupling of methane by Billaud et al.[27] and Gueret et al.[28] at similar conditions. The thermal-coupling upon conversion of organic material is enhanced by the partial pressure of hydrogen.[29] In our setup, thermal-coupling of ethylene was observed to occur from about 800 °C (results not shown) and reached highest intensities at 1100−1200 °C (Supporting Information). Also, hydrogen is suspected to stabilize the byproducts during pyrolysis, since benzene concentrations were observed to be about 5−10 times higher in the presence of H_2 as reactant gas at 1300 °C. Once formed, benzene could not be completely pyrolyzed at 1300 °C (Figure 2C and Supporting Information). Therefore, previous instabilities of chorine isotope composition could be assigned to overlapping ion signals of $H^{37}Cl$ and fragment ions of pyrolysis byproducts at m/z 38. In conclusion, the conversion temperature of 1300 °C was insufficient to prevent interfering byproduct formation during HTC of chlorinated hydrocarbons.

Parameters Affecting Byproduct Formation. Byproduct formation during HTC of hydrocarbons was characterized using ethylene as a nonchlorinated model compound. Effect of reactor load (9−45 nmol of ethylene on column), reactant gas (H_2) concentration (up to 0.5 mL/min), and conversion temperature (1100−1500 °C) were investigated in order to quantify and optimize HTC. Ion clusters of m/z 14−16, m/z 25−27, m/z 37−42, m/z 49−54, and m/z 74−79 were identified as the main molecular or fragment ions arising from inefficient conversion (Figure 2). Therefore, all ions associated with hydrocarbons were quantified as distinct ion clusters as described above. A special focus was put on ions

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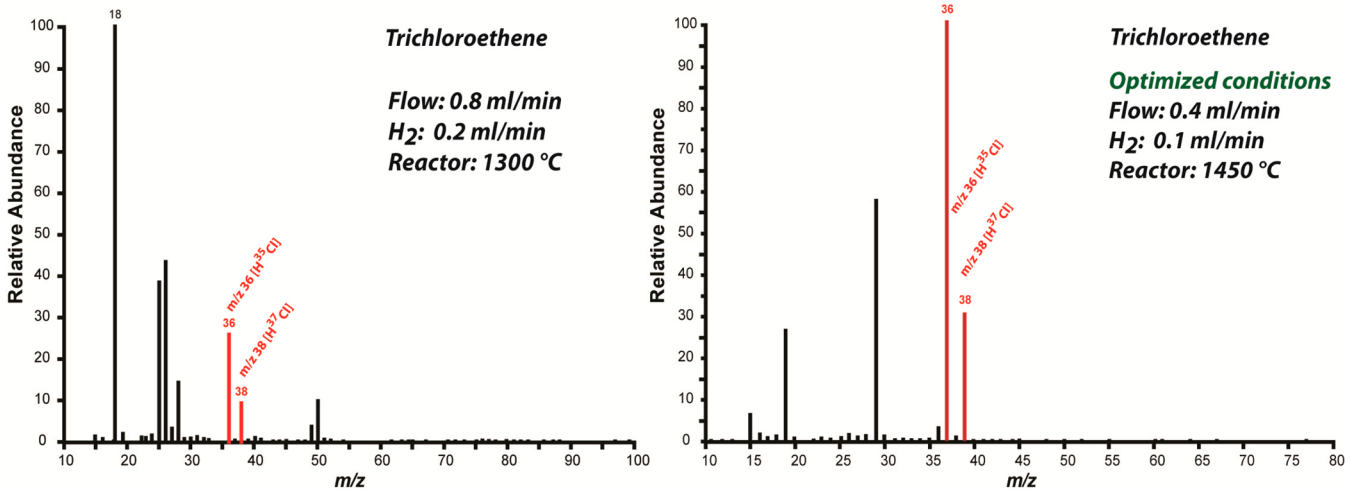

Figure 4. Conversion quality of trichloroethene (TCE) at insufficient conversion conditions (flow 0.8 mL/min, H_2 0.2 mL/min, HTC at 1300 °C) vs optimized conditions (flow 0.4 mL/min, H_2 0.1 mL/min, HTC at 1450 °C). The HTC background was monitored via ion trap MS.

which may interfere with the analysis of the chlorine isotope target compounds [$H^{35}Cl$ and $H^{37}Cl$].

All investigated parameters (reactor load, reactant gas concentration, and conversion temperature) were observed to affect byproduct formation to a certain extent (Figure 3). Increasing reactor load as well as reactant gas (H_2) concentration was observed to enhance the formation of byproducts. The increase of reactor load resulted in formation of hydrocarbons with higher molecular masses (C_4H_x and C_6H_x). In comparison, increasing H_2 concentration enhanced the formation of short hydrocarbon chains (C_1H_x and C_2H_x), which is in agreement with previous reports on thermal-coupling of methane by Billaud et al.,[27] as well as Gueret et al.[28] Increasing conversion temperature (Figure 3) resulted in a significant reduction of byproduct formation also in agreement with a previous report.[29]

According to our results, formation of byproducts interfering with the detection of HCl ions could be significantly reduced by increasing the conversion temperature from 1300 to 1500 °C (Figure 2B,D and Supporting Information). Alternatively, increasing residence time of the compound in the reactor at 1450 °C was also observed to be sufficient for a complete conversion (Figure 4 and Supporting Information). However, overload of the reactor has to be considered; therefore, analyte amount on the column has to be limited to avoid the risk of interfering byproduct formation. In order to prove complete conversion of chlorinated hydrocarbons, subsequent experiments were performed at optimized conditions (GC flow 0.4 mL/min, H_2 flow 0.1 mL/min, HTC temperature 1450−1500 °C). Quantitative conversion without interference of byproducts could be confirmed for chlorinated methane, ethylene, ethane, benzene, and acetic acid methyl ester (Supporting Information). At optimized conditions, byproduct formation for all tested compounds could be reduced, whereby no interfering fragment ions were detected (Figure 2B, D and Supporting Information). Meanwhile, formation of the HCl target ions m/z 36 and 38 was not affected by the new conversion conditions and remained in a similar range (Supporting Information).

Validation of Chlorine Isotope Measurements via GC-HTC-IRMS. Trichloroethene (TCE) and tetrachloroethene (PCE) reference materials were used for online measurement of chlorine isotope composition via GC-HTC-IRMS. All reference materials were analyzed in an amount of approximately 30 nmol of Cl on column, a helium carrier gas flow rate of 0.4 mL/min, reactant gas (H_2) flow of 0.1 mL/min, and at a conversion temperature of 1500 °C (optimized HTC conditions, see paragraph above). The corresponding chlorine isotope composition $\delta^{37}Cl_{raw}$ was determined as described in the methods part.

The stability of the GC-HTC-IRMS setup was tested by using two TCE standards (TCE references 2 and 6). The injection of 30 nmol on column resulted in a peak intensity of 1500−2000 mV (m/z 36). Both compounds were measured over 5 days under the same conditions. The corresponding chlorine isotope values ($\delta^{37}Cl_{raw}$) were observed to be very stable over 5 days and an additional replacement of the reactor tube (Figure 5). Average chlorine isotope composition ($\delta^{37}Cl_{raw}$) during this period was measured to be 23.6 ± 0.3‰ and 25.5 ± 0.3‰ for TCE reference 2 and TCE reference 6 ($n \geq 5$), respectively. In comparison, reported

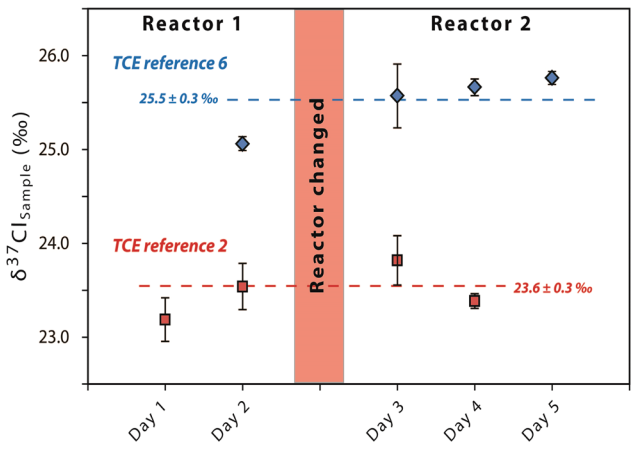

Figure 5. Evaluation of the stability of chlorine isotope analysis. Trichloroethene (TCE) reference no. 2 and 6 ($n \geq 5$) were converted at 1500 °C and total flow rate of ~0.5 mL/min (GC flow 0.4 mL/min, H_2 flow 0.1 mL/min). The stability of chlorine isotope composition was evaluated over 5 days and with two reactors.

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Anal. Chem. 2015, 87, 2832−2839

Table 1. Chlorine Isotope Composition of Reference Compounds[a]

Reference compounds	DI-IRMS	GC-HTC-IRMS				
		Method validation			Analysis	
	$\delta^{37}Cl$ [‰ vs. SMOC]	$\delta^{37}Cl_{Sample}$ [‰]	$\delta^{37}Cl$ [‰ vs. SMOC]	n	$\delta^{37}Cl$ [‰ vs. SMOC]	n
TCE ref. 2	**-1.19 ± 0.01**	23.47 ± 0.30	**-1.20**	19	**anchor**	
TCE ref. 6	**2.17 ± 0.2**	25.65 ± 0.24	**2.10**	16	**anchor**	
PCE ref. 1	**-0.49 ± 0.12**	23.89 ± 0.37	**-0.57**	16	**-0.49 ± 0.17**	10
PCE ref. 5	**1.03 ± 0.08**	25.01 ± 0.40	**1.14**	16	**0.64 ± 0.15**	9
MeCl ref. (Linde)	**6.03 ± 0.02**				**6.85 ± 0.51**	10
HCH ref. 1 (HiMedia)	**-0.18 ± 0.03**				**0.27 ± 0.32**	9
HCH ref. 2 (Greyhound)	**-5.49 ± 0.12**				**-5.20 ± 0.27**	10
TCAA ref. (Sigma)	**-5.11 ± 0.17**				**-4.60 ± 0.28**	10

[a]Chlorine isotope composition measured off-line via DI-IRMS ($\delta^{37}Cl$ [‰ vs SMOC]) and online via GC-HTC-IRMS. Online measurements were validated (red) and the corresponding chlorine isotope composition determined as $\delta^{37}Cl_{Sample}$ [‰] and $\delta^{37}Cl$ [‰ vs SMOC]. Chlorine isotope composition of several reference compounds was analyzed (blue) and converted to SMOC-scale.

instabilities for GC-HTC-qMS were in a range of $1\sigma \leq 1.4$‰ ($n = 3$) and shifts of 3.0−4.0‰ over 3 days of measurements.[17]

Because of the observed stability of the system during analysis of the chlorine isotope composition of the TCE standards, four reference compounds (PCE reference 1, TCE reference 2, PCE reference 5, and TCE reference 6) were subsequently analyzed at similar conditions. Memory effects were observed during successive analysis of different compounds. Therefore, for each compound at least 16 data points were measured after stabilization of the corresponding isotope value. The chlorine isotope composition of the reference compounds $\delta^{37}Cl_{raw}$ is given in Table 1 and Figure 6. Linear

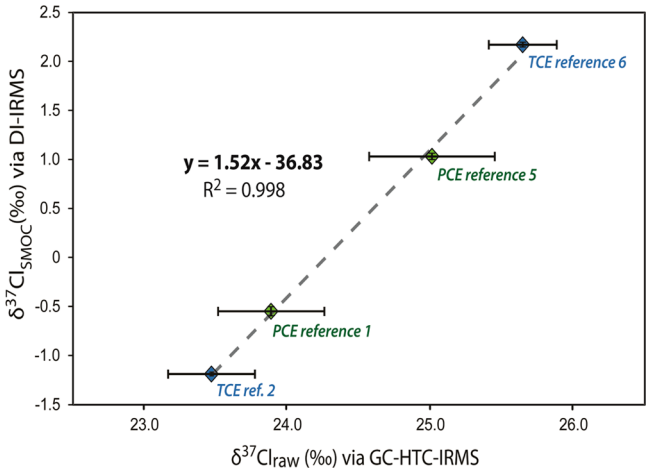

Figure 6. Validation of chlorine isotope measurement via GC-HTC-IRMS. Normalization of measured chlorine isotope composition was done using TCE and PCE reference material. True isotope composition $\delta^{37}Cl_{SMOC}$(‰) is given on the *y*-axis (determined off-line via DI-IRMS). Measured isotope composition $\delta^{37}Cl_{raw}$(‰) (online via GC-HTC-IRMS) is plotted at the *x*-axis. The "expansion factor" of the GC-HTC-IRMS setup was determined by a linear regression fit.

regression of the measured chlorine isotope composition ($\delta^{37}Cl_{raw}$) to the true chlorine isotope composition ($\delta^{37}Cl_{SMOC}$) resulted in an "expansion factor" of $m = 1.52$ with an "additional correction factor" of $b = -36.83$.

The normalization of the online chlorine isotope values to the SMOC-scale were in a linear agreement ($R^2 = 0.980$) to chlorine isotope composition determined via an off-line method. $\delta^{37}Cl_{SMOC}$ determined via GC-HTC-IRMS were −0.57 ± 0.37‰ ($n = 16$) for PCE reference 1, −1.20 ± 0.30‰ ($n = 19$) for TCE reference 2, 1.14 ± 0.40‰ ($n = 16$) for PCE reference 5, and 2.10 ± 0.24‰ ($n = 16$) for TCE reference 6 (Table 1). In summary, chlorine isotope composition ($\delta^{37}Cl_{SMOC}$) determined via GC-HTC-IRMS was in a very good agreement with the "true" chlorine isotope composition measured via DI-IRMS with a maximum deviation of ≤0.1‰.

