Investigating the Variability of Stable Isotope Fractionation (C, Cl) During Microbial Reductive Dehalogenation of Chlorinated Ethenes

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Zusammenfassung

Chlorierte Ethene, wie z.B. Tetrachlorethen (PCE) oder Trichlorethen (TCE), sind weit verbreitete Schadstoffe im Grundwasser. Bioremediation, bei der man sich die mikrobielle reduktive Dehalogenierung zu Nutze macht, ist eine nützliche *in situ* Sanierungsstrategie. Wichtige Mikroorganismen für eine erfolgreiche Sanierung sind Organohalid-respirierende Bakterien (OHRB), die die reduktive Dehalogenierung mittels reduktiver Dehalogenasen (RdhAs) katalysieren. Die komponentenspezifische Isotopenanalyse (CSIA) ermöglicht es, den *in situ* Schadstoffabbau zu verfolgen und sogar zu quantifizieren. Außerdem kann CSIA zur Identifizierung der dabei zugrundeliegenden Reaktionsmechanismen genutzt werden. Die Anwendung von CSIA für die Untersuchung des *in situ* Bioabbaus chlorierter Ethene wird durch sehr variable, kinetische Isotopenfraktionierung (ausgedrückt als ε) bei der mikrobiellen Dehalogenierung dieser Substanzen erschwert. Neueste Studien mit chemischen Modellsystemen haben gezeigt, dass die duale Isotopenanalyse von Kohlenstoff und Chlor (ausgedrückt als $\Lambda_{C/Cl}$) ein vielversprechendes Instrument darstellt, um die Gründe für die variable Isotopenfraktionierung chlorierter Ethene, wie z.B. verschiedene Reaktionsmechanismen, aufzuklären.

Diese Arbeit untersuchte die Faktoren, die für die Variabilität der Isotopenfraktionierung verantwortlich sind, die bei der Dehalogenierung chlorierter Ethene durch OHRB-Reinkulturen beobachtet wurde. Weiterhin wurden mögliche Ursachen für die variable Isotopenfraktionierung dehalogenierender Anreicherungskulturen (d.h. mikrobieller Gemeinschaften) unter Verwendung eines vereinfachenden Mischkulturansatzes analysiert. Zu diesem Zweck wurde die duale Isotopenanalytik mit mikrobiellen Dehalogenierungsexperimenten kombiniert, wobei Stämme der Gattung *Desulfitobacterium* als Modellorganismen und PCE bzw. TCE als Modellstoffe verwendet wurden.

Frühere Studien mit chemischen Modellsystemen zeigten, dass verschiedene Reaktionsmechanismen bei der Dehalogenierung von PCE in signifikant unterschiedlichen ε-Werten resultierten. Im ersten Teil dieser Studie wurde untersucht, ob verschiedene Reaktionsmechanismen von PCE-abbauenden Enzymen (PCE-RdhAs) die variablen PCE ε-Werte verschiedener OHRB-Reinkulturen verursachen. Die duale Isotopenfraktionierung (ε_C, ε_{Cl}, $\Lambda_{C/Cl}$) wurde für die PCE-Dehalogenierung durch verschiedende, Gram-positive Stämme von *Desulfitobacterium* gemessen, die sich hinsichtlich ihres PCE-abbauenden Enzyms unterscheiden. Obwohl sich die ε-Werte stark unterschieden, konnten basierend auf ähnlichen $\Lambda_{C/Cl}$-Werten den verschiedenen

Enzymen derselbe Reaktionsmechanismus zugeordnet werden. Durch die ähnlichen Zellwandeigenschaften der Stämme, konnte ein limitierender PCE-Massentransfer zu den Enzymen als Grund für die variablen ε-Werte ausgeschlossen werden. Die Ergebnisse zeigten daher, dass eher verschiedene raten-limitierende Schritte in der mehrstufigen Enzymreaktion (z.B. Diffusion im Substratkanal) und nicht verschiedene Reaktionsmechanismen die Magnitude der PCE-Isotopenfraktionierung der Gattung *Desulfitobacterium* bestimmen.

Für einzelne OHRB-Reinkulturen, die mehrere RdhAs besitzen, wurde eine variable Isotopenfraktionierung beobachtet. Daher wurde im zweiten Teil dieser Studie der Effekt verschiedener Expressionsmuster von RdhA-Enzymen eines OHRB auf die Isotopenfraktionierung untersucht. Für PCE wurde die Kohlenstoff- und Chlorisotopenfraktionierung für lebende Zellen von *Desulfitobacterium dehalogenans* strain PCE1 bestimmt, die sich in der exprimierten RdhA vor dem PCE-Abbau unterschieden (i.e.S. enzymatischer Phänotyp). Ähnliche duale Isotopenfraktionierung (ε_C, ε_{Cl}, $\Lambda_{C/Cl}$) und signifikante Verzögerungsphasen wurden für alle enzymatischen Phänotypen während des PCE-Abbaus beobachtet. Dies zeigte, dass in allen Experimenten die Dehalogenierung von PCE hauptsächlich von der spezialisierten PCE-RdhA nach der *de novo* Synthese katalysiert wurde. Die Ergebnisse weisen darauf hin, dass OHRB die Dehalogenierung chlorierter Ethene vorwiegend mit der RdhA katalysieren, die für den jeweiligen Transformationsschritt spezialisiert ist, auch wenn andere RdhAs präsent sind. Daher sind verschiedene Expressionsmuster von RdhAs wahrscheinlich nicht für die variable Isotopenfraktionierung einzelner Reinkulturen verantwortlich.

Typischerweise sind mehrere OHRB in mikrobiellen Gemeinschaften an kontaminierten Standorten vorhanden und es ist wahrscheinlich, dass sie gleichzeitig zum Abbau eines einzelnen chlorierten Ethens beitragen. Im dritten Teil wurde untersucht, wie sich ändernde Dehalogenierungsaktivitäten von Stämmen, die ein chloriertes Ethen gleichzeitig abbauen, auf die Isotopenfraktionierung mikrobieller Gemeinschaften auswirken. Zu diesem Zweck wurden in binären Mischkulturen aus zwei *Desulfitobacterium*-Stämmen, die unterschiedliche intrinsische ε-Werte für PCE besitzen, die PCE-Isotopenfraktionierung und Zellzahlen der Stämme gemessen. Sehr variable ε_C-Werte wurden für PCE bei unterschiedlichen, initialen Abundanzen der Stämme und unter sich ändernden Kultivierungsbedingungen (z.B. vorherige Zufuhr von TCE) gemessen. Dies wurde durch unterschiedliche Stamm spezifische Beiträge zum PCE-Abbau (d.h. Dehalogenierungsaktivitäten) verursacht, die aus den gemessenen Isotopeneffekten berechnet wurden. Die Dehalogenierungsaktivitäten der Stämme wiesen keine Korrelation mit den Zellzahlen der Stämme auf. Dies unterstreicht die Notwendigkeit für aussagekräftige Biomarker zum Nachweis der Dehalogenierungsaktivität von OHRB innerhalb mikrobieller Gemeinschaften. Unter sich ändernden Kultivierungsbedingungen veränderte sich die Dehalogenierungsaktivität der Stämme. Dies legt den Schluss nahe, dass an kontaminierten Standorten die Aktivität einzelner Stämme von den Umweltbedingungen abhängig ist. Daher sollten für kontaminierte Standorte spezifische ε-Werte in Laborversuchen ermittelt und in regelmäßigen Abständen oder unter verschiedenen Kultivierungsbedingungen nachgemessen werden. Dies würde ermöglichen, potenzielle Änderungen der beobachtbaren Isotopenfraktionierung der

gegenwärtigen mikrobiellen Gemeinschaft abzuschätzen.