Determination of Chlorine Isotope Composition of Different Compound Classes via GC-HTC-IRMS. Available materials with known chlorine isotope composition, including trichloroethylene (TCE refs 2 and 6), tetrachloroethylene (PCE refs 1 and 5), methyl chloride (MeCl ref.), hexachlorocyclohexane (HCH refs 1 and 2), and trichloroacetic acid methyl ester (TCAA ref.), were used for validation of the performance of the online chlorine isotope analysis by the GC-HTC-IRMS. The system was operating with a helium carrier gas flow of 0.4 mL/min, reactant gas (H_2) flow of ∼0.1 mL/min, and at a conversion temperature of 1450 °C. Reference compounds were injected in a concentration of approximately 30−40 nmol of Cl on column. The corresponding peak intensities were in a range of 1500−2500 mV (m/z 36) (Supporting Information). The determined chlorine isotope composition was normalized using TCE references 2 and 6 as an anchor for calibration to the SMOC-scale and are presented as $\delta^{37}Cl_{SMOC}$ (Figure 7, Table 1). The chlorine isotope composition of all reference compounds determined online via GC-HTC-IRMS was in very good agreement with chlorine isotope values determined off-line via DI-IRMS with a maximum deviation of ≤0.8‰. Further, observed precision was very satisfying with standard deviations below ±0.5‰ for all reference compounds.

Limitations and Challenges. Though online analysis of chlorine isotopes ($^{37}Cl/^{35}Cl$) via GC-HTC-MS/IRMS is a promising universal compound-specific approach, several limitations and challenges remain as discussed in the following.

Memory Effects. Online isotopes analysis of chlorine via HTC is thus far limited by serious memory effects, as shown in Figure 8. Therefore, stabilization time and repetition of measurements until stabilization of values is required for accurate chlorine isotope analysis. In order to reduce memory effects, all transfer-lines were continuously heated to avoid potential condensation of HCl or H_2O in cold sections. In addition, the ion source was heated to approximately 120 °C (personal communication ThermoFinnigan) to reduce sorption of HCl at metal surfaces. However, the success was limited;

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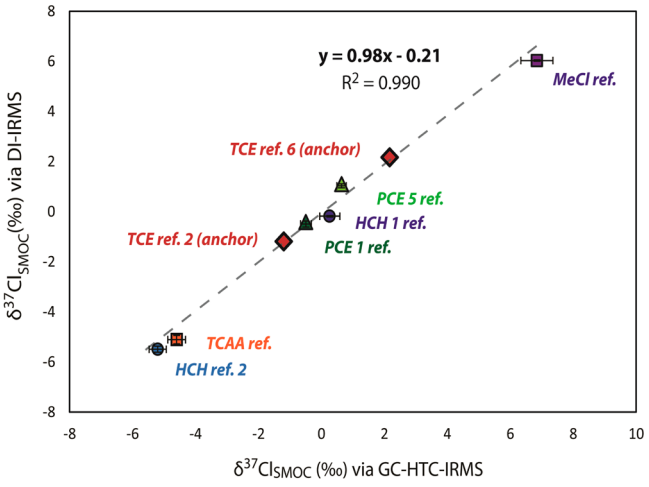

Figure 7. Determination of chlorine isotope composition of several chlorinated reference compound via GC-HTC-IRMS. True isotope composition $\delta^{37}Cl_{SMOC}(‰)$ is given on the *y*-axis (determined off-line via DI-IRMS). Measured chlorine isotope composition (online via GC-HTC-IRMS) was normalized to the SMOC-scale using TCE references 2 and 6 is plotted at the *x*-axis.

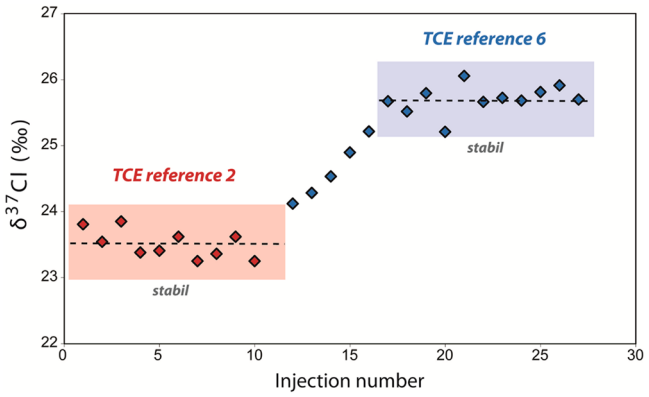

Figure 8. Memory effect during chlorine isotope measurement demonstrated for TCE reference no. 2 and 6.

conclusively, memory effects could only be assigned to the HTC reactor. Therefore, further attempts are needed to prevent memory effects, e.g., investigation of different HTC reactor materials.

Stability of the Aluminum Oxide HTC Reactor. The lifetime of the aluminum oxide ceramic was at applied conditions less than 1 week. The increasing alteration of the ceramic material resulted in leakage after a few days of operation. Therefore, a careful monitoring of the tightness of the system is needed. In our setup, the air water background was monitored online via ion trap MS.

Thus, far, however, it is not clear if only high temperature (1450−1500 °C) or high temperature in combination with HCl is responsible for the relative short lifetime of the ceramic reactor. Conclusively, reactor material with higher stability is required to overcome the limitations of aluminum oxide ceramics in the future.

Water Formation. Because of the high availability of hydrogen as reactant gas in our setup (∼20%), oxygen preferably reacted to H_2O (for details see the Supporting Information), which can potentially lead to memory effects, since it may act as a trap for hydrochloric acid. Several sources of oxygen were considered. On the one hand, oxygen was observed to penetrate the system due to leakages, small cracks of the ceramic structure due to fatigue of the material; on the other hand, oxygen was suspected to leach out permanently from the aluminum oxide ceramic reactor at high conversion temperature.[30] In both cases H_2O may be produced during HTC. However, H_2O background was observed to remain stable and did not significantly interfere with chlorine isotope measurements. Furthermore, efforts to reduce H_2O formation, including the increase of HTC temperature and carbon deposition in the ceramic rector, were of minor success. Therefore, no other efforts were undertaken to reduce the water formation. Though H_2O in a limited range was observed not to affect isotope measurements, sufficient background monitoring and maintenance remained essential to provide stable chlorine isotope analysis.

Effect of Hydrochloric Acid on Instrumental Setup. Hydrochloric acid, a highly reactive and aggressive agent, could potentially damage the IRMS ion source. Visual inspection, however, revealed no obvious effect or damage of the IRMS ion source, even after 1 year of extensive use of high-temperature conversion and HCl measurements. These findings are in accordance to the ones by Hitzfeld et al.[17] reporting no effects of HCl on the qMS source. Thus, HCl seems to be a reasonable analyte for chlorine stable isotope determination via GC-HTC-IRMS.

Expansion Factor. The expansion factor in isotope analysis is usually MS or IRMS specific. For instance, Bernstein et al.[11] reported for nine MS detectors (IRMS or qMS) an expansion factor between 0.72 and 1.31.[11] However, determined expansion for GC-HTC-IRMS was particularly high (m = 1.52). Thus far, high hydrogen content in the carrier gas flow (∼20 vol %) is suspected to affect the ionization in the ion source, similar to the H^{3+} factor in hydrogen isotope analysis.[31] This observation is not completely understood and further investigation is need in the future.

CONCLUSIONS

The analysis of chlorine isotopes via high-temperature conversion to HCl is a promising tool for routine analysis of chlorinated compounds. Online analysis via GC-HTC-IRMS reduces significantly sample preparation time in comparison to off-line conversion and the amount of analyte needed for chlorine isotope analysis. Furthermore, we demonstrate that a range of GC amendable chlorinated compound classes is now accessible for chlorine isotope analysis via IRMS. The performance of chlorine isotope analysis was shown for chloromethanes, chloroethenes, chlorocyclohexanes, and chloroacetic acid methyl ester. In comparison to available techniques chlorine isotope analysis via GC-HTC-IRMS showed low detection limits (∼10−15 nmol Cl on column). Lower detection limit was thus far only reported for GC-qMS (<10 pmol).[13] However, detection limit via GC-HTC-IRMS is still lower as reported for MC-ICPMS coupled to gaschromatography (≥200 nmol Cl on column),[15] TIMS (<85 nmol Cl on column)[10] and comparable to GC-IRMS (∼10 nmol Cl on column).[14] Achieved precision with our setup (1σ < 0.3‰, Figure 6) is inferior to that of GC-IRMS (1σ = 0.1‰),[14] and MC-ICPMS (1σ = 0.06‰[15]) but still sufficient for an universal online method. Quantitative conversion and HTC performance were demonstrated and characterized in detail for a range of chlorinated and nonchlorinated compounds. The parallel characterization of multi collector mass spectrometry for

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isotope analysis and ion trap mass spectrometry for characterization for products opens perspectives to evaluated HTC interfaces for CSIA.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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

Notes

The authors declare no competing financial interest.

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Publication III

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Substrate Hydrophobicity and Cell Composition Influence the Extent of Rate Limitation and Masking of Isotope Fractionation during Microbial Reductive Dehalogenation of Chlorinated Ethenes

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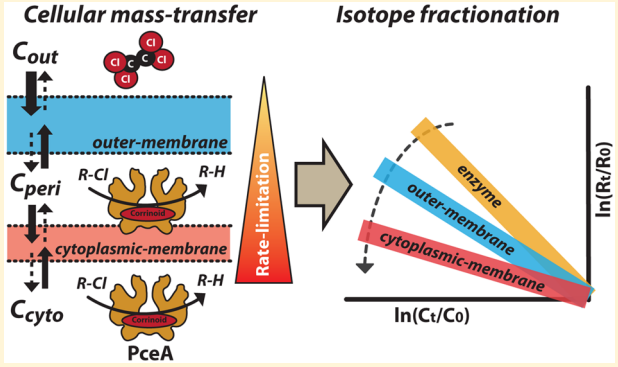
Supporting Information

ABSTRACT: This study investigated the effect of intracellular microscale mass transfer on microbial carbon isotope fractionation of tetrachloroethene (PCE) and trichloroethene (TCE). Significantly stronger isotope fractionation was observed for crude extracts vs intact cells of *Sulfurospirillum multivorans*, *Geobacter lovleyi*, *Desulfuromonas michiganensis*, *Desulfitobacterium hafniense* strain PCE-S, and *Dehalobacter restrictus*. Furthermore, carbon stable isotope fractionation was stronger for microorganisms with a Gram-positive cell envelope compared to those with a Gram-negative cell envelope. Significant differences were observed between model organisms in cellular sorption capacity for PCE (*S. multivorans*-$K_{d\text{-}PCE}$ = 0.42−0.51 L g^{-1}; *D. hafniense*-$K_{d\text{-}PCE}$ = 0.13 L g^{-1}), as well as in envelope hydrophobicity (*S. multivorans* 33.0° to 72.2°; *D. hafniense* 59.1° to 60.8°) when previously cultivated with fumarate or PCE as electron acceptor, but not for TCE. Cell envelope properties and the tetrachloroethene reductive dehalogenase (PceA-RDase) localization did not result in significant effects on observed isotope fractionation of TCE. For PCE, however, systematic masking of isotope effects as a result of microscale mass transfer limitation at microbial membranes was observed, with carbon isotope enrichment factors of −2.2‰, −1.5 to −1.6‰, and −1.0‰ ($CI_{95\%}$ < ± 0.2‰) for no membrane, hydrophilic outer membrane, and outer + cytoplasmic membrane, respectively. Conclusively, rate-limiting mass transfer barriers were (a) the outer membrane or cell wall and (b) the cytoplasmic membrane in case of a cytoplasmic location of the RDase enzyme. Overall, our results indicate that masking of isotope fractionation is determined by (1) hydrophobicity of the degraded compound, (2) properties of the cell envelope, and (3) the localization of the reacting enzyme.



INTRODUCTION

In recent years, compound specific stable isotope analysis (CSIA) has become a routine approach for monitoring and quantification of in situ biodegradation of contaminants at polluted sites.[1,2] CSIA has the advantage that it can provide estimates of degradation which are less affected by dilution, dispersion and adsorption along the groundwater flow path compared to conventional concentration analysis. For these reasons, it is a useful approach for evaluation and assessment of (bio)degradation processes in situ.[1,3] CSIA makes use of the measurable isotope fractionation between lighter (e.g., ^{12}C, ^{35}Cl) and heavier (e.g., ^{13}C, ^{37}Cl) isotopes during (bio)-degradation of contaminants. Since molecules/bonds with lighter isotopes react faster, heavier isotopes become enriched in the residual fraction of the reactant. The extent of isotope fractionation can be quantified using the Rayleigh approach and expressed as isotope enrichment factor (ε).[2,4] The magnitude of the isotope enrichment factor depends on the nature of the (bio)chemical bond cleavage during degradation and can be related to a certain reaction mechanism. However, fractionation can be significantly masked due to rate-limitation in mass transfer, that is, at microbial membranes, as was observed for *Sulfurospirillum multivorans*, but also as result of superimposed isotope effects of multiple step reactions.[5−10]

Various microbial strains from the phyla of Proteobacteria, Firmicutes, and Chloroflexi are capable of energy conservation via organohalide respiration using the chlorinated ethenes as electron acceptors (EA). Despite strong similarities of the reductive dehalogenase enzyme (RDase) in different microorganisms, isotope analysis of carbon revealed highly variable isotope fractionation for different strains during dehalogenation of tetrachloroethene (PCE) and trichloroethene (TCE), making characterization of the reaction via CSIA difficult.[8,9,11]

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In general, variability may be the result of different elementary reactions at the catalytic site inside an enzyme, commitment to catalysis in the enzyme reaction, multiple chemical reaction steps, limited bioavailability or limited mass transfer.[12] On this basis, observed microbial variability of carbon isotope fractionation was assumed to be related to either different reaction mechanisms, specific corrinoid incorporated into the RDase enzymes, microbial cell envelope properties or growth conditions.[8,9,13] Dual-element isotope analysis applied in recent studies, however, supported a similar reaction mechanism for several strains capable of reductive dehalogenation.[10,14,15] Further, we recently could show that the modification of corrinoid cofactor did not affect the observed isotope fractionation by a specific enzyme.[10] Additionally, Harding et al.[16] showed that growth conditions did not significantly affect the observed isotope fractionation, though, only isotope fractionation of TCE for *Dehalococcoides* was investigated and the effect of the microbial membrane was not considered.