Diese Arbeit erbringt Beweise, dass variable PCE ε-Werte von OHRB nicht durch verschiedene Reaktionsmechanismen, sondern durch unterschiedliche raten-limitierende Schritte in der Reaktionsfolge bedingt sind. Des Weiteren legt die ähnliche PCE-Isotopenfraktionierung unterschiedlicher enzymatischer Phänotypen eines OHRB den Schluss nahe, dass verschiedene Expressionsmuster von RdhAs nicht für die variablen ε-Werte von OHRB mit mehreren RdhAs verantwortlich sind. Dies muss in weiteren Studien mit OHRB, die während des PCE-Abbaus mehrere RdhAs gleichzeitig exprimieren, bestätigt werden. Mischkulturen zweier Stämme zeigten, dass die Isotopenfraktionierung chlorierter Ethene in mikrobiellen Gemeinschaften in Abhängigkeit von der Dehalogenierungsaktivität einzelner Stämme sehr variabel sein kann. Basierend auf dieser Arbeit können zukünftige Studien mit komplexeren Mischkulturen oder Anreicherungskulturen konzipiert werden, um die Variabilität der Isotopenfraktionierung von chlorierten Ethenen in mikrobiellen Gemeinschaften weiter zu untersuchen.

Summary

Chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), are common groundwater contaminants. Bioremediation harnessing microbial reductive dehalogenation provides a valuable *in situ* clean-up strategy. Key microorganisms for successful remediation are organohalide-respiring bacteria (OHRB) which catalyze reductive dehalogenation by reductive dehalogenases enzymes (RdhAs). Compound specific isotope analysis (CSIA) allows to track and even quantify *in situ* contaminant transformation. In addition, CSIA can be used to identify the underlying reaction mechanism. Application of CSIA to assess *in situ* biotransformation of chlorinated ethenes is impeded by highly variable kinetic isotope fractionation (expressed as ε) during microbial dehalogenation of these compounds. Recent studies with chemical model systems showed that dual isotope analysis of carbon and chlorine (expressed as $\Lambda_{C/Cl}$) is a promising tool to elucidate the causes (e.g. different reaction mechanisms) that lead to variable isotope fractionation of chlorinated ethenes.

This work investigated the underlying factors responsible for the variability of chlorinated ethene isotope fractionation reported for pure cultures of OHRB. Furthermore, potential causes for variable isotope fractionation of dehalogenating enrichment cultures (i.e. microbial communities) were assessed using a simplifying mixed culture approach. To this end, dual isotope analysis was combined with microbial dehalogenation experiments using strains of the *Desulfitobacterium* genus as model organisms and PCE/TCE as model compounds.

Previous studies with chemical model systems proved that different reaction mechanisms of PCE dehalogenation resulted in significant different ε values. In the first part of this study, it was investigated whether different reaction mechanisms of PCE-transforming enzymes (PCE-RdhAs) cause the variable PCE ε values of different pure cultures of OHRB. Dual isotope fractionation (ε_C, ε_{Cl}, $\Lambda_{C/Cl}$) was determined for PCE dehalogenation by several Gram-positive *Desulfitobacterium* strains differing in their PCE-RdhA. Although ε values varied considerably, similar $\Lambda_{C/Cl}$ values allowed to assign the same reaction mechanism to the different PCE-RdhAs. Due to similar cell envelope properties of the strains, PCE mass transfer limitations to the enzymes were excluded to cause varying ε values. The results thus revealed that different rate-limiting steps (e.g. substrate channel diffusion) in the enzymatic multistep reactions of individual PCE-RdhAs rather than different reaction mechanisms determine the extent of PCE isotope fractionation in the *Desulfitobacterium* genus.

Variable isotope fractionation was reported for single pure cultures of OHRB carrying multiple RdhAs. Thus, the effect of different RdhA expression patterns of an OHRB on isotope fractionation was evaluated in the second part of this study. Carbon and chlorine isotope fractionation of PCE was determined for living cells of *Desulfitobacterium dehalogenans* strain PCE1 differing in their expressed RdhA prior to PCE transformation (i.e. enzymatic phenotype). Similar dual isotope fractionation (ε_C, ε_{Cl}, $\Lambda_{C/Cl}$) and significant lag-phases were observed during PCE dehalogenation for all enzymatic phenotypes. This suggested that in all experiments, PCE dehalogenation was predominantly catalyzed by the specialized PCE-RdhA after *de novo* synthesis. The results indicate that OHRB catalyze chlorinated ethene dehalogenation predominantly by the specialized RdhA for the individual transformation step despite the presence of other RdhA. Thus, differences in RdhA expression patterns are presumably not responsible for variable isotope fractionation of single pure cultures of OHRB.

Multiple OHRB are typically present in microbial communities at contaminated sites and their simultaneous contribution to transformation of a single chlorinated ethene is likely to occur. In the third part, it was investigated how changing dehalogenation activities of strains that simultaneously transform the same chlorinated ethene affect isotope fractionation of microbial communities. To this end, PCE isotope fractionation and strain cell numbers were monitored in binary mixed cultures of two *Desulfitobacterium* strains, which differ in their strain intrinsic ε_C value for PCE. Highly variable PCE ε_C values were obtained for different initial strain abundances and under changing cultivation conditions (e.g. prior provision of TCE). This was attributed to different strain specific contributions to PCE transformation (i.e. dehalogenation activities) which were calculated from measured isotope effects. Dehalogenation activities of the strains did not correlate with strain cell numbers. This emphasizes the need for conclusive biomarkers to assess dehalogenation activities of OHRB in microbial communities. The dehalogenation activity of the single strains changed during the deployed cultivation conditions. This indicates that at contaminated sites the activity of single OHRB depends on environmental conditions. Thus, specific ε values for contaminated sites should be determined in laboratory experiments and re-measured on a regular basis or under different cultivation conditions. This would allow to estimate potential changes in observable isotope fractionation of the present microbial community.

This work provides evidence that variable PCE ε values of OHRB do not result from different reaction mechanisms but different rate-limiting steps in the reaction sequence. Furthermore, similar PCE isotope fractionation of different enzymatic phenotypes of an OHRB suggests that different RdhA expression patterns do not cause variable ε values of OHRB carrying multiple RdhAs. This needs to be substantiated in further studies with OHRB which simultaneously express multiple RdhAs during PCE dehalogenation. Mixed cultures of two OHRB showed that chlorinated ethene isotope fractionation in microbial communities can be highly variable depending on dehalogenation activities of single strains. Based on this work, future studies can be designed with more complex mixed cultures or enrichment cultures to further assess the variability of chlorinated ethene isotope fractionation in microbial communities.

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1

General Introduction

1.1 Environmental Significance of Halogenated Organic Compounds and Chlorinated Ethenes

Halogenated organic compounds (HOCs, organohalides, organohalogens) are hydrocarbons containing one or more covalently bound halogen substituents (F, Cl, Br, I).[1] The halogen substituents affect the physicochemical properties of HOCs increasing their density and hydrophobicity. Consequently, such compounds exhibit low water solubility and tend to accumulate in organic phases including organisms. In addition, halogen substituents can increase the toxicity of a molecule.[1–3] Organohalogens are ubiquitous in the environment which results from their natural production from abiotic and biotic sources, such as volcanoes, combustion, plants and fungi.[4] However, a large fraction of organohalides in the environment can be attributed to anthropogenic production and releases.[1] Their physicochemical properties make them beneficial for various applications in agriculture as pesticides or in industry as solvents, degreasing agents and additives for chemical syntheses. Over the last century, large-scale production of primarily organochlorine compounds resulted in increasing emissions into the environment and contamination of all major environmental compartments (i.e. air, water, soil).[1,3]
Chlorinated solvents and among them chlorinated ethenes belong to the most frequently detected groundwater pollutants.[5–9] Chlorinated ethenes consist of two double-bonded carbon centers with one to four chlorine substituents (Figure 1.1).[10] Tetrachloroethene (PCE) and trichloroethene (TCE) are mainly used for dry-cleaning or degreasing and enter the environment due to improper storage, accidental spills but also by intentional disposal in the past.[6] When introduced into an aquifer, most chlorinated ethenes form dense non-aqueous phase liquids (DNAPLs) in deeper layers of the aquifer due to their high density and limited water solubility (Table 1.1).[6,10] Despite their restricted water solubility, chlorinated ethenes leach from DNAPLs and often exceed statutory maximum contaminant levels for drinking water.[6] All chlorinated ethenes can cause liver and/or kidney damage. In addition, TCE and VC are classified as human carcinogens. Thus, exposure to chlorinated ethenes poses a risk to human health[2,11,12] raising the need for contaminated field site remediation. For this purpose, bioremediation taking advantage of microbial *in situ* chlorinated ethene transformation is a valuable option. Bioremediation is often considered as a more cost-effective approach than *ex situ* remediation techniques following pump-and-treat and has lower environmental impact.[13]

1.2 Organohalide-Respiring Bacteria as Key Microorganisms for Chlorinated Ethene Transformation and *in situ* Bioremediation

All chlorinated ethenes are susceptible to anaerobic (reductive) microbial transformation under anoxic conditions.[8] Aerobic (oxidative) PCE transformation under oxic conditions does not occur and TCE oxidation proceeds only at slow rates.[18] In contrast, DCE and VC are readily biodegradable by microbial oxidation, but these compounds mainly occur as metabolites at contaminated sites from reductive PCE and TCE transformation.[19] Thus, anaerobic reductive transformation of chlorinated ethenes is the most frequently applied clean-up strategy for

Table 1.1. Physicochemical properties and selected human health effects of chlorinated ethenes.

Chlorinated Ethene	**Molecular Weight (g/mol)**	**Density (g/L)**	**Water Solubility (g/L)**	**K_{OW}**	**Selected Human Health Effects**	**Ref**
Tetrachloroethene (PCE)	165.83	1.62	0.15	3.4	Liver & kidney damage, likely carcinogenic	[14]
Trichloroethene (TCE)	131.39	1.46	1.28	2.6	Liver & kidney damage, carcinogenic	[15]
cis-1,2-Dichloroethene (*cis*-DCE)	96.95	1.28	6.41	1.86	Liver & kidney damage	[16]
Vinyl Chloride (VC)	62.5	0.91	2.76	1.36	Liver damage, carcinogenic	[17]

bioremediation of contaminated sites.[18] Under anoxic conditions, organohalide-respiring bacteria (OHRB) use chlorinated ethenes and other organohalides as terminal electron acceptors for the generation of respiratory energy via electron transport phosphorylation. This process is denoted as reductive dehalogenation and results in cleavage of a carbon-halogen bond. The halogen atom is released as halide and substituted by a proton (hydrogenolysis).[20,21] For chlorinated ethenes, this leads to sequential dechlorination of PCE into less chlorinated products and innocuous ethene as the end-product (Figure 1.1).[18]

OHRB emanate from the phyla Firmicutes, Proteobacteria as well as Chloroflexi and can be grouped into obligate OHRB and metabolically versatile OHRB.[20,22–24] Metabolically versatile OHRB are found within genera of Firmicutes (*Desulfitobacterium*) and Proteobacteria (e.g. *Sulfurospirillum*, *Desulfuromonas*). They dehalogenate PCE and TCE into *cis*-DCE and are able to use other organohalides as terminal electron acceptors including chlorophenols and chlorobenzenes. Versatile OHRB are not restricted to reductive dehalogenation for energy conservation and can use other electron acceptors such as fumarate, nitrate or ferric iron.[22–24] In contrast, obligate OHRB are restricted to reductive dehalogenation for energy conservation and occur within genera of Chloroflexi (e.g. *Dehalococcoides*, *Dehalogenimonas*) and Firmicutes (*Dehalobacter*).[23] Obligate OHRB dehalogenate a broader spectrum of organohalides than versatile OHRB including chlorobenzenes, chlorophenols but also polychlorinated biphenyls and dioxins. In addition, the capability to completely dechlorinate PCE into ethene is restricted to obligate OHRB such as *Dehalococcoides mccartyi*.[22] *Dehalococcoides* are perceived as one of the most important microbial groups for successful groundwater remediation based on their ability to completely dehalogenate organohalides.[25] They are metabolically restricted to organohalides as terminal electron acceptors, H_2 as electron donor and acetate as carbon source.[26] Thus, they rely on supply of these substrates from other members within their microbial community or on

exogenous supply within laboratory cultures.[25]

The capability of versatile and obligate OHRB to dehalogenate chlorinated ethenes makes them key microorganisms for bioremediation.[27] Successful dechlorination and production of innocuous ethene at contaminated sites depends on the presence and dehalogenating activity of OHRB especially *Dehalococcoides*. This can be promoted by injection of OHRB containing cultures into the underground (bioaugmentation) if no autochtonous OHRB populations are present and/or injection of fermentable substrates such as molasse or plant oil to generate acetate and H_2 for stimulation of *Dehalococcoides* activity (biostimulation).[28–31]