Thus far several studies reported different carbon isotope fractionation for TCE vs PCE, though catalyzed by the same enzymes or microorganisms. Additional effects of substrate properties, that is, hydrophobicity, were suggested to be responsible for variability in isotope fractionation, since rate-limiting steps prior to the catalytic C−Cl cleavage may mask the real magnitude of isotope effects of the reaction.[7,17] Therefore, we aimed to investigate the microbial cell envelope composition, as well as the properties of the substrate (PCE and TCE), as contributing factor for the observed variability in carbon isotope fractionation. On the one hand different cell wall types, such as the Gram-negative (outer membrane | peptidoglycan layer | cytoplasmic membrane) and the Gram-positive (peptidoglycan layer | cytoplasmic membrane) cell walls may affect the transport of chlorinated ethenes, due to a lower or higher sorption capacity.[18] On the other hand solubility and hydrophobicity of chlorinated ethenes may affect microscale mass transfer processes, that is, the solubility in water and the partition coefficient K_{OW} are significantly different for TCE (solubility = 1280 mg L^{-1} and K_{ow} = 407) in comparison to PCE (solubility = 192.3 mg L^{-1} and K_{ow} = 2512).[19,20] In both cases limitation in microscale mass transfer would affect the observable isotope fractionation.[7] Thus, better evaluation and understanding of potential rate-determining steps and the role of intracellular microscale mass-transfer during (bio)catalysis is required for reliable application of CSIA in lab experiments and at field site.

Therefore, we investigated the effect of the microbial cell type and composition on stable isotope fractionation. Representative microorganisms for different cell envelope types, including the Gram-negative *Sulfurospirillum multivorans*, *Geobacter lovleyi*, and *Desulfuromonas michiganensis,* as well as Gram-positive *Desulfitobacterium hafniense* strain PCE-S, and *Dehalobacter restrictus*, were used as intact cells or crude extracts in order to assess the effect of different cell envelopes on carbon isotope fractionation of PCE and TCE. The specific sorption capacity for PCE and TCE of Gram-positive vs Gram-negative microorganisms was evaluated using *S. multivorans* and *D. hafniense* strain PCE-S as model organisms. Furthermore, variability of cell-envelope properties due to different cultivation conditions and the corresponding effect on isotope fractionation for the Gram-negative *S. multivorans* was analyzed. Masking of isotope effects by the cytoplasmic-membrane in addition to the outer-membrane, was investigated for the first time in detail for *S. multivorans* making use of the previously observed growth dependent localization (periplasmic or cytoplasmic) of PceA reductive dehalogenase.[21]

MATERIALS AND METHODS

Chemicals. All chemicals were purchased either from Merck (Darmstadt, Germany) or Sigma-Aldrich Chemie (Munich, Germany) in highest purity available. Gasses were purchased by either from Linde Gas AG (Pullach, Germany) or AirProducts (Hattingen, Germany).

Cultivation. *Dehalobacter restrictus* PER-K23,[22] *Geobacter lovleyi* strain SZ,[23] as well as *Desulfuromonas michiganensis*[24] were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *D. restrictus*, *G. lovleyi*, and *D. michiganensis* were cultivated using DSMZ medium M732 with tetrachloroethene (PCE) or trichloroethene (TCE) as electron acceptor. Hydrogen (used by *D. restrictus* and *G. lovleyi*) or acetate (used by *G. lovleyi* and *D. michiganensis*) served as electron donor. All cultures were incubated at 30 °C in a rotary shaker at 120 rpm.

Sulfurospirillum multivorans and *Desulfitobacterium hafniense* strain PCE-S from laboratory stocks were cultivated in an anoxic mineral medium as previously described by Scholz-Muramatsu et al.[25] Pyruvate (40 mmol L^{-1}) or formate (40 mmol L^{-1}) and acetate (10 mmol L^{-1}) were used as electron donor and carbon source, respectively. Fumarate (40 mmol L^{-1}), TCE or PCE were applied as electron acceptors during cultivation, as indicated. PCE or TCE were added as pure substance (0.5 mmol L^{-1}) or dissolved in hexadecane (TCE: 1 mmol L^{-1} or PCE: 18 mmol L^{-1}). Both strains were incubated at 30 °C in a rotary shaker.

Preparation of Cell Suspension and Bacterial Crude Extract. Bacterial cells were harvested at the end of the exponential growth-phase by centrifugation at 8.000*g* and 4 °C for 20 min. The cells were washed in two cycles via sequential centrifugation and resuspending with 100 mmol L^{-1} Tris-HCl buffer at pH 7.5 and used as cell suspension or crude extract in subsequent experiments. Bacterial crude extract was prepared under anoxic conditions as described by Nijenhuis et al.[8] Additionally, 10 mg Lysozyme and 1 mg DNase I were added and incubated for 10−15 min at room temperature prior to treatment with French press (Thermo Fisher Scientific, Bremen) at 20.000 psi. The produced crude extract was stored under anoxic conditions at −20 °C until further use.

Determination of Cell Sorption Capacity for PCE and TCE. Sorption experiments were performed to determine the equilibrium partition coefficient (K_d) for the sorption of PCE and TCE to cells. All sorption experiments were conducted under oxic conditions to avoid dehalogenation of the chlorinated ethenes during the experiment. Control experiments were done to exclude sorption to septa. No dehalogenation products were found, therefore, decreasing chloroethene concentration could be only addressed to the sorption by cellular biomass. Bacterial cells were harvested as described above and the total biomass in cell suspension was determined according to Bradford (1976) using the Bio-Rad Protein Assay Dye reagent concentrate (Bio-Rad, Munich, Germany). The sorption experiment was performed in 20 mL crimp-neck head space vials containing 10 mL of cell suspension in Tris-HCl buffer at pH 7.5 and control vials containing Tris-HCl buffer only. Different concentrations of PCE (35−360 μmol L^{-1}) or TCE (60−600 μmol L^{-1}) were applied in triplicates and incubated overnight at room temperature on a rotary shaker at 150 rpm. Subsequently,

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Table 1. Stable Isotope Enrichment Factors for Growing Cells vs Crude Extracts of *Geobacter lovleyi*, *Desulfuromonas michiganensis*, *Sulfurospirillum multivorans*, *Dehalobacter restrictus*, and *Desulfitobacterium hafniense* Strain PCE-S As Well As Representative Corrinoids[a]

		PCE				TCE			
		ε_C	CI (95%)	R^2	ref	ε_C	CI (95%)	R^2	ref
ε-proteobacteria gram-negative	***S. multivorans***								
	crude extract	−1.4	±0.1‰	0.986	10	−20.0	±0.5‰	1	10
	crude extract	−1.4	±0.1‰	0.924	17	−16.2	±3.7‰	0.920	17
	crude extract	−1.0	±0.2‰	0.949	8	−13.2	±1.8‰	0.990	8
	growing culture	−0.4	±0.2‰	0.668	8	−18.7	±4.2‰	0.840	17
	growing culture					−16.4	±1.5‰	0.970	11
δ-proteobacteria gram-negative	***G. lovleyi* SZ**								
	crude extract	−2.3	±0.4‰	0.854	this study	−9.3	±0.6‰	0.970	this study
	growing culture		ns		9	−8.5	±0.6‰	0.980	9
	growing culture					−12.2	±0.5‰		15
	D. michiganensis								
	crude extract	−2.6	±0.4‰	0.878	this study	−7.1	±0.4‰	0.980	this study
	growing culture	−1.7	±0.1‰	0.993	this study	−4.4	±0.3‰	0.999	this study
	growing culture		ns		9	−3.5	±0.3‰	0.990	9
firmicutes gram-positive	***D. restrictus***								
	crude extract	−6.3	±0.2‰	0.993	this study	−7.7	±0.4‰	0.980	this study
	growing culture	−4.0	±0.2‰	0.996	this study	−8.3	±0.9‰	0.990	this study
	growing culture					−3.3	±0.3‰	0.980	11
	***D. hafniense* strain PCE-S**								
	crude extract	−7.6	±0.6‰	0.991	this study	−12.9	±0.4‰	0.999	this study
	crude extract	−8.9	±1.8‰	0.933	8	−10.9	±1.1‰	0.990	17
	growing culture	−5.2	±1.5‰	0.931	8	−12.2	±2.3‰	0.880	17
corrinoids	cyanocobalamin	−16.2		0.990	51	−16.5		0.990	51
	cyanocobalamin	−13.2	±1.5‰	0.983	8	−15.4	±2.1	0.961	17
	cyanocobalamin	−22.4	±0.8‰	0.998	10	−15.0	±2.0	0.979	10
	cyanocobalamin					−16.1	±0.9		15
	norpseudo-B12	−25.3	±0.8‰	0.998	10	−18.5	±2.8	0.961	10
	dicyanocobinamide	−25.2	±0.5‰	0.999	10	−16.5	±0.7	0.997	10
	cobaloxime					−21.3	±0.5		15

[a]ns: Not significant.

concentrations of chlorinated ethenes were analyzed by taking 0.5 mL head space sample and measured as described elsewhere.[8] The sorption coefficient K_d for PCE and TCE to the biomass was determined by fitting the results to a linear sorption model $K_d = A_i/C_i$ with A_i as mass of chloroethenes sorbed per cell mass in solution and C_i as mass of chloroethene remained in solution.[26] The concentration of PCE and TCE in the liquid phase was determined according to the equation $f_l = 1/(1 + H^*(V_{hs}/V_l))$ using the dimensionless Henry's law constant (H), head space volume (V_{hs}), and liquid volume (V_l). For applied conditions the dimensionless equilibrium constant f_l for PCE and TCE in liquid phase was determined to be 0.570 and 0.703, respectively.

Determination of Cell Envelope Hydrophobicity. Cell hydrophobicity was analyzed determining the contact angle of a water drop on a cell layer as described elsewhere.[27,28] Bacterial cells were harvested in late exponential growth phase by centrifugation (8000*g*, 4 °C, and 20 min) and washed under oxic conditions twice in 30 mL 10 mmol L^{-1} KNO_3 at pH 6.2. Subsequently, the cell pellet was resuspended in 1−2 mL 10 mmol L^{-1} KNO_3. A homogeneous cell layer was brought on a 25 mm nitrocellulose membrane filter, pore size 0.45 μm (Whatman, GE Healthcare Bio-Sciences Corp.) by filtering 100−200 μL cell suspension solved in 20 mL KNO_3, using a vacuum filtration device (Sartorius, Germany). The hydrophobicity of the cell envelope was determined with the DSA 100 drop shape analyzer (Krüss, Germany). For each culture at least five points were measured and given as average value of the contact angle, plus standard deviation.

Dehalogenation Experiments. *Dehalogenation Using Methyl Viologen As Artificial Electron Donor.* Enzymatic dehalogenation was done with bacterial cells or bacterial crude extracts harvested in exponential growth phase, as previously described by Nijenhuis et al.[8] and Renpenning et al.[10] Degradation reaction was done in 20 mL crimp-neck head space vials containing 10 mL methyl viologen buffer (100 mmol L^{-1} Tris-HCl, pH 7.5; 1.6 mmol L^{-1} methyl viologen; 0.2 mmol L^{-1} $(NH_4)_2SO_4$) with methyl viologen as artificial electron donor.[29,30] As electron acceptor 0.5 mmol L^{-1} or 1 mmol L^{-1} PCE or TCE in ethanol solution was added to the reaction buffer, respectively. The dehalogenation reaction was started by adding 100 μL titanium(III)citrate solution, to reduce methyl viologen, and subsequently 5−100 μL of cells suspension or crude extracts to the reaction vial followed by incubation at room temperature on a rotary shaker (160 rpm). The reactions were stopped at different reaction time points by

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adding 1 mL saturated Na_2SO_4 solution (adjusted to pH 1 with H_2SO_4). Concentrations of chlorinated ethenes were analyzed by taking 0.5 mL head space sample. Remaining sample volume was used for stable isotope analysis as described below.

Dehalogenation Using Hydrogen As Physiological Electron Donor. Dehalogenation with physiological ED was done as described by Neumann et al.[31] The degradation reaction was performed in 20 mL crimp-neck head space vials containing 10 mL MOPS-buffer (100 mmol L^{-1} MOPS, pH 7.5, 0.4 mmol L^{-1} cysteine; 1 mg L^{-1} resazurin). As physiological electron donor 3% hydrogen in vial head space was used. As electron acceptor PCE or TCE with a final concentration of 0.5 mmol L^{-1} was added to the reaction buffer. The dehalogenation reaction was started by adding 100−500 μL freshly harvested cell suspension (resting cells) to the reaction vial and subsequently incubated at room temperature on a rotary shaker (160 rpm). The reaction was stopped as described in the previous section.