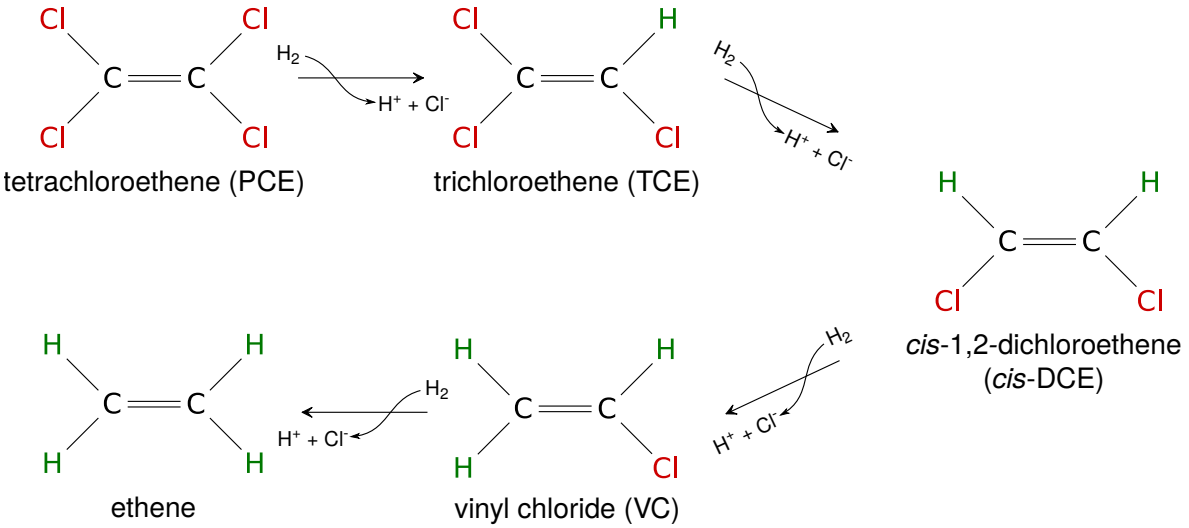

Figure 1.1. Sequential microbial reductive dehalogenation of tetrachloroethene to ethene.

1.3 Enzymes Catalyzing Reductive Dehalogenation

Reductive dehalogenation of organohalides is catalyzed by membrane-associated reductive dehalogenases enzymes (RdhA).[20] All but one RdhA contain a cobalt-bearing corrinoid (cobamide) in the active center which transfers the electrons to the organohalide via a super-reduced cobalt ion (Co^I).[20,24,32] Two iron-sulfur clusters are commonly encoded in protein sequences of RdhAs which aid electron transfer from the electron donor towards the active center.[20,24] In addition, a twin-arginine translocalization (TAT) signal is usually present in RdhA protein sequences which is responsible for protein transport across the cytoplasmic membrane.[20,33] Catalytically active RdhAs are bound to the outer side of the cytoplasmic membrane via the accessory protein RdhB.[24,33,34]

RdhAs are encoded in the genomes of OHRB within *rdh*-gene operons. The number of *rdh*-gene operons varies between 1 to 7 for versatile OHRB and 5 to 38 for obligate OHRB.[20,35] *Rdh*-gene operons typically comprise the *rdhAB* cluster encoding the catalytic subunit (*rdhA*) and the putative membrane anchor (*rdhB*).[36–39] This gene cluster is frequently accompanied with variable assortments of accessory genes comprising one or more of *rdhCTKZED* genes.[24,39] While for most of these genes the exact function is unknown, some are involved in enzyme maturation and transcriptional regulation of *rdhA*- and *rdhB*-genes.[24,39–43] *De novo* synthesis of RdhAs

is initiated by transcriptional regulators of the NosR- and NirI-type, CRP/FNR-type and MarR-type (*rdhC*, *rdhK*, *rdhR*) upon the presence of organohalides that specifically induce gene expression.[40,41,43,44] This results in up-regulation of *rdhA*-gene transcription and an increased RdhA enzyme amount per cell.[45–48]

RdhAs can be grouped in orthologous groups based on amino acid sequence identity. Correlations between substrate specificity and catalytic mechanisms of single groups have proved difficult since only few RdhAs were biochemically characterized.[20,32] Variable substrate specificities were reported for catalytically active RdhAs (s. refs[24,27,33] for recent reviews).[20,24,27,33,49] Thus, the range of possible halogenated electron acceptors of a single OHRB is determined by the specific RdhAs and corresponding *rdhA*-genes present in its genome.[20] For several characterized RdhAs, the substrate spectrum triggering *rdhA*-gene expression is narrower than the spectrum of dehalogenated organohalides of the catalytically active RdhA. This can result in overlapping substrate spectra of different RdhAs of a single organism.[24,49] In addition, simultaneous transcription of multiple *rdhA*-genes in OHRB was observed in the presence of single organohalides. However, relative *rdhA*-gene transcription levels differed depending on the provided organohalide.[49–51]

RdhAs specifically catalyzing metabolic dehalogenation of chlorinated ethenes (CE-RdhAs) can be found in versatile and obligate OHRB.[33] In *Dehalococcoides*, CE-RdhAs comprise enzymes catalyzing multiple or single dechlorination steps from TCE into ethene (e.g. VcrA, BvcA, TceA) or enzymes dechlorinating PCE into TCE (PceA).[25,38,52] Among versatile OHRB such as *Desulfitobacterium*, the PceA enzyme catalyzes dehalogenation of PCE and TCE into *cis*-DCE while the PrdA enzyme catalyzes dechlorination of PCE into TCE.[24,53] In addition, cometabolic PCE dehalogenation was reported for a variety of RdhAs of *Desulfitobacterium* transforming chloroaromatic compounds (CprA/CrdA).[49,54–56] However, *de novo* synthesis of these RdhAs was not induced in the presence of PCE and required the prior exposure of the strains to chloroaromatics.[49,54,55]

1.4 OHRB in Microbial Communities

Characterization and monitoring of OHRB in microbial enrichment cultures and at contaminated sites is routinely carried out by quantitative real-time PCR (qPCR) targeting CE-*rdhA*-genes or 16S rRNA-genes of OHRB.[57–70] Due to their high importance for complete site remediation, numerous studies evaluated CE-*rdhA*-gene abundances of *Dehalococcoides* (*bvcA*-, *vcrA*-, *tceA*- and *pceA*-gene) at contaminated sites or in enrichment cultures.[64–68] In addition, several studies analyzed CE-*rdhA*- and 16S rRNA-gene abundances of different OHRB from multiple genera.[59–61,69–71] These studies indicate that at contaminated sites typically several strains of *Dehalococcoides* coexist with strains of *Desulfitobacterium*, *Dehalobacter Desulfuromonas* and others.[59–62,67,69–71] Results of laboratory enrichment cultures suggest that multiple OHRB contribute to complete dehalogenation of PCE to ethene. It was hypothesized that dechlorination of PCE into TCE and *cis*-DCE is catalyzed by strains of *Desulfitobacterium*, *Dehalobacter*, *Geobacter* and/or *Dehalococcoides*, while dehalogentation of *cis*-DCE to ethene can generally be

attributed to *Dehalococcoides*.[59–62] However, changing *rdhA*-gene abundances of different OHRB were observed in enrichment cultures upon changes in provided electron donors and chlorinated ethenes.[60,67,72] This suggest that abundances of single OHRB strains in microbial communities can vary depending on environmental conditions which may affect their dehalogenating activities. Although CE-*rdhA*- and 16S rRNA-gene quantification is an indispensable tool to monitor and characterize OHRB in microbial communities, this technique does not allow to infer dehalogenating activities.[57] In laboratory cultures, chlorinated ethene concentrations can be tracked and correlated with dehalogenation activity of different OHRB strains based on increasing gene abundances and exclusion of other metabolic pathways. However, this is not conclusive at field sites where chlorinated ethene concentrations can be biased by dilution, sorption and dissolution from a DNAPL[73] or other metabolic pathways of versatile OHRB may occur. Therefore, other techniques are required that can provide explicit evidence for ongoing chlorinated ethene transformation at contaminated sites.

1.5 Compound Specific Isotope Analysis and Isotope Fractionation During Reductive Dehalogenation of Chlorinated Ethenes

Compound specific isotope analysis (CSIA) has the potential to prove and quantify *in situ* contaminant transformation in the field. CSIA can also be applied to identify contaminant sources, transformation pathways and the nature of the underlying reaction mechanism.[73,74] Application of CSIA takes advantage of different chemical reaction rates of molecules containing the light isotope (e.g. ^{12}C) or the heavy isotope (e.g. ^{13}C) of an element at the reactive position. The difference in chemical reaction rates of these isotopologues is expressed by the kinetic isotope effect (KIE)[75]:

$$KIE = \left(\frac{^{L}k}{^{H}k}\right) \tag{1}$$

With ^{L}k reflecting the reaction rate of molecules containing the light isotope at the reactive position (light isotopologue) and ^{H}k reflecting the reaction rate of molecules containing the heavy isotope at the reactive position (heavy isotopologue). Reaction rates for light isotopologues are typically faster because they require less activation energy than heavy isotopologues to reach the transition state. Consequently, the heavy isotopes accumulate in the remaining compound fraction and the light isotopes are enriched in the transformation product which is denoted as isotope fractionation.[73] For chlorinated ethene transformation, the resulting shifts in compound specific isotope ratios (^{13}C/^{12}C, ^{37}Cl/^{35}Cl) can be tracked at natural isotopic abundance by established analytical techniques such as gas chromatography (GC) coupled to isotope ratio mass spectrometry (for carbon and chlorine) and quadrupole mass spectrometry (for chlorine).[76] Isotope ratios are expressed in δ-notation (e.g. for carbon):

$$\delta^{13}C_{sample} = \frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{reference}} - 1 \tag{2}$$

as relative difference between the isotope ratio of the measured sample $(^{13}C/^{12}C)_{sample}$ and a reference standard $(^{13}C/^{12}C)_{reference}$.[76] The magnitude of changes in isotope ratios during transformation is quantified by the isotope enrichment factor (ε) according to the Rayleigh equation:

$$ln\left(\frac{\delta^{13}C_t + 1}{\delta^{13}C_0 + 1}\right) = \varepsilon * ln(f) \quad (3)$$

with $\delta^{13}C_t$ as measured compound isotope ratio ($^{13}C/^{12}C$) during transformation, $\delta^{13}C_0$ as initial compound isotope ratio and f as the remaining compound fraction.[73,75] Measured ε values can be applied to quantify *in situ* contaminant transformation. However, the applied ε value must accurately describe the shift in isotope ratios at the specific field conditions because inappropriate ε values yield wrong estimates of *in situ* transformation.[77] In addition, ε values can be used to elucidate the underlying processes and mechanisms of compound transformation.[73] This is based on the fact that the magnitude of ε values reflects differences in reaction rates of light and heavy isotopologues and thus reflect the KIE.[75] The magnitude of the KIE is determined by the manner of bond cleavage (i.e. reaction mechanism) in the transition state and different KIEs can usually be expected for different mechanisms. To relate ε values to a certain reaction mechanism, the position specific apparent kinetic isotope effect (AKIE) is calculated from measured ε values and compared with theoretical or measured KIEs for a given mechanistic scenario.[75] Thus, combined determination of ε values in elementary chemical reactions and biochemical reactions allows to elucidate enzymatic transformation mechanisms.[73]

For microbial dehalogenation of chlorinated ethenes, highly variable carbon ε values (ε_C) have been reported for different enrichment cultures and pure strains of OHRB. In addition, different ε_C values were reported for dehalogenation of single chlorinated ethenes by the same OHRB.[47,78–88] This hampers elucidation of enzymatic transformation mechanisms and quantification of *in situ* transformation emphasizing the need to unravel the underlying factors for the apparent variability.

Different reaction mechanisms have been proposed for reductive dehalogenation of chlorinated ethenes including nucleophilic substitution (S_N2)[89], (nucleophilic) addition-elimination and addition-protonation[90,91] as well as inner- or outer-sphere single electron transfer (SET).[92–94] Several studies showed that different reaction mechanisms of chlorinated ethene dehalogenation are associated with different ε_C values.[72,90,95,96] Distinct enzymatic reaction mechanisms of chlorinated ethenes may therefore provide a possible explanation for the reported variability of ε_C values. However, the intrinsic KIE of bond cleavage is only reflected in measured ε values if this step is rate-limiting for the overall reaction sequence.[73] Microbial transformation reactions comprise a cascade of multiple reaction steps aside from bond cleavage that may become rate-limiting and display a bottleneck for the overall reaction rate. In this case, the intrinsic ε value is diminshed (i.e. masked) and can vary even for the same reaction mechanism.[73] On the level of the catalyzing enzyme, compound transformation comprises multiple reaction steps such as enzyme-substrate association that may become rate-limiting and mask the intrinsic isotope

effect of bond cleavage.[75] For microbial PCE dehalogenation, masking effects by slow reaction steps within in the enzymatic multistep reaction were reported.[97] The potential association of different ε values with different enzymatic transformation mechanisms and/or the presence of rate-limiting enzymatic reaction steps indicate that microbial chlorinated ethene isotope fractionation is enzyme specific.