Analytical Methods. *Chlorinated Ethene Concentration.* Analysis of chlorinated ethenes was done as described elsewhere using GC-FID.[8]

Stable Carbon Isotope Analysis. All samples were measured via GC-C-IRMS in at least triplicates and the standard deviation was generally below 0.5‰ as previously described by Renpenning et al.[10] Samples were injected automatically via head space (200 μL) using an autosampler (TriPlus RSH Autosampler, Thermo, Germany). All injections were done in split mode 1:5 to 1:10 and 250 °C injector temperature. The GC (Trace 1320, Thermo, Germany) was equipped with a Zebron ZB-1 capillary column (60 m × 0.32 mm, 0.5 μm film; Phenomenex Inc.). After separation and combustion of the compounds isotope composition was measured via IRMS (MAT 253 IRMS, ThermoFinnigan, Germany)

Calculation and Definitions. The carbon isotope composition is reported in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard.[32] The isotope fractionation was calculated by applying the Rayleigh-equation [eq 1] with R_0 and R_t as isotope values, and C_0 and C_t as concentrations at time 0 and time t, respectively.[33]

$$\ln(R_t/R_0) = \varepsilon \times \ln(C_t/C_0) = \varepsilon \times \ln(C_t/C_{total}) \quad (1)$$

In alternative to C_0, the total chloroethene concentration C_{total} in each sample was calculated from the mass balance, as described elsewhere.[10]

$$C_0 = C_{total} = C_{PCE} + C_{TCE} + C_{DCE} + C_{VC} + C_{Ethene} \quad (2)$$

Here the concentrations were corrected using the mass balance approach. The corresponding slope ratios and the confidence intervals ($CI_{95\%}$) were determined via linear regression using Excel Analysis Toolpak (Microsoft).

RESULTS

Microbial Isotope Fractionation of PCE and TCE by Growing Cells vs Crude Extracts. The effects of different cell envelope types, that is, Gram-negative and Gram-positive type, on masking of PCE and TCE carbon isotope fractionation was determined using growing cells and the corresponding bacterial crude extracts of several representative microorganisms (Table 1).

Significant differences in isotope enrichment factors were observed between growing cells and the corresponding bacterial crude extracts during PCE dehalogenation, as presented in Table 1 for *Sulfurospirillum multivorans* (ε_{cells} = −0.4 ± 0.1‰,[8] $\varepsilon_{extract}$ = −1.4 ± 0.2‰[10]), *Geobacter lovleyi* (ε_{cells} = not significant,[9] $\varepsilon_{extract}$ = −2.3 ± 0.4‰), *Desulfuromonas michiganensis* (ε_{cells} = −1.7 ± 0.1‰, $\varepsilon_{extract}$ = −2.6 ± 0.4‰), *Dehalobacter restrictus* (ε_{cells} = −4.0 ± 0.2‰, $\varepsilon_{extract}$ = −6.3 ± 0.2‰), and *Desulfitobacterium hafniense* strain PCE-S (ε_{cells} = −5.2 ± 1.5‰,[8] $\varepsilon_{extract}$ = −7.6 ± 0.6‰). Systematically higher isotope fractionation using crude extracts pointed to the cellular membrane as a rate-limiting barrier, resulting in reduction of the observed isotope fractionation in intact cells, as previously observed by Nijenhuis et al.[8] Moreover, significantly higher isotope fractionation of PCE was observed for Gram-positive bacteria (−4.0‰ to −5.2‰ for growing cells of *D. restrictus* and *D. hafniense* strain PCE-S) compared to the bacteria containing Gram-negative outer-membrane (not significant to −1.7‰ for growing cells of Gram-negative *S. multivorans*, *G. lovleyi*, and *D. michiganensis*).

In contrast, for TCE dehalogenation only modest differences in isotope enrichments between growing cells and the corresponding bacterial crude extracts were measured for all microorganisms and cell wall types: *S. multivorans* (ε_{cells} = −18.7 ± 4.2‰,[17] $\varepsilon_{extract}$ = −20.0 ± 0.5‰[10]), *G. lovleyi* (ε_{cells} = −8.5 ± 0.6‰,[9] $\varepsilon_{extract}$ = −9.3 ± 0.6‰), *D. michiganensis* (ε_{cells} = −4.3 ± 0.2‰, $\varepsilon_{extract}$ = −7.0 ± 0.7‰), *D. restrictus* (ε_{cells} = −8.4 ± 0.9‰, $\varepsilon_{extract}$ = −7.7 ± 0.4‰), and *D. hafniense* strain PCE-S (ε_{cells} = −12.2 ± 2.3‰,[9] $\varepsilon_{extract}$ = −12.9 ± 0.4‰) (Table 1). In addition, isotope enrichment for TCE dehalogenation was observed to be generally stronger in comparison to PCE.

Our results indicate that cell envelope composition has a major effect on observed variabilities of microbial isotope fractionation. Moreover, properties of the degraded substrate may significantly affect the intracellular microscale mass transfer. This may mask the magnitude of detected isotope fractionation, as observed for the highly hydrophobic PCE.

Properties of Cell Envelope and Effect on PCE/TCE Interaction. In order to investigate effects of cell envelope type and properties on isotope fractionation, Gram-negative *S. multivorans* and Gram-positive *D. hafnienese* strain PCE-S were used as model organisms and different cultivation conditions were tested. Either fumarate or pure chlorinated ethene (PCE or TCE) was applied as electron acceptor. The corresponding cells were used for evaluation of cellular response to different growth conditions by determining chloroethene sorption capacity by cellular membranes, hydrophobicity of the cell envelope and fatty acid composition of the membrane.

Sorption Capacity by Cellular Membranes. No significant cellular sorption of TCE was observed for *S. multivorans*, as well as for *D. hafniense* in all experiments, however, sorption of PCE was present. Further, cellular sorption was observed to be about three times higher for *S. multivorans* with K_d = 0.42 ± 0.09 L g^{-1} (cultivated with fumarate) and K_d = 0.51 ± 0.27 L g^{-1}(cultivated with PCE), in comparison to *D. hafniense* with K_d = 0.13 ± 0.09 L g^{-1} (cultivated with fumarate) (Supporting Information Figure S1).

Hydrophobicity of the Cell. Similar cell hydrophobicity was observed for *D. hafniense* strain PCE-S cells cultivated with fumarate (contact angle = 59.1 ± 3.7°) or PCE (60.8 ± 3.0°) as electron acceptor (Table 2), indicating that the Gram-positive *D. hafniense* did not modify the cell surface hydrophobicity during cultivation. Remarkably, a significant change of cell hydrophobicity was observed already after one subcultivation for the Gram-negative *S. multivorans,* since cells grown with

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Environ. Sci. Technol. 2015, 49, 4293−4301

Table 2. Sorption Properties and Cell Envelop Hydrophobicity of *S. multivorans* and *D. hafniense* Strain PCE-S[a]

	cultivation ED/EA	K_d for PCE [L g^{-1}]	K_d for TCE [L g^{-1}]	contact angle (°)
S. multivorans	Pyr/Fum	0.42 ± 0.09	0.05 ± 0.02	72.2 ± 2.0
	Pyr/PCE	0.51 ± 0.27	ns	33.0 ± 3.4
***D. hafniense* strain PCE-S**	Pyr/Fum	0.13 ± 0.09	ns	59.1 ± 3.7
	Pyr/PCE	–	–	60.8 ± 3.0

[a]Sorption of chlorinated ethenes at the microbial membrane is given as sorption coefficient K_d. The hydrophobicity of the cell envelope was determined via contact angle (°) of a water drop on a cell surface layer; conclusively, higher contact angle represents higher hydrophobicity. ns: not significant, –: not determined.

fumarate were considerably more hydrophobic (contact angles 72.2 ± 2.0°) compared to when grown with PCE (33.0 ± 3.4°). This result indicates that *S. multivorans* adapts as response to different cultivation conditions and that the cell surface was more hydrophilic in the presence of hydrophobic chloroethenes, as it was already observed for other Gram-negative microorganisms.[34] Consequently, transport across the hydrophilic cell surface may become more difficult for more hydrophobic compounds such as PCE.

Fatty Acid Composition of the Membrane. Similar to previous reports with using different bacterial strains and solvents,[35−39] alteration of the fatty acid composition was observed for *S. multivorans* and *D. hafniense* as a response to PCE vs fumarate as electron acceptor. The ratio of saturated fatty acids increased about three times for *D. hafniense* and more than four times for *S. multivorans* in the presence of PCE as electron acceptor (Supporting Information Figure S3). Saturated fatty acids result in better alignment of the cellular membrane and reduce the permeability for hydrophobic compounds.[40] Our results indicate that cultivation of microbes with chlorinated ethenes as electron acceptors may result in modification of the cell envelope composition, which apparently affects mass transfer across microbial membranes.

Effect of Cell Envelope Composition of *S. multivorans* on Isotope Fractionation. The effect of bacterial cell envelope composition on isotope fractionation was investigated using resting cells of *S. multivorans.* The cells were previously cultivated with pyruvate/PCE (Pyr/PCE), formate/PCE (Form/PCE) and pyruvate/fumarate (Pyr/Fum) as electron donor/acceptor pairs, respectively. For the subsequent determination of isotope fractionation methyl viologen was used as artificial ED in all dehalogenation experiments, addressing only the periplasmic located PceA (Supporting Information Figure S4A).

Carbon isotope enrichment factors of TCE were similar to all combinations (ε_C = −26.7 ± 0.6‰ for Pyr/PCE cultivation, ε_C = −24.9 ± 0.3‰ for Form/PCE cultivation and ε_C = −26.0 ± 0.4‰ for Pyr/Fum cultivation) (Table 3 and Supporting Information Figure S5). In contrast, significantly lower isotope fractionation was measured for PCE dehalogenation by cells previously cultivated with chlorinated ethenes (ε_C = −1.5 ± 0.2‰ for Pyr/PCE cultivation; −1.6 ± 0.1‰ for Form/PCE cultivation) in comparison to cells cultivated with fumarate (ε_C = −2.2 ± 0.2‰) (Table 3 and Supporting Information Figure S5).

Prior experiments already demonstrated that TCE was insignificantly affected by cellular sorption, and therefore supposed to be less affected by intracellular mass transfer. Determined isotope fractionation for TCE catalyzed by resting cells supported this observation. However, this was not the case for PCE. Isotope fractionation of PCE is not only much smaller, but also significantly different according to the cultivation conditions. The observed masking of isotope fractionation for PCE may be explained by the decrease in microscale mass transfer of PCE through the outer-membrane prior to the dehalogenation reaction by PceA RDase in periplasm. Cells previously cultivated with Pyr/Fum were measured to be more hydrophobic in comparison to cells cultivated on Pyr/PCE. Therefore, microscale mass transfer of hydrophobic PCE may be more rate-limited by hydrophilic cell envelope resulting in a higher masking of isotope effects.

Effect of RDase Localization of *S. multivorans* on Isotope Fractionation. In order to investigate the effect of the cytoplasmic membrane as a second rate-limiting barrier, *S. multivorans* was cultivated with pyruvate (ED) and fumarate (EA) only. As reported by John et al.,[21] cultivation of *S. multivorans* with fumarate as EA resulted in the localization of PceA-RDase in the cytoplasm compared to the mainly periplasmic location when cultivated with PCE (Supporting Information Figure S4B). In our case, however, Western blot analysis revealed only a partial localization of PceA in the cytoplasm even after 15th subcultivations (Supporting Information Figure S6).

Dehalogenation experiments and the corresponding measurements of isotope fractionation of PCE and TCE were done with resting *S. multivorans* cells previously cultivated for 15 subcultivations with pyruvate (ED) and fumarate (EA). In order to address only the membrane-bound PceA RDase in the periplasm methyl viologen (MV) was used as an artificial electron donor, since MV cannot penetrate the cytoplasmic

Table 3. Carbon Isotope Enrichment Factors for Crude Extract (CE) and Resting (RC) Cells Cultivated under Different Conditions[a]

cultivation	dehalogenation assay			PCE			TCE		
ED/EA	cells/extract	ED	localization of reacting PceA	εC [‰]	CI [95%]	R^2	εC [‰]	CI [95%]	R^2
pyruvate/fumarate	CE	MV	free	−2.2	±0.2	0.984	−24.9	±0.4	0.999
pyruvate/fumarate	RC	MV	peri	−2.2	±0.2	0.993	−26.0	±0.4	0.999
pyruvate/PCE	RC	MV	peri	−1.5	±0.2	0.985	−26.7	±0.6	0.999
formate/PCE	RC	MV	peri	−1.6	±0.1	0.993	−24.9	±0.3	0.999
pyruvate/PCE	RC	H_2	peri	−1.5	±0.2	0.976	−24.5	±3.1	0.983
pyruvate/Fumarate	RC	H_2	peri + cyto	−1.0	±0.1	0.990	−25.3	±0.4	0.999

[a]The reacting PceA was localized either in the periplasm (peri), cytoplasm (cyto) or was free accessible in bacterial crude extracts. Either MV (methyl viologen) or hydrogen were used as electron donor (ED).