In addition, isotope fractionation can be masked by limited substrate transfer towards and into the cells. Low substrate bioavailability at the catalyzing enzymes results in a high amount of unsaturated enzyme binding sites. Thus, each substrate molecule reaching the cell interior is immediately transformed irrespective of its isotopic composition and enzyme intrinsic isotope fractionation is masked.[73,98] Microbial cell walls and the cytoplasmic membrane were previously reported to act as rate-limiting barriers during diffusive uptake of PCE. This was deduced from smaller isotope fractionation measured for PCE dehalogenation by whole cells compared to protein crude extracts and purified enzymes.[78,82,99]

The potential impact of both different reaction mechanisms and masking effects on single element ε values (or AKIEs) hampers their application for mechanistic considerations of microbial transformation reactions. Dual element isotope analysis allows to assign varying ε values to different reaction mechanisms or masking effects.[73] Masking effects do not show element specific isotope effects and therefore affect observable isotope fractionation of both elements to the same extent. Thus, masking effects cancel out when isotope values of two elements are considered relative to each other in a dual isotope plot (e.g. δ^{13}C vs δ^{37}Cl) and dual isotope slopes (Λ) reflect the ratio of the intrinsic isotope effect of both elements.[75] An association of different Λ values with different ε values typically indicates different reaction mechanisms, whereas similar dual isotope slopes suggest the impact of masking.[73] Previous studies proved the applicability of dual isotope slopes ($\Lambda_{C/Cl}$) to distinguish different reaction mechanisms of reductive dehalogenation of chlorinated ethenes.[72,90,96] Thus, dual element isotope analysis in combination with systematic microbiological studies opens the opportunity to assess the underlying causes for variable isotope fractionation during microbial chlorinated ethene dehalogenation.

1.6 Objectives and Structure of the Thesis

CSIA is a promising tool to prove and quantify *in situ* transformation of chlorinated ethenes but also to decipher transformation pathways of reductive dehalogenation under laboratory and field conditions. Reliable application of CSIA to assess microbial reductive dehalogenation requires a detailed understanding of the factors determining the magnitude of isotope fractionation during this metabolic process. The main goal of this study was to investigate potential causes for the observed variability of chlorinated ethene isotope fractionation of pure cultures of OHRB and microbial communities mediating dehalogenation. For pure cultures, the effects of (i) different RdhA enzyme properties and (ii) changing RdhA expression patterns on chlorinated ethene isotope fractionation were evaluated. For microbial communities, (iii) the variability of ε and $\Lambda_{C/Cl}$ values during simultaneous dehalogenation activity of multiple OHRB strains

was investigated. To this end, laboratory experiments were conducted under defined conditions using organohalide-respiring strains from the *Desulfitobacterium* genus as model organisms and PCE/TCE as model compounds. *Desulfitobacterium* strains were chosen because they show stable growth yields in pure cultures.[27] In contrast, pure culture studies of *Dehalococcoides* are impeded by low growth rates and stable growth is usually obtained in more complex co- or enrichment cultures.[9,20,25,27,35]

Chapter 1 provides the theoretical background for the proposed research work and introduces the concepts of compound specific isotope analysis (CSIA) and quantitative real-time PCR to assess microbial chlorinated ethene transformation.

In chapter 2 it was investigated whether PCE-transforming RdhAs differing in their transformation products and their specificity for PCE transformation (metabolic vs cometabolic transformation) dehalogenate PCE via different reaction mechanisms. To this end, ε_C, ε_{Cl} and $\Lambda_{C/Cl}$ values were compared for PCE dehalogenation by five different pure strains of *Desulfitobacterium* comprising a total of three different types of PCE-RdhAs (PceA, PrdA, CrdA). The results were contrasted with reported isotope effects for microbial, enzymatic and abiotic PCE dehalogenation to carve out the impact of different reaction mechanisms or masking on the measured isotope effects. The results of this chapter provide implications for the variability of PCE ε values reported for different pure cultures of OHRB.

Chapter 3 investigated potential effects of different RdhA expression patterns on PCE isotope fractionation. Most OHRB carry multiple *rdhA*-genes and transcription can vary depending on environmental stimuli. Thus, the enzymatic phenotype of a single OHRB with regard to the relative abundance of catalytically active RdhAs may vary due to different experimental conditions. Substrate spectra of different RdhAs can overlap and ε values are enzyme specific. Therefore, measured ε values may reflect contributions of several RdhAs and may change depending on relative RdhA abundances and activities. This was investigated by contrasting PCE isotope fractionation of different enzymatic phenotypes of *Desulfitobacterium dehalogenans* strain PCE1. Different enzymatic phenotypes were generated by exposing strain PCE1 to either chloroaromatic compounds or PCE. During subsequent PCE transformation by the different phenotypes, dual element isotope fractionation was monitored. Isotope effects were compared to decipher potential contributions of RdhAs expressed on chloroaromatic compounds to PCE dehalogenation. Dehalogenation profiles, cell growth and isotope fractionation were compared to previous studies to explain the results in the context of RdhA expression upon exposure to different organohalides. The results of this study contribute to decipher the underlying cause for variable ε values measured for single pure cultures of OHRB.

Chapter 4 evaluates the variability of chlorinated ethene isotope fractionation in microbial communities during simultaneous transformation of a single chlorinated ethene by multiple strains. Multiple OHRB able to contribute to contaminant transformation are typically present at contaminated sites and pure culture ε values differ. During simultaneous chlorinated ethene transformation by multiple strains, the apparent ε value represents a lumped parameter affected by dehalogenation activity and the intrinsic ε value of each contributing strain.[100] Changing

environmental conditions were shown to alter the abundance of different OHRB strains. Consequently, their individual contribution to transformation may change and apparent ε values of microbial communities differ depending on the prevailing cultivation/environmental conditions. This was studied within various PCE transformation experiments using binary mixed cultures of *Desulfitobacterium dehalogenans* strain PCE1 and *D. hafniense* strain Y51, which show significantly different strain specific ε_C values for PCE. Isotope fractionation and strain abundances were quantified for different initial mixed culture compositions and a continuously cultivated batch culture under changing cultivation conditions. Strain specific contributions and dehalogenation profiles were compared for the different experiments and contrasted with cell abundances of the strains. In addition, the applicability of strain specific abundances as quantitative proxy for dehalogenation activities of the strains was investigated. The results of this study provide first insights into underlying causes for the variability of ε values measured for microbial enrichment cultures (i.e. microbial communities).
In chapter 5, a general conclusion of this research work is provided and concepts for future studies are proposed.