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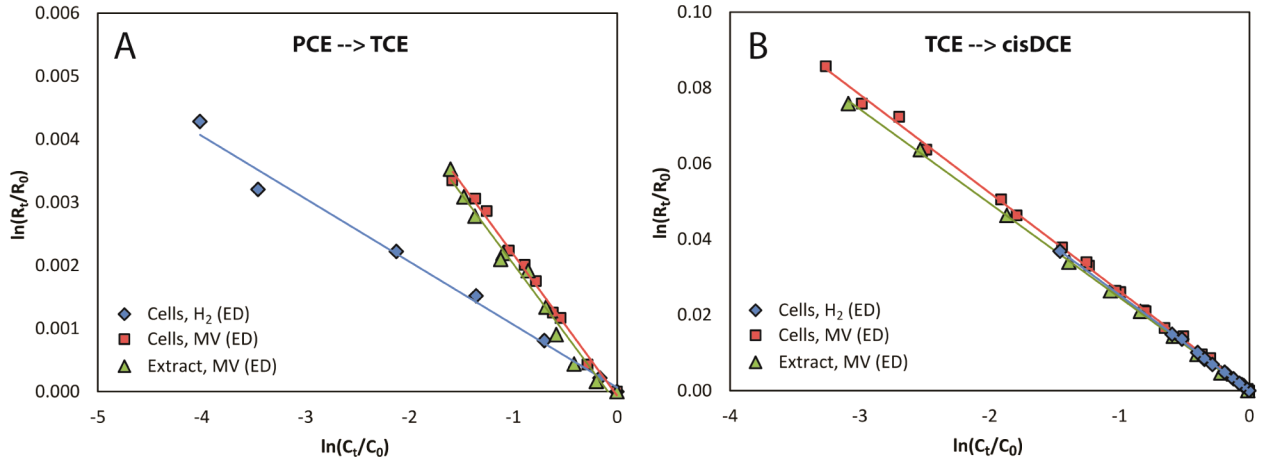

Figure 1. Carbon isotope fractionation of PCE (A) or TCE (B) during dehalogenation by *S. multivorans*. The effect of the cellular membranes was investigated using crude extracts (no barrier) in combination with MV as electron donor, resting cells in combination with MV as electron donor (outer membrane as only barrier) or resting cell in combination with hydrogen as physiological electron donor (outer + cytoplasmic membrane barriers). Effect of cytoplasmic-membrane on masking of isotope fractionation is linked to the substrate dependent localization of the PceA RDase enzyme.

membrane.[29,30] The corresponding carbon isotope enrichment factors were $\varepsilon_C = -2.2 \pm 0.2‰$ for PCE and $\varepsilon_C = -26.0 \pm 0.4‰$ for TCE (Table 3). Likewise, the application of crude extract instead of resting cells at the same conditions resulted in similar carbon isotope enrichment factors $\varepsilon_C = -2.2 \pm 0.2‰$ for PCE and $\varepsilon_C = -24.9 \pm 0.4‰$ for TCE. Alternatively, hydrogen was used as a native electron donor (in combination with resting cells only) to address also the cytoplasmic PceA RDase. Corresponding carbon isotope enrichment was measured to be weaker for PCE ($\varepsilon_C = -1.0 \pm 0.1‰$), but did not affect TCE ($\varepsilon_C = -25.3 \pm 0.4‰$). Additional control experiments ruled out specific effects of methyl viologen or hydrogen as electron donors on measured isotope fractionation, since different electron donor (MV vs hydrogen) did not result in any change of isotope fractionation in cells harboring exclusively periplasmatic PceA-RDase (Table 3). Therefore, reduced isotope fractionation of PCE for *S. multivorans* is most probably related to the additional PceA-RDase activity localized in the cytoplasm and the corresponding rate-limiting transport trough the cell membranes.

DISCUSSION

Role of Substrate Hydrophobicity and Cell Envelope Composition on Microscale Mass Transfer. In this study, limitation in microscale mass transfer of chlorinated ethenes during microbial dehalogenation was considered to be determined by (1) the hydrophobic properties of the degraded compound and (2) the properties of the cell envelope. This hypothesis is supported by several observations:

(a) Isotope fractionation for dehalogenation of TCE, in comparison to the more hydrophobic PCE, was overall higher in all experiments and more similar to abiotic isotope fractionation catalyzed by pure corrinoids (Table 1). This indicates an effect of compound hydrophobicity on mass transfer and masking of isotope fractionation.

(b) Higher carbon isotope enrichment factors were obtained in experiments with resting cells of *S. multivorans* cultivated with fumarate, with concurrently higher cell surface hydrophobicity and lower relative content of saturated fatty acids, compared to PCE cultivated cells (Table 3).

(c) For *S. multivorans* the cytoplasmic membrane was observed to be an additional mass transfer barrier for PCE, if the RDase enzyme is localized in the cytoplasm.

(d) Microbial dehalogenation of PCE for growing cultures could be distinguished in two groups: the Gram-negative Proteobacteria with εC −0.4 to −1.7‰ and Gram-positive Firmicutes with εC −4.0 to −5.2‰, suggesting a general effect of the cell envelope composition (Table 1), though an effect of the RDase enzyme cannot be excluded.

Masking of isotope fractionation for PCE indicates microscale mass transfer limitation across the membrane. Differences between PCE and TCE, therefore, may be partly explained by different hydrophobicity of both compounds. Since TCE is a less hydrophobic compound, transport across the cell envelope might be less affected as discussed below. Therefore, hydrophobicity of the degraded compound is probably an important factor that determines microscale mass transfer and the corresponding masking of the isotope fractionation.

Effect of Microscale Mass Transfer on Microbial Isotope Fractionation. During microbial reductive dehalogenation, three main, potentially rate- or mass transfer limiting, barriers can be considered for the chlorinated substrate prior to the catalytic cleavage of C−Cl bond: (1) the reductive dehalogenase enzyme, (2) the cytoplasmic membrane in case of a cytoplasmic location of the enzyme as well as (3) the outer membrane or cell wall.

Reductive Dehalogenase. Evidence for considerable masking of isotope fractionation at the active site of the PceA enzyme was already demonstrated in our earlier dual-element study.[10] In addition, the first crystal structure of PceA of *S. multivorans* was recently published by Bommer et al.[41] and revealed an enzyme structure with an active site inside the core of the protein. To get access to the active site chlorinated hydrocarbons have to pass a 12 Å long and 3 × 5.5 Å wide hydrophobic channel. The channel forms a restriction filter and is thought to disfavor access for molecules much larger than halogenated ethenes. Therefore, Rdh-enzymes may restrict the mass-transfer for highly hydrophobic compounds to the active site and enhance the masking of observable isotope fractionation. Evidence for rate-limitation at the active-site of

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PceA-RDase was provided by Renpenning et al.[10] by comparing abiotic reaction rates mediated by several corrinoids (i.e., cyanocobalamin, dicyanocobinamide, norpseudo-B_{12} and nor-B_{12}) to enzymatic reaction rates catalyzed by PceA of *S. multivorans*. Using corrinoids abiotic dehalogenation rates were observed to be about 10-times faster for PCE vs TCE, while catalyzed by enzymes dehalogenation rates were similar for both chlorinated ethenes. In addition to *S. multivorans*, isotope fractionation determined for *G. lovleyi* and *D. michiganensis* crude extracts provides evidence to support this hypothesis, since enrichment factors for PCE were measured to be −2.3 to −2.6‰, whereas for TCE significantly higher enrichment factors of −7.1 to −9.3‰ were observed. Rate-limitation at the active-site of the enzyme would explain the overall low isotope fractionation of hydrophobic PCE by *S. multivorans*, *G. lovleyi*, and *D. michiganensis*, whereas the less hydrophobic TCE was not observed to be affected. Nonetheless, mass transfer of TCE may be affected. However, since the abiotic dehalogenation is reported to be about 10-times slower for TCE in comparison to PCE, the rate of transport apparently exceeds the overall rate of the reaction.[10,42] Therefore, rate-limitation of the catalytic step (C−Cl cleavage) and corresponding masking of isotope effects cannot be observed for TCE.

Cytoplasmic-Membrane. The cytoplasmic membrane was shown to result in a significant rate-determining step prior to dehalogenation. Isotope enrichment of PCE for the same *S. multivorans* cells was significantly reduced from $\varepsilon_C = -2.2 \pm 0.2$‰ (only periplasmic PceA-RDase active) down to $\varepsilon_C = -1.0 \pm 0.1$‰ (cytoplasmic and periplasmic PceA-RDase active) (Figure 1 and Table 3). Assuming a simplified case of approximate 1:1 share of PceA-RDase at both sides of the cytoplasmic membrane (Supporting Information Figure S6), isotope enrichment factor of exclusively cytoplasmic PceA-RDase can be estimate to be close to 0‰. Therefore, cytoplasmic dehalogenation may explain partly the variability of isotope enrichment factors observed in different studies for growing cells in comparison to crude extracts or purified enzyme, as for instance isotope enrichment factors for PCE dehalogenated by mixed culture KB-1 (−2.6 to −5.5‰), *Desulfitobacterium* strain PCE-S (−5.2 to −8.9‰), and *Geobacter lovleyi* (not significant to −2.3‰).[8−10,43]

Outer-Membrane. In contrast to Harding et al.,[16] different growth conditions were observed to affect cell envelope properties of *S. multivorans*, as well as the extent of isotope fractionation of PCE. Microscale mass transfer induced masking of isotope fractionation was already observed in bioavailability studies at low substrate concentration and concentration gradients.[44,45] Furthermore, the effect of rate-limiting mass transfer on isotope fractionation was demonstrated in several studies using high biomass concentration.[46−48] Therefore, our results suggest an additional rate-limiting mass transfer at the outer-membrane of *S. multivorans*.

Since no outer-membrane is present, rate-limiting effect on isotope fractionation is supposed to be negligible for Firmicutes and Chloroflexi. Still, isotope fractionation was measured to be significantly different between growing cells and crude extracts for Gram-positive *D. hafniense* and *D. restrictus* (Table 1). These results cannot be explained by the presence or absence of an intact outer-membrane alone, but may also be due to cytoplasmic activity of the RDase.

In summary, our experiments revealed significant masking of isotope fractionation of PCE by the outer- and cytoplasmic-membrane, as well as the RDase of *S. multivorans* (Table 3). The effects were mainly observed for PCE, but not for TCE. Nevertheless, as discussed above, TCE mass transfer may still be limited at the membranes, however, does not limit the rate of the overall reaction due to lower rate of catalytic reaction for TCE compared to PCE.[10,42] Further, we were able to confirm the in vivo activity of the cytoplasmic PceA-RDase in *S. multivorans*. Though thus far only investigated for *S. multivorans*,[21] active cytoplasmic dehalogenase may occur frequently in organohalide respiring bacteria during the initial growth, and therefore, affect observed isotope fractionation. Microscale mass transfer of chlorinated ethenes in this case will be limited by outer-membrane, cytoplasmic-membrane and RDase enzyme. Similarly to the effect of mass transfer and bioavailability discussed above, determined enrichment factors will not elucidate the real magnitude of isotope fractionation. Conclusively, isotope fractionation may be underestimated in microbial systems compared to the actual chemical reaction and not appropriate for evaluation of reaction mechanisms.

Implication of Using Single Element CSIA for Evaluation of Microbial Reactions. Though CSIA was proven to be a useful method for monitoring in situ remediation and characterization of reaction mechanisms, for application of single-element isotope analysis one has to consider the limitations in biological systems. This study was able to demonstrate the systematic masking of isotope fractionation, due to microbial microscale mass transfer limitation as a result of different cultivation conditions and the corresponding modification of bacterial membrane of *S. multivorans* and the localization of the PceA RDase. Furthermore, hydrophobic properties of the degraded compound and type of membrane were observed to affect mass transfer at the membrane and mask the magnitude of the real isotope fractionation significantly, as shown for PCE. Therefore, our results offer an explanation for the previously observed variability in stable isotope fractionation.

Overall, characterization of microbial reactions may be improved by application of bacterial crude extracts or purified enzymes. In addition, application of multielement isotope analysis by combining different element involved in the reaction provided more valuable information.[10,14,15,49,50] Therefore, characterization of reductive dehalogenation enzymes may be achievable with CSIA by addressing multiple elements. However, for the monitoring and quantification of in situ biodegradation application of enrichment factors determined from reactions with bacterial crude extracts and purified enzymes are most probably inappropriate, as isotope fractionation may be overestimated. Here enriched mixed cultures or growing cells, with the according substrates, have to be used for determination of appropriate enrichment factors.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest

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Publication IV

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On-Line Hydrogen-Isotope Measurements of Organic Samples Using Elemental Chromium: An Extension for High Temperature Elemental-Analyzer Techniques

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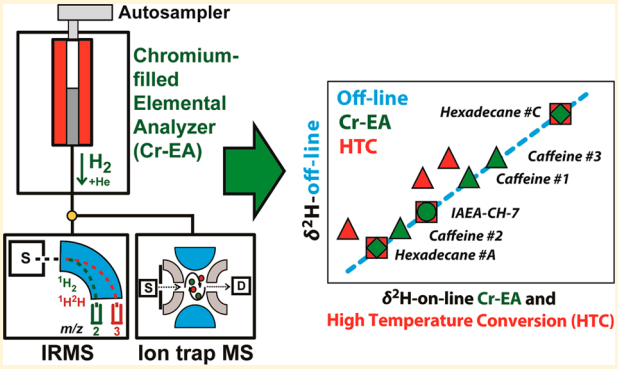
Supporting Information

ABSTRACT: The high temperature conversion (HTC) technique using an elemental analyzer with a glassy carbon tube and filling (temperature conversion/elemental analysis, TC/EA) is a widely used method for hydrogen isotopic analysis of water and many solid and liquid organic samples with analysis by isotope-ratio mass spectrometry (IRMS). However, the TC/EA IRMS method may produce inaccurate δ^2H results, with values deviating by more than 20 mUr (milliurey = 0.001 = 1‰) from the true value for some materials. We show that a single-oven, chromium-filled elemental analyzer coupled to an IRMS substantially improves the measurement quality and reliability for hydrogen isotopic compositions of organic substances (Cr-EA method). Hot chromium maximizes the yield of molecular hydrogen in a helium carrier gas by irreversibly and quantitatively scavenging all reactive elements except hydrogen. In contrast, under TC/EA conditions, heteroelements like nitrogen or chlorine (and other halogens) can form hydrogen cyanide (HCN) or hydrogen chloride (HCl) and this can cause isotopic fractionation. The Cr-EA technique thus expands the analytical possibilities for on-line hydrogen-isotope measurements of organic samples significantly. This method yielded reproducibility values (1-sigma) for δ^2H measurements on water and caffeine samples of better than 1.0 and 0.5 mUr, respectively. To overcome handling problems with water as the principal calibration anchor for hydrogen isotopic measurements, we have employed an effective and simple strategy using reference waters or other liquids sealed in silver-tube segments. These crimped silver tubes can be employed in both the Cr-EA and TC/EA techniques. They simplify considerably the normalization of hydrogen-isotope measurement data to the VSMOW-SLAP (Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation) scale, and their use improves accuracy of the data by eliminating evaporative loss and associated isotopic fractionation while handling water as a bulk sample. The calibration of organic samples, commonly having high δ^2H values, will benefit from the availability of suitably ^{2}H-enriched reference waters, extending the VSMOW-SLAP scale above zero.



Hydrogen stable isotope analysis of organic compounds has been a valuable tool for hydrology, earth sciences, ecology, biochemistry, environmental research, and many other disciplines for more than 60 years.[1] The traditional measurement of hydrogen isotopes of water involved either conversion of water to gaseous elemental hydrogen (H_2) or equilibration with gaseous hydrogen, and subsequent mass spectrometric analysis. Analysis of the H_2 gas has been performed with a mass spectrometer, a dual-inlet isotope-ratio mass spectrometer (DI-IRMS), or a continuous-flow isotope-ratio mass spectrometer (CF-IRMS). The hydrogen-isotope measurement of substances other than water has included (1) oxidation to water, reduction by zinc to gaseous H_2, and analysis by DI-IRMS,[1,2] (2) oxidation to water, reduction by uranium, and analysis by DI-IRMS,[3,4] (3) high-temperature pyrolysis and analysis by DI-IRMS,[5] (4) reduction by chromium directly in a DI coupled reactor and subsequent analysis by DI-IRMS,[6] (5) high-temperature combustion

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of organic mixtures, gas chromatographic separation, and analysis by CF-IRMS,[7] (6) reduction by carbon on an alumina pyrolysis tube,[8−10] (7) high-temperature pyrolysis in an elemental analyzer and analysis by CF-IRMS,[11−14] (8) reduction by chromium in an elemental analyzer and analysis by CF-IRMS,[15,16] and (9) combustion of organic materials and hydrous minerals to water followed by hydrogen isotope-ratio analysis using a laser.[17]

In recent years, the predominant method for hydrogen-isotope measurements of water, liquids, and solids has been on-line CF-IRMS, utilizing a helium carrier gas and either high-temperature conversion (HTC) or chromium reduction. HTC involves conversion of the hydrogen-bearing sample to molecular hydrogen in a glassy carbon filled inert reactor (tube-in-tube construction) at a temperature of 1100 to 1500 °C. Commercial HTC systems are available from Thermo Fisher Scientific (TC/EA, Waltham, Massachusetts, USA), HEKAtech GmbH (HTO, Wegberg, Germany), and Elementar Analysensysteme GmbH (Vario Pyro Cube, Hanau, Germany). Elementar provides commercial chromium reduction systems (HDChrome) as an option to their line of elemental-analyzer systems. In these systems, the EA is used at first to oxidize hydrogen-bearing samples to water, followed by on-line chromium reduction of this water to molecular hydrogen.

In an HTC system, water is reduced following eq 1.

$$H_2O + C \rightarrow H_2 + CO \quad (1)$$

Reduction of hydrogen-bearing samples to molecular hydrogen with chromium can be performed in the range of 1050 to 1270 °C in a quartz reactor (as high as 1400 °C in a ceramic reactor) containing chromium metal; the stoichiometry of the reaction for water follows eq 2.

$$3H_2O + 2Cr \rightarrow 3H_2 + Cr_2O_3 \quad (2)$$

Both methods are useful for water. The HTC glassy carbon technique has the advantage that it can also be used for analyzing oxygen isotopic compositions by monitoring the ion currents of the produced carbon monoxide at m/z 28 and 30, often even within the same run. Direct injection of liquid samples by an autosampler through a septum into the reactor offers convenience and superior reproducibility.

The handling of 0.15−1 μL liquid samples by manual injection into silver or tin capsules, rapid crimping of capsules, and measurement in an EA without sample loss are challenging due to the risk of partial evaporation and isotopic fractionation. In spite of these technical difficulties, the use of standard waters anchored to the VSMOW-SLAP (Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation) hydrogen-isotope scale remains an essential step for properly calibrating hydrogen isotopic results.

In practice, the HTC method can give problematic results when organic samples with nitrogen or halogen atoms are measured due to interference with quantitative conversion of organic hydrogen to molecular hydrogen. Resulting erroneous delta values are likely due to nonquantitative yields of molecular hydrogen.[16,18−21]

In this contribution, we make an effort to (i) scrutinize the yield of molecular hydrogen in reactions of chemically different organic samples, (ii) describe an improved method to reduce the isotopic fractionation during conversion to molecular hydrogen, (iii) enhance the quality of hydrogen-isotope measurements of solid and encapsulated liquid samples, and (iv) develop an improved strategy for hydrogen isotopic calibration of solid and encapsulated liquid samples.

To comply with guidelines for the International System of Units (SI), we follow the proposal of Brand and Coplen[22] and use the term urey, after H. C. Urey[23] (symbol Ur), as the isotope-delta[24] value unit. In such a manner, an isotope-delta value expressed traditionally as −25 per mil can be written −25 mUr (or −2.5 cUr or −0.25 dUr; use of any SI prefix is permissible).

EXPERIMENTAL SECTION

Materials. At the Leipzig Laboratory for Stable Isotopes (LSI), chromium powder (<0.3 mm, Patinal, Merck, Germany), quartz chips (2−3 mm, HEKAtech GmbH, www.hekatech.com), and quartz wool (HEKAtech) were used to fill an empty quartz reactor for an EA-system (Euro EA 3000, Euro Vector, Milan, Italy). The Centre for Isotope Research (CIO) laboratory at the University of Groningen used a similar technique.

Solid samples were loaded into 4 × 6 mm silver capsules or 3.6 × 5 mm tin capsules (HEKAtech). For volatile samples, e.g., *cis*-dichloroethylene (DCE # 2) and trichloroethylene (TCE), approximately 5 mg of Com-Aid for liquids (aluminum oxide powder, Leco Corp., St. Joseph, Michigan, USA) was added together with the sample in a 2 × 5 mm tin cup for liquids. This reduced the evaporation of the sample on the metal surface.

At the Reston Stable Isotope Laboratory (RSIL), chromium powder (200 μm, 99.0% purity, Goodfellow), glassy carbon chips (3−4 mm, www.microanalysis.co.uk), and silver wool (part No. 22131365, Elementar) were used to pack a ceramic tube (C1101, www.microanalysis.co.uk) for the TC/EA. Solid samples were loaded into 3.5 × 5 mm silver capsules (Costech, part No. 41066).

At the Jena MPI laboratory, chromium chips of roughly 2 mm size (CAS 7440-47, Aldrich 255610) were used as the top ∼2 cm layer. Solid samples were loaded into 4 × 6 mm silver capsules. Liquids were loaded into thick-walled, 3.6 × 6 mm silver capsules (IVA Analysentechnik, Meerbusch, Germany), followed by crimping with a standard pair of crimping pliers.

Reference Materials and Samples. Reference waters, VSMOW, VSMOW2, SLAP2, USGS47, and organic liquids (e.g., oils) with volumes from 0.15 to 0.25 μL were crimp-sealed in silver-tube segments (crimped silver tubes) for convenient use in EA applications.[25] The availability of reliably, preloaded and sealed aliquots of primary reference waters from the U.S. Geological Survey (USGS) in Reston, Virginia, USA (http://isotopes.usgs.gov/lab/referencematerials.html) facilitates routine and robust hydrogen isotopic calibration without risking isotopic fractionation during injection of water samples by hand. These waters and UC04[28] ($\delta^2H_{VSMOW\text{-}SLAP}$ = +113.6 mUr, prepared by G. Olack and A. Colman at University of Chicago) reference water were sealed in silver tubes. Additionally, we used IAEA-CH-7 polyethylene foil and IAEA-600 caffeine from the International Atomic Energy Agency (IAEA) in Vienna, Austria (http://nucleus.iaea.org/rpst/ReferenceProducts/ReferenceMaterials/Stable_Isotopes/index.htm), and NBS 22 oil from the U.S. National Institute of Standards and Technology (NIST) (see Table 3). Additional materials were acquired from commercial sources: urea (>99.5%, Fluka, Switzerland), cysteine (>97%, Merck, Germany), 4,4′-DDT (98%, Sigma-Aldrich, Germany), and 4,4′-DDE (98%, ABCR, Germany). Three caffeine samples with different deuterium enrichments (caffeine #1, caffeine #2, and caffeine #3, with purities ≥99%), *cis*-dichloroethylene (DCE #2, purity ≥99.5%), and

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trichloroethylene (TCE, purity ≥99.5%) had been prepared and characterized isotopically at Indiana University using off-line combustion in quartz ampules, uranium reduction of water to molecular hydrogen, and analysis by DI-IRMS (http://mypage.iu.edu/~aschimme/compounds.html).

Instrumental Setup. *Off-Line Method.* Any nonquantitative conversion of the elements hydrogen, carbon or nitrogen in organic compounds to analyte gases H_2, CO_2, and N_2 for measurement of stable isotope ratios can potentially entail isotopic fractionation. The traditional off-line combustion of milligram amounts of organic material with copper oxide (CuO) at 800 °C in evacuated and sealed "quartz" ampules overnight offers sufficient time at high temperature to yield quantitatively H_2O, CO_2, and N_2 after cooling to 300 °C. Cryogenic separation of combustion products in a vacuum line yielded pure N_2 (collected with a Toepler pump and flame-sealed into a glass tube) and CO_2 (collected and sealed in a glass tube at the temperature of liquid N_2). A subsequent step of reducing H_2O to H_2 in the presence of uranium metal at 800 °C is a classical method for preparation of H_2 for stable isotope mass spectrometry[29] and was applied uniformly to reference waters (VSMOW2, SLAP2, and IAEA-604 (+800 mUr) generated reference H_2 samples) and for waters from combustion of organics.[30] We found no isotopic difference between reference waters that were either "pre-combusted" with CuO in "quartz" or directly admitted to hot uranium in the vacuum line.

On-Line Methods. At Leipzig, a Euro EA 3000 (EuroVector SpA, Milan, Italy) was modified to a single-oven system by connecting the exit of the chromium-reduction oven to a GC column (Figure 1). An empty quartz reactor was filled with

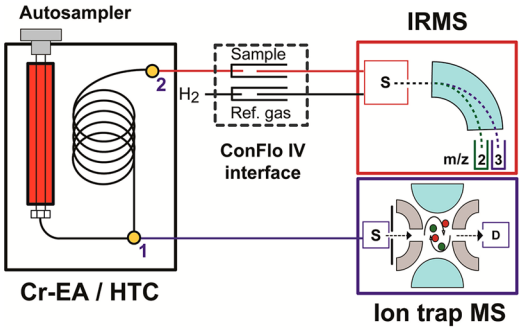

Figure 1. Diagram of instrumentation for converting organic samples to molecular hydrogen for subsequent measurement of the hydrogen stable isotopic composition by isotope-ratio mass spectrometry, with parallel simultaneous identification of byproduct formation (ion-trap mass spectrometry) connected at postreactor position #1 (or post-GC column position #2) used in Leipzig.

20 mm of quartz wool at the bottom, overlain by a ~120 mm column of quartz chips combined with an 80 to 200 mm column of untreated chromium powder (i.e., to the same filling height as in a CHN reactor, 220 mm; see Supporting Information Figure S-16). The chromium layer was separated with 5 mm of quartz wool, and it was topped off with a 3 to 5 mm layer of quartz wool to separate the chromium from accumulating silver and tin residues and to facilitate emptying and cleaning of the top section of the reactor. Silver and tin residues were removed every 130 to 150 samples. The chromium-filled section of the reactor occupied the hottest zone (1050 °C, a maximum temperature of 1270 °C is possible) in the EA. The oxygen valve remained permanently closed, and the helium carrier gas flow was set to 75 mL min^{-1}. The carousel of a Thermo AS200 autosampler attached to the EA was loaded with samples and received a helium purge flow of 50 mL min^{-1}. Helium carrier gas (grade 5.0) swept the generated molecular hydrogen through a GC column and into the high-flow port of a ConFlo IV interface (Thermo Fisher, Bremen, Germany), that was connected via an open split to a MAT 253 IRMS (Thermo Fisher, Bremen, Germany) (Figure 1). Undiluted effluent samples yielded a signal of 1.5 to 13 V for m/z 2 (feedback resistor m/z 2: 1GΩ; m/z 3: 1TΩ). The additional coupling of an organic Ion Trap MS (PolarisQ, Thermo Finnigan, Bremen, Germany) in parallel with the IRMS allowed the simultaneous identification of byproduct formation (Figure 1).

At the RSIL, solid samples were weighed and loaded into silver capsules (Costech, 41066). The δ^2H measurements were made using a HTC (also known as TC/EA for temperature conversion/elemental analysis, Thermo Finnigan, Bremen, Germany) reduction unit equipped with a Costech (Valencia, California) Zero-Blank 100-position autosampler, a ConFlo IV gas introduction system (Thermo Fisher, Bremen, Germany), and a Delta^{plus} XP isotope-ratio mass spectrometer (Thermo Fisher, Bremen, Germany). The glassy carbon tube inside of the ceramic tube normally used for the TC/EA was removed. The ceramic tube was filled from bottom to top with 5 cm of silver wool, 10 cm of glassy carbon chips, 0.8 cm of quartz wool, and 3 cm of chromium powder. The filling height was the same as in the TC/EA. The silver and sample residues were removed every 150 to 200 samples. The helium carrier gas (106 mL min^{-1}) was fed from the top, as originally supplied. The original 0.6 m, 6.35 mm, 5-Å-zeolite GC column of the TC/EA was replaced by a 1.0 m GC column of the same diameter and zeolite GC column. The reactor temperature was set at 1150 °C, and the GC temperature was maintained at 90 °C.