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2

Deciphering the Variability of Stable Isotope (C, Cl) Fractionation of Tetrachloroethene Biotransformation by Desulfitobacterium strains Carrying Different Reductive Dehalogenases Enzymes

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2.1 Abstract

Kinetic isotope effects have been used successfully to prove and characterize organic contaminant transformation on various scales including field and laboratory studies. For tetrachloroethene (PCE) biotransformation, however, causes for the substantial variability of reported isotope enrichment factors (ε) are still not deciphered (ε_C = -0.4 to -19.0 ‰). Factors such as different reaction mechanisms and masking of isotope fractionation by either limited intracellular mass transfer or rate-limitations within the enzymatic multistep reaction are under discussion. This study evaluated the contribution of these factors to the magnitude of carbon and chlorine isotope fractionation of *Desulfitobacterium* strains harboring three different PCE-transforming enzymes (PCE-RdhA). Despite variable single element isotope fractionation (ε_C = -5.0 to -19.7 ‰; ε_{Cl} = -1.9 to -6.3 ‰), similar slopes of dual element isotope plots ($\Lambda_{C/Cl}$ values of 2.4 ± 0.1 to 3.6 ± 0.1) suggest a common reaction mechanism for the different PCE-RdhAs. Cell envelope properties of the *Desulfitobacterium* strains allowed to exclude masking effects due to PCE mass transfer limitation. Our results thus revealed that different rate-limiting steps (e.g. substrate channel diffusion) in the enzymatic multistep reactions of individual PCE-RdhAs rather than different reaction mechanisms determine the extent of PCE isotope fractionation in the *Desulfitobacterium* genus.

2.2 Introduction

Contamination of groundwater by tetrachloroethene (PCE) and other chlorinated ethenes is a persistent environmental problem and calls for reliable and cost-effective remediation techniques such as *in situ* microbial transformation.[1–3] Compound specific isotope analysis (CSIA) provides a tool that allows to track and quantify *in situ* transformation processes of chlorinated ethenes. In addition, it enables to differentiate between transformation pathways (e.g. anaerobic vs aerobic microbial transformation of vinyl chloride) and to decipher the nature of the underlying (bio)chemical reaction mechanism.[4–9] Substitution of a light isotope (e.g. ^{12}C) with a heavy isotope (e.g. ^{13}C) at the reactive position of a molecule typically results in slightly faster reaction rates for molecules containing the light isotope compared to molecules containing the heavy isotope which is denoted as kinetic isotope effect (KIE). During progressing compound transformation, the KIE leads to an increasing enrichment of the heavy carbon and chlorine isotopes (^{13}C as well as ^{37}Cl) in the remaining substrate pool.[4] The magnitude of the KIE is quantified by the isotope enrichment factor (ε) using the Rayleigh equation.[4] For microbially mediated reductive dehalogenation of PCE, highly variable carbon ε values (ε_C) have been reported for different bacterial isolates and microbial enrichment cultures covering a range of -0.4 to -19.0 ‰.[10–13] These variations can be attributed either to different reaction mechanisms or masking effects.

In general, the magnitude of the intrinsic KIE of a chemical reaction is determined by the manner of chemical bond cleavage and therefore related to the transition state structure. However, the intrinsic KIE can be considerably lowered (i.e. masked) by preceding reaction steps

that are non-isotope sensitive but rate-limiting for the overall reaction, i.e. act as a bottleneck. Depending on the magnitude of such masking effects, ε values for a given reaction mechanism can therefore significantly differ.[14]

For microbial PCE dehalogenation, different reaction mechanisms have been proposed including (nucleophilic) addition-elimination[15], nucleophilic substitution (SN2)[16] and inner- or outer-sphere single electron transfer (SET).[17–19] Recently, the application of CSIA provided evidence for the addition-elimination mechanism for enzymatic PCE transformation, while computational studies favored the (inner-sphere) SET mechanism.[20–23] The key enzymes for microbial PCE transformation are PCE reductive dehalogenases (PCE-RdhAs). They are terminal reductases in the respiratory chain of organohalide-respiring bacteria (OHRB) involved in energy conservation by anaerobic reductive dehalogenation of organohalides.[24,25] PCE-RdhA enzymes contain two-iron sulfur clusters and a cobalt bearing corrinoid cofactor at the active site.[26] PCE-RdhAs can be found in different bacterial taxa including *Dehalococcoides*, *Sulfurospirillum* or *Desulfitobacterium* and dehalogenate PCE either into TCE ($\mathrm{RdhA_{TCE}}$) or *cis*-DCE ($\mathrm{RdhA_{cDCE}}$).[25]

Previous studies with chemical model systems proved that different reaction mechanisms resulted in very different ε_C values, e.g. -16.6 to -17.0 ‰ for the addition-elimination mechanism[21], -28.0 ‰ for S_N2[27] and -7.6 to -9.3 ‰ for an outer-sphere SET.[28] PCE transformation by whole cells, crude protein extracts and purified enzymes showed smaller isotope effects for whole cell experiments which was attributed to rate-limiting PCE mass transfer through the bacterial cell walls and/or the cytoplasmic membrane.[11,29,30] The observed isotope masking effect was stronger for Gram-negative bacteria, which was attributed to their hydrophilic outer membrane that is absent in Gram-positive bacteria.[30] In addition to mass transfer into the cells, masking of PCE isotope fractionation was also proposed within the enzymatic multistep reaction by slow reaction steps preceding C-Cl bond cleavage such as enzyme-substrate association.[31]

While single element ε values can reflect both, masking effects cancel out in a dual isotope plot (e.g. δ^{13}C vs δ^{37}Cl) because the processes leading to masking effects do not cause isotope effects themselves.[14] Different dual isotope slopes ($\Lambda_{C/Cl}$) of chlorinated ethene transformation therefore indicate different reaction mechanisms, whereas similar slopes despite different single element ε values indicate masking effects.[9,21,22]

A previous study suggested that varying PCE ε values and varying transformation products arise from different reaction mechanisms of the catalyzing enzymes. This was inferred from significantly different ε and $\Lambda_{C/Cl}$ values determined for two enrichment cultures transforming PCE to either TCE or *cis*-DCE.[32] However, the authors argued that the observed deviation between ε values and $\Lambda_{C/Cl}$ may also arise from different rate-limiting steps in the reaction sequence.[32] In a recent study, varying PCE ε and $\Lambda_{C/Cl}$ values were predominantly attributed to masking effects of preceding reaction steps and only one $\Lambda_{C/Cl}$ value of living cell suspensions was proposed to reflect the isotope effect of C-Cl bond cleavage according to the addition-elimination reaction mechanism.[22] Thus, further experimental evidence is needed to evaluate whether the reported variability of PCE ε values is caused by different reaction mechanisms.

An association of distinct PCE reaction mechanisms with characteristic $\Lambda_{C/Cl}$ values, ε values and transformation products will help to constrain the prevailing PCE transformation pathway in the field and allow to choose an appropriate ε value for the quantification of *in situ* transformation.