The stable isotope laboratory at the Jena MPI (BGC-IsoLab) slightly modified an existing setup that was described in detail in an earlier publication on $\delta^{18}O$ analysis and reference materials.[31] In short, the equipment was comprised of a HEKAtech HTO high temperature reaction system with GC separation (HEKAtech, Wegberg, Germany) coupled via a ConFlo III[32] open split to a Delta^{plus} XL (Thermo Fisher, Bremen, Germany). The reactor was built using a silicon carbide (SiC) outer tube and an inner glassy carbon tube with reverse carrier gas flow.[33] The reaction zone was kept at a constant temperature of 1400 °C with a continuous helium carrier flow of 60 mL min^{-1}. Samples were placed in a 50-position Zero-Blank autosampler (Costech, Milan, Italy). The upper ~2 cm of glassy carbon chips was replaced with chromium chips; after initial tests, we left the top layer bare, i.e., without quartz wool. A temperature of 1400 °C was necessary to prevent early clogging of the reactor, to ensure availability of of the sample within the hot zone and to analyze >200 samples with a single filling. The upper sample residues were vacuumed out after every complete sequence of 50 reactions. The filling was renewed after the fourth sequence.

At the CIO/Groningen, solid samples were weighed and loaded into silver capsules (OEA laboratories, C12240,100p). The δ^2H measurements were made using a HTC (also known as TC/EA for temperature conversion/elemental analysis, Hekatech, Germany) reduction unit equipped with a Zero-Blank 40-position autosampler, an open split gas introduction system (Isoprime, Cheadle Hulme, England), and an Isoprime isotope-ratio mass spectrometer (Isoprime, Cheadle Hulme, England). The glassy carbon tube normally used for the TC/EA was replaced by a quartz tube conventionally used on a TC/EA.

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Anal. Chem. 2015, 87, 5198−5205

Table 1. Reactions of Various Elements in Organic Compounds with Hot Chromium

element	reaction	result	melting point (°C)
chromium			1907
hydrogen	no reaction with Cr	H_2	
oxygen	$3H_2O + 2Cr \rightarrow Cr_2O_3 + 3H_2$	chromium(III) oxide	2435
carbon	$3Cr + C_2H_4 \rightarrow Cr_3C_2 + 2H_2$	chromium(III) carbide	1890
nitrogen	$2Cr + N_2 \rightarrow 2CrN$	chromium(III) nitride	1500 dissociation to Cr and N_2
sulfur	$2Cr + 3S \rightarrow Cr_2S_3$	chromium(III) sulfide	1350
phosphorus	$Cr + P \rightarrow CrP$	chromium(III) phosphide	
chlorine	$2Cr + 6HCl \rightarrow 2CrCl_3 + 3H_2$	chromium(III) chloride	1152
back-reaction of chromium chloride at high temperature	$6CrCl_3 + 12H_2O + 8Cr \rightarrow 4Cr_2O_3 + 6CrCl_2 + 6HCl + 9H_2$	chromium(II) chloride HCl forms as a byproduct	824

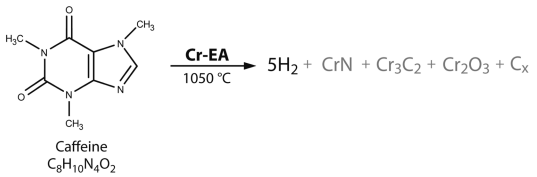
Table 2. Hydrogen Isotopic Comparison of Selected Waters and Organic Samples Using HTC and Chromium Techniques for Determining $\delta^2H_{VSMOW\text{-}SLAP}$ Values[a]

sample	chemical nature	H_2 yield via HTC H_2 [%][b]	H_2 yield via Cr H_2 [%][b]	results via HTC δ^2H/mUr	results via Cr-EA δ^2H/mUr	reference values or via uranium method (AS) δ^2H/mUr
VSMOW2	water, H_2O	100	100	0	0	0
SLAP2	water, H_2O	100	100	−427.5	−427.5	−427.5
IAEA-604	water, H_2O	100	100	+800	+800.9	+800
NBS 22	oil, $(CH_2)_n$	100	100	−116.9	−116.9	−116.9
IAEA-CH-7	polyethylene foil, $(CH_2)_n$	100	100	−100.3	−100.3	−100.3
IAEA-600	caffeine, $C_8H_{10}N_4O_2$	70	101	−186	−157.6	−153 (AS)
urea	urea, CH_4N_2O	92	102	−210	−202	n.d.
cysteine	amino acid, $C_6H_{12}N_2O_4S_2$	91	101	−60	−38	n.d.
cis-DCE	*cis*-dichloroethylene, $C_2H_2Cl_2$	n.d.	n.d.	n.d.	723.5	+728.7 (AS)
TCE	trichloroethylene, C_2HCl_3	n.d.	n.d.	n.d.	505	+505.6 (AS)
DDT	dichlorodiphenyl-trichloroethane, $C_{14}H_9Cl_5$	91	98	−23	−23	n.d.
4,4′-DDE	dichlorodiphenyl-dichloroethane, $C_{14}H_{10}Cl_4$	90	96	−77	−92	n.d.

[a]Results calibrated to the VSMOW-SLAP scale by assigning 0 and −427.5 mUr to VSMOW2 and SLAP2 reference waters, respectively. The standard deviation is ∼± 2 mUr for both methods. Caffeine (IAEA-600) and *cis*-DCE show the limit of the achievable accuracy of hydrogen isotopic measurements. Values marked (AS) were measured at Indiana University using the traditional off-line uranium method. All other results measured in the Leipzig Laboratory of Stable Isotopes (LSI). [b]Calculated assuming a 100% yield of molecular hydrogen from IAEA-CH-7 polyethylene foil. n.d. = not determined.

The quartz tube (OEA laboratories G11116.001e) was filled with chromium powder (Patinal, Merck). The filling height was the same as in the TC/EA. The silver and sample residues were removed every approximately 90 samples. The helium carrier gas (90 mL/min) was fed from the top as originally supplied. The reactor temperature was set at 1030 °C, and the GC temperature was maintained at 90 °C.

Principles. Samples from the carousel fall into the hot zone of a furnace, where the organic material is pyrolyzed and/or reacts with chromium; the tin or silver containers are melted. Except for excess carbon, most of the organic matter volatilizes and is swept through the hot chromium bed by the helium carrier gas. Further high-temperature reactions of the volatile organic remnants with chromium ensure irreversible and quantitative uptake of carbon, nitrogen, oxygen, sulfur, phosphorus, and halogens (eq 3), while molecular hydrogen passes on with the carrier gas. As no chemical byproducts like hydrogen cyanide (HCN) and hydrogen chloride (HCl) are formed, the hydrogen yield will be virtually quantitative. Therefore, the hydrogen isotopic composition of molecular hydrogen should not be affected by hydrogen bearing byproducts (Table 1). For example, the hydrogen in caffeine is chemically converted quantitatively to molecular hydrogen in a chromium EA according to the following nonstoichiometric expression:



(3)

As described above, the chromium reactor can also be installed in a standard HTC system (HTO by HEKAtech or TC/EA by Thermo Scientific, Bremen) inside a quartz or ceramic reactor tube. The chromium-filled segment should occupy the zone with the highest temperature, which can be as high as feasible for the equipment. Care must be taken, though, when quartz wool is part of the packing. Then, the temperature should not exceed the annealing point of quartz wool (∼1200 °C). Samples must fall into a zone with sufficiently high temperature for rapid and quantitative thermal decomposition. The temperature of the lower chromium bed should be within the thermal stability range

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Table 3. Comparison of the Hydrogen Isotopic Two-Point Calibration with VSMOW2 and SLAP2 Reference Waters (427.5 mUr Difference) versus SLAP2 and a ^{2}H-Enriched IAEA-604 Reference Water (1227.5 mUr Difference)

sample	raw data δ^2H/mUr	standard deviation δ^2H/mUr	n	calibrated via VSMOW2 to SLAP $y = 1.011x - 5.4$ δ^2H/mUr	calibrated via SLAP to IAEA-604 $y = 1.010x - 5.7$ δ^2H/mUr	reference value δ^2H/mUr
SLAP2[a]	−417.4	1.3	6	−427.5[b]	−427.5[b]	−427.5[c]
VSMOW2[a]	5.4	1.2	11	0[b]	−0.3	0[c]
UC04[a]	118.2	1.1	5	114.1	113.8	113.6[d]
NBS 22[a]	−110	0.6	5	−116.9	−117.2	−116.9[c]
IAEA-604[a]	797.4	1.1	6	+801	+800[b]	799.9[e]
IAEA-CH-7	−94.0	0.2	5	−100.5	−100.7	−100.3[c]
IAEA-600	−147.9	0.4	5	−157.6	−157.8	−153

[a]Liquids sealed in silver-tube segments from the U.S. Geological Survey (USGS). [b]Internationally defined anchors for calibration. [c]Reference values from IAEA. [d]Reference values from USGS. [e]Value and uncertainty from quantitative, gravimetrical mixing at CIO, University of Groningen.[26]

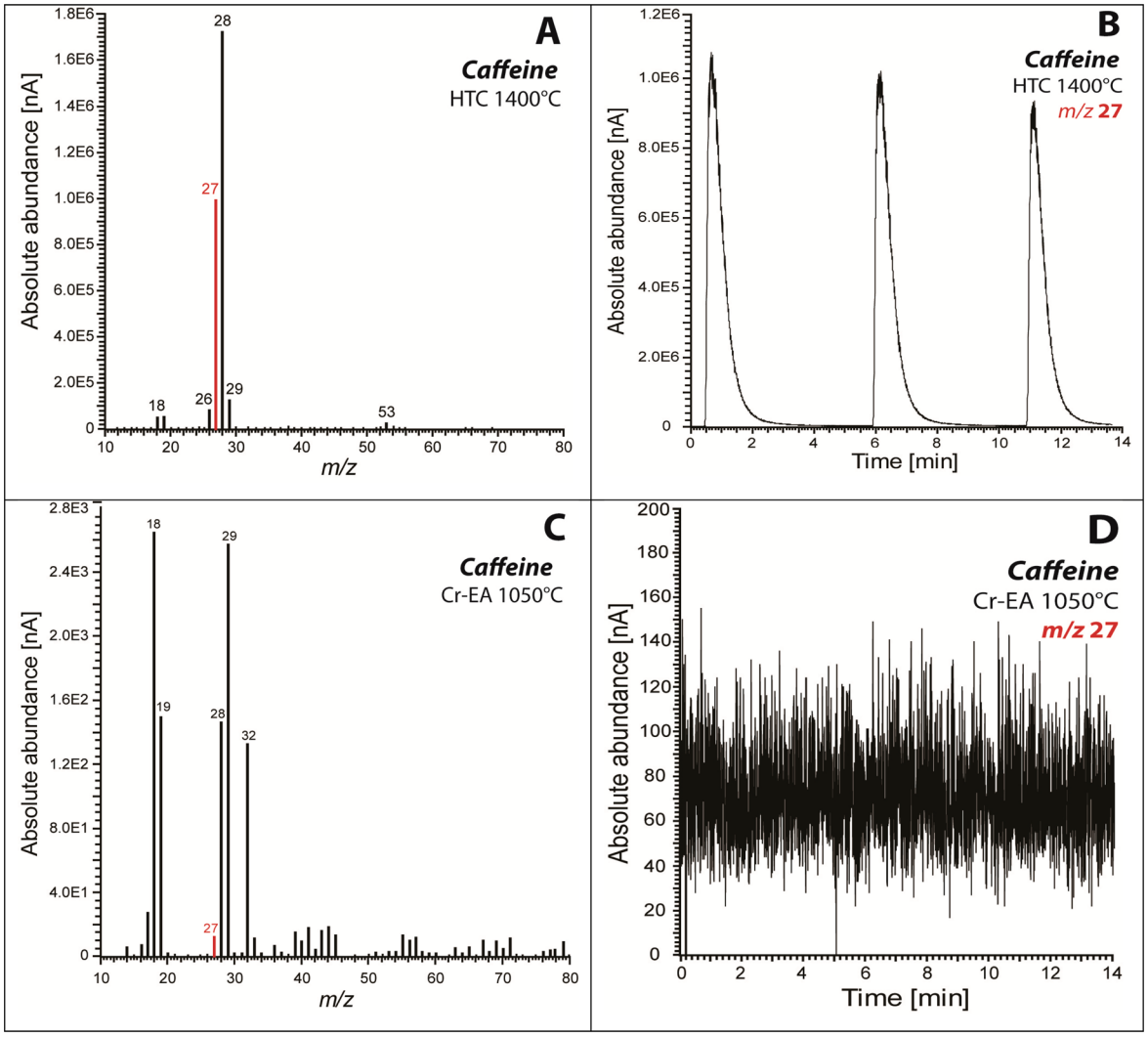

Figure 2. (A) Background scans during high-temperature conversion of caffeine to molecular hydrogen; (B) detection of m/z 27 for measurement of hydrogen cyanide gas, HCN, signal strength >1 mA; (C) background during Cr-EA thermal conversion of caffeine to generate molecular hydrogen (air water background ion trap); (D) detection of m/z 27 for HCN, signal strength <0.1 μA.

of the various carbon, nitrogen, oxygen, sulfur, phosphorus, or halogen-reaction products with chromium (Table 1).

RESULTS AND DISCUSSION

The chromium-elemental analyzer (Cr-EA) method was independently evaluated in four laboratories in direct comparison with the on-line HTC-method (Leipzig LSI, Jena MPI, RSIL, and the CIO/Groningen). In addition, results were compared against hydrogen isotopic data generated by the Bloomington, Indiana laboratory using an off-line manual uranium-based, gaseous hydrogen-preparation method followed by DI-IRMS analysis.

Evaluation of Hydrogen-Bearing Byproducts Affecting the Hydrogen Yield. Long-term experience with HTC systems for hydrogen- and oxygen-isotope measurements shows that some organic substances yield results that are substantially different from those measured by other laboratories. The reasons

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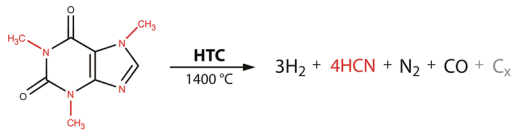
for such behavior have not always been understood; they could be hidden in matrix effects that depend on specific analytical operating conditions or they could be a more systemic effect related to the molecular composition. For example, the presence of organically bound oxygen and nitrogen in caffeine has long been suspected to be responsible for poor comparability of hydrogen isotopic data among laboratories. At the Leipzig LSI, we therefore investigated cases with nonquantitative high-temperature conversion of organic hydrogen to molecular hydrogen with a ion trap mass spectrometer, which was suitable for identifying the expected range of byproducts. In the first experiment, the exhaust of a HTC-EA reactor was sampled immediately at the postreactor position (Figure 1). As a suitable candidate, we selected caffeine, where the yield of molecular hydrogen in the HTC reaction is known to be mostly ~60−70% of the theoretical value.[34] The missing hydrogen must therefore reside in different molecules, probably HCN, as may be inferred from the following reaction (eq 4, non-stoichiometric, Table 2).



Caffeine
$C_8H_{10}N_4O_2$ (4)

Hence the three methyl groups seem to give off one H_2 molecule each, with the third hydrogen remaining bound to nitrogen, producing HCN. The fourth hydrogen in HCN in eq 4 arises from the ring-nitrogen bearing a hydrogen atom. Thus, a theoretical H_2 yield would be 60% exactly. A nonquantitative nitrogen peak can often be seen in the HTC reaction of caffeine when m/z 28 is monitored,[31] and this nitrogen must arise from partial conversion of the instantaneously formed HCN in contact with carbon or other residues in the reactor.

In another HTC experiment involving the ion trap mass spectrometer, we sampled the helium stream after the GC column (Figure 1). Here, we detected only minor signals related to previously observed byproducts. Apparently, most of these pyrolytic HTC byproducts had been effectively trapped on the active GC column. As an example, high-temperature decomposition of caffeine generated HCN as an HTC byproduct that was detected in the postreactor position with an intensity of ~10^6 nA (Figure 2 A, and B). No trace of HCN (m/z 27) could be detected after the GC. In contrast, the Cr-EA system yielded an HCN intensity of only ~100 nA in the postreactor position (Figure. 2 C, and D) with abundances of other byproducts being reduced as well. The water−air background of the Cr-EA system showed an intensity of ~10^3 nA, whereas the equivalent background was elevated at ~10^4 nA for the HTC system (for further results, see the Supporting Information).

The observed yields of molecular hydrogen in the Cr-EA system reached nearly 100% in most cases we studied, and no significant amounts of byproducts were observed, except for halogen-bearing compounds (Table 2). The presence of chlorine caused a relatively small HCl intensity of ~10^3 nA for the chromium EA and a much larger signal of ~10^5 nA for the HTC (see the Supporting Information). In general, reduced yields of molecular hydrogen seem to produce more negative δ^2H values (Table 2).

Reference Materials and Two-Point Calibration to the VSMOW-SLAP Scale. International primary reference waters, laboratory standard waters, and NBS 22 oil were used for calibration with reference $\delta^2H_{VSMOW\text{-}SLAP}$ values as recommended in the IUPAC 2014 tables.[35] The materials were made available by the USGS in crimp-sealed silver-tube segments as described above that hold, for example, 0.25 μL of water (i.e., 0.25 mg of water, or 28 μg of H, or 314 μmol of H_2). Suitable amounts of IAEA-CH-7 polyethylene foil, IAEA-600 caffeine, and other solid materials were weighed into silver or tin capsules and contained the hydrogen equivalent of 0.25 μL of water each.

The development of the silver-tube technique for isotopic-fractionation-free loading of small volumes of liquids (e.g., water, oil[25]) offers an invaluable opportunity to load hydrogen and oxygen isotopic reference materials with well-defined isotope-delta values, interspersed among samples in a carousel. The USGS, in collaboration with the IAEA and the University of Groningen in the Netherlands, is currently expanding their repertoire of available standard waters in crimp-sealed silver-tube segments to include ^{2}H-enriched waters, for example, with a δ^2H value of +800 mUr (IAEA-604) for 2-point calibrations

Table 4. Between-Sample Memory Test by the Reston Stable Isotope Laboratory

analysis sequence	sample	raw data δ^2H/mUr	mean values and standard deviation/mUr
1	USGS47	−145.3	
2	USGS47	−145.8	
3	USGS47	−146.0	
4	USGS47	−145.5	−145.5 ± 0.3
5	VSMOW	4.6	
6	VSMOW	3.9	
7	VSMOW	3.1	3.9 ± 0.7
8	water, +400 mUr	403.2	
9	water, +400 mUr	403.0	
10	water, +400 mUr	405.3	403.8 ± 1.3
11	VSMOW	4.7	
12	water, +400 mUr	403.9	
13	caffeine #3	177.4	
14	caffeine #3	177.7	
15	caffeine #3	176.7	
16	caffeine #3	176.9	
17	caffeine #3	177.7	
18	caffeine #3	177.5	177.3 ± 0.4
19	caffeine #1	99.4	
20	caffeine #1	99.6	
21	caffeine #1	99.0	
22	caffeine #1	99.4	
23	caffeine #1	98.7	99.2 ± 0.4
24	caffeine #2	−150.9	
25	caffeine #2	−150.8	
26	caffeine #2	−151.8	
27	caffeine #2	−150.6	
28	caffeine #2	−151.7	
29	caffeine #2	−151.0	−151.1 ± 0.5
30	USGS47	−146.0	
31	USGS47	−146.7	
32	USGS47	−146.7	−146.5 ± 0.4
33	VSMOW	2.9	
34	VSMOW	3.6	
35	VSMOW	4.8	3.8 ± 1.0
36	water, +400 mUr	403.8	
37	water, +400 mUr	404.6	
38	water, +400 mUr	403.8	404.0 ± 0.4

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Anal. Chem. 2015, 87, 5198−5205

Table 5. $\delta^2H_{VSMOW\text{-}SLAP}$ Results Obtained with On-line HTC, On-line Cr-EA and Off-line Uranium Reduction

laboratory	sample	on-line HTC (TC/EA) δ^2H/mUr	on-line Cr δ^2H/mUr	off-line[a] δ^2H/mUr
Leipzig	IAEA-600	−181.5 ± 1.2, n = 10	−157.8 ± 1.2, n = 15	
RSIL	IAEA-600	−177.9 ± 0.8, n = 4	−158 ± 1.0, n = 4	
Jena MPI	IAEA-600	−175.52 ± 2.4, n = 24	−155.8 ± 1.2, n = 8	
Groningen	IAEA-600	n.d.	−158.3 ± 2, n = 5	
		−178.3 ± 3	−157.5 ± 1.1	−152.4 ± 1.3, n = 8
Leipzig	caffeine #1	74.0 ± 1, n = 10	96.9 ± 1.0, n = 15	
RSIL	caffeine #1	72.0 ± 0.9, n = 6	95.8 ± 0.7, n = 13	
Jena MPI	caffeine #1	71.6 ± 1.1, n = 24	97.6 ± 0.9, n = 4	
Groningen	caffeine #1	73.5 ± 0.7, n = 6	98.6 ± 2.0, n = 10	
		72.8 ± 2.1	97.2 ± 1.2	98.0 ± 1.5, n = 8
Leipzig	caffeine #2	−180 ± 1.4, n = 10	−157.3 ± 1.1, n = 15	
RSIL	caffeine #2	−177.5 ± 2.4, n = 17	−155.3 ± 0.3, n = 14	
Jena MPI	caffeine #2	−174.0 ± 1.6, n = 24	−154.0 ± 0.8, n = 4	
Groningen	caffeine #2	n.d.	−161.1 ± 2.0, n = 10	
		−177.2 ± 3	−156.9 ± 3.1	−152.4 ± 1.5, n = 17
Leipzig	caffeine #3	151.2 ± 1.6, n = 10	175.3 ± 1.4, n = 15	
RSIL	caffeine #3	148.9 ± 1.6, n = 24	173.9 ± 0.6, n = 14	
Jena MPI	caffeine #3	148.1 ± 1.4, n = 24	175.6 ± 1.2, n = 4	
Groningen	caffeine #3	152.4 ± 0.3, n = 6	170.9 ± 2, n = 10	
		150.2 ± 3	173.9 ± 2.1	171.2 ± 4.2, n = 9

[a]Off-line analyses were performed at Indiana University. n.d. = not determined.

substantially above the traditional confines of the VSMOW-SLAP range (Table 2).

Accuracy and Precision of the Cr-EA Method. The quality of hydrogen-isotope results is composed of two parts. First, a highly linear and stable IRMS ion source with a very stable H_3^+ factor is essential. At the UFZ, the H_3^+ factor was determined twice a day within an interval from 1 to 12 nA in a fully automated fashion and proved stable within 6.4 ± 0.05 ppm nA^{-1} over several weeks. Second, the conversion method needs to be quantitative for hydrogen and should be checked by suitable reference materials.[30] The recovery of hydrogen on weighing and the integration of the area is a simple, accurate, and necessary method for evaluation.

The reproducibility of δ^2H values was better than ±2 mUr for all laboratories. At Leipzig, a small between-sample memory effect of <2% was observed (see the Supporting Information) and corrected. In the Jena, a small memory effect was also observed, similar to the one observed in water analysis[33] and routinely corrected for the effect. The CIO/Groningen laboratory had similar findings, but the memory effect depended on the specific material combusted and was therefore harder to correct for in a systematic way than when analyzing water samples only, such as in Guidotti et al.[36] This led to sample peaks frequently missing after a jump in delta value.

No between-sample memory or drift over time was observed at the RSIL. A typical analysis sequence with measured δ^2H values is shown in Table 4.

Different samples with and without nitrogen or chlorine were examined with HTC-EA and Cr-EA systems. Yields of molecular hydrogen and δ^2H values were compared with the established off-line method (uranium reduction). The overall results are given in Table 2. Comparative experiments were carried out by analyzing three types of caffeine with different levels of deuterium enrichment using three different methods, namely, (i) off-line reduction, (ii) an on-line HTC or TC/EA with a glassy carbon chip-filled reactor, and (iii) an on-line, chromium-filled reactor. Table 5 summarizes $\delta^2H_{VSMOW\text{-}SLAP}$ results obtained by the three methods. The hydrogen-isotope results obtained via on-line Cr-EA closely resemble the results determined by the off-line method within analytical uncertainty. The reproducibility of the δ^2H analyses on caffeine samples from different laboratories can be as good as ±1.5 mUr. However, the hydrogen-isotope results from three caffeine samples via on-line HTC (or TC/EA) are too low by about 20 to 26 mUr, albeit with reasonably good reproducibility (<2.6 mUr). This surprising reproducibility, indicating a rather reproducible isotopic fractionation, can be deleterious to achieving high-quality results. For instance, a laboratory might not become suspicious of something going wrong when analyzing such nitrogen-bearing compounds on an HTC system.

Diffusion of Hydrogen Halides into Chromium. Hydrogen chloride gas, as well as other hydrogen halides, can form byproducts and temporarily diffuse into hot chromium. This unaccounted-for hydrogen leads to reduced molecular hydrogen yields, memory effects, and potential isotopic fractionation. It may even affect subsequent nonhalogenated samples in a sequence when hydrogen halides diffuse back out of the chromium. Chromium binds strongly with chlorine to form chromium(III) chloride. However, above ~800 °C, chromium(III) chloride can back-react with molecular hydrogen to form hydrogen chloride (HCl, back-reaction) (see the Supporting Information).

■ CONCLUSIONS

The chromium-elemental analyzer (Cr-EA) method for converting organic hydrogen to molecular hydrogen is a powerful tool for the measurement of hydrogen isotopic compositions of organic matter that may contain heteroelements (e.g., nitrogen, chlorine) in addition to carbon and hydrogen. The Cr-EA method is far less susceptible to matrix effects that may limit the usefulness of high-temperature thermal conversions in glassy carbon environments (e.g., TC/EA). The convenient availability of ready-to-use aliquots of isotopic reference waters from the U.S. Geological Survey (USGS, http://isotopes.usgs.gov/lab/referencematerials.html) in which water is securely crimp-sealed in silver tubes greatly simplifies two-point calibration to the hydrogen-isotope VSMOW-SLAP scale. The Cr-EA method

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works well with such preloaded water references and enables laboratories to tie hydrogen isotopic measurement results of organic substances directly to the VSMOW-SLAP scale. Further testing will be required to evaluate the suitability of the Cr-EA method for on-line measurements of stable hydrogen isotope ratios in chemically unusual samples that may be encountered in biological, environmental, industrial, pharmaceutical, and geological contexts.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental information, chemicals, references, and δ^2H determination, with a focus on the quantitative conversion to molecular hydrogen using both techniques, HTC and Cr-EA, and on the background and/or different hydrogen bearing byproducts for CHO-, CHNO(S)-, and CHCl-bearing compounds using the Ion Trap MS are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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This manuscript includes contributions from all authors. All authors have given their approval of the present version of this manuscript.

Notes

The authors declare no competing financial interest.

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