Using dual element isotope analysis, the present study systematically investigates the factors responsible for the highly variable ε values reported for microbial reductive dehalogenation of PCE on whole cell level. To this end, we compared carbon and chlorine ε values and $\Lambda_{C/Cl}$ values for PCE transformation in cell suspensions of different *Desulfitobacterium* strains carrying different PCE-RdhAs. *Desulfitobacterium hafniense* strain Y51 and strain PCE-S dehalogenate PCE to *cis*-DCE via the PceA enzyme[33,34] (further denoted as $RdhA_{cDCE}$) whereas *D. dehalogenans* strain PCE1 dehalogenates PCE to TCE via the PrdA enzyme[35,36] (further denoted as $RdhA_{TCE}$) and *D. hafniense* strain PCP-1 transforms PCE only cometabolically via CrdA (further denoted as $RdhA_{co}$) in the presence of 2,4,6-trichlorophenol.[37] Different stable products of the RdhAs and the absence of iron-sulfur clusters in $RdhA_{co}$[37] point towards structural differences in the enzymes (e.g. conformation of the active sites) and therefore open the possibility for enzyme specific isotope effects (e.g. different reaction mechanisms). *Desulfitobacterium* strains are classified as Gram-positive bacteria lacking a second outer membrane in comparison to Gram-negative bacteria.[38] The identical cell wall structure for strains carrying different RdhAs allows us to assign varying ε values to either different reaction mechanisms or different degrees of masking by rate-determining steps in the enzymatic multistep reactions, while reported mass transfer limitations over the second membrane can be neglected. In addition, different Gram stainings of $RdhA_{cDCE}$-carrying strains were reported (Y51: Gram-negative staining vs PCE-S: Gram-positive staining)[34,39] which points to slight structural differences in the Gram-positive cell wall (i.e. thickness of the peptidoglycan layer).[40] This allows to assign potential differences in ε values of strains carrying the same enzyme ($RdhA_{cDCE}$) to mass transfer limitations into the cells.

2.3 Materials and Methods

Cultivation of *Desulfitobacterium* Strains and Experimental Setup

Desulfitobacterium dehalogenans strain PCE1 (No. 10344), *D. hafniense* strain PCE-S (No. 14645) and *D. hafniense* strain PCP-1 (No. 12420) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *D. hafniense* strain Y51 was kindly provided by Prof. Furukawa of the Department of Food and Bioscience Beppu University (Japan). All strains were cultivated in 250 mL serum bottles sealed with butyl rubber stoppers and contained 200 mL of an anoxic, sodium bicarbonate buffered medium described elsewhere.[41] Precultures of strains Y51, PCE1 and PCE-S were grown with 1 mM pyruvate and 200 μM PCE, whereas strain PCP-1 was inoculated with 1 mM pyruvate and 100 μM of an anoxic and autoclaved 2,4,6-trichlorophenol (TCP) stock solution. All cultures were grown in the dark at 20 °C. Precultures grown on PCE were degassed using an N_2/CO_2 gas mixture (80:20) to avoid a carryover of volatile dehalogenation products during inoculation. This was confirmed

by GC-MS measurements prior to inoculation. For each experiment, inocula yielding a final concentration of 2.5 % (v/v) were added from the respective preculture to a total of three replicate bottles, to which 1 mM pyruvate and 200 μM were previously added. Additionally, 25 μM TCP were provided to replicate bottles of strain PCP-1 and resupplied when completely converted to 4-monochlorophenol as determined by HPLC-UV/VIS. Each experiment contained an additional, cell free control microcosm. For *D. dehalogenans* strain PCE1 and *D. hafniense* strain Y51 two degradation experiments, each comprising three living replicates and an abiotic control were conducted.

Sample Extraction and Analytical Methods

Sampling. At each sampling point, 5 mL of liquid was extracted using gas-tight glass syringes (Innovative Laborsysteme GmbH, Stützerbach, Germany). For immediate chlorinated ethene concentration analysis, 1 mL was equally divided into two 10 mL crimp glass vials (each 0.5 mL) which were previously filled with 4.5 mL ultrapure water (Merck Millipore, Burlington, MA) and 100 μL 1 M phosphoric acid. The latter was added to stop the microbial activity. Sample vials were sealed with aluminum crimp caps containing silicone/PTFE septa and directly measured. Four aliquots of 1 mL were stored in 1.5 mL screw neck HPLC vials to which 50 μL of 10 M NaOH were added to stop the microbial activity. HPLC vials were closed with PTFE-lined screw caps, frozen and stored upside down at -20 °C for later isotope analysis.

Chlorinated Ethene Concentrations. Analysis of chlorinated ethenes was performed in duplicates on a GC-MS system (Agilent Technologies 7890A GC coupled to an Agilent Technologies 5975VL MS Detector, Santa Clara, CA) via static headspace injection of 500 μL using a Gerstel MultiPurpose Sampler MPS (Mülheim an der Ruhr, Germany). Injections were done in split mode 1:10 with a Split/Splitless injector operated at 240 °C. Chromatographic separation of *cis*-DCE, *trans*-DCE, TCE and PCE was achieved using a Rtx-VMS fused silica column (60 m x 0.25 mm, 1.4 μm film; Restek, Bellefonte, PA) applying the following temperature program: 40 °C held for 1 min, 30 °C/min to 110 °C and afterwards 25 °C/min up to a final temperature of 200 °C which was held for 2 min. Helium was used as carrier gas with a constant flow of 1 mL/min. For each measurement run, calibration curves were analyzed at the beginning and the end of the sequence.

Stable Carbon Isotope Analysis. Stable carbon isotopes of PCE, TCE and *cis*-DCE were determined using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) comprising a Trace GC Ultra (Thermo Finnigan, San Jose, CA) coupled to a Delta Plus XP IRMS detector (ThermoFinnigan) via a GC Combustion III interface (ThermoFinnigan). For analysis, duplicate samples of selected time points (frozen in 1.5 mL HPLC vials) were diluted with ultrapure water to a total volume of 3 mL in glass screw vials and sealed with magnetic screw caps containing a silicone/PTFE septum. Dilution was adjusted to obtain peak amplitudes of 2000 to 4000 mV for precise δ^{13}C determination. Based on current instrumental performance this corresponded to a PCE concentration of 200 μg/L. Samples were enriched and extracted using solid phase microextraction (SPME) via a StableFlex-Fiber covered with 85 μm

Carboxen/Polydimethylsiloxan (Supelco, Bellefonte, PA) and a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). Enrichment was done at 40 °C for 20 min after which the sample was desorbed at 270 °C for 1 min in an OPTIC 3 PTV injector (GL Sciences, Eindhoven, Netherlands). For the chromatographic separation of PCE, TCE and *cis*-DCE, a Rtx-VMS capillary column (60 m x 32 mm; 1.8 μm film) and the following temperature program were applied: 40 °C held for 4 min, 8 °C/min to 180 °C, afterwards 20 °C/min up to 200 °C which was held for 2 min. Helium was used as carrier gas with an initial flow of 2 mL/min for 2 min during desorption and 1.5 mL/min for the rest of the measurement run. Compound specific standards with known carbon isotope signatures (δ^{13}C) of -27.35 ‰, -26.68 ‰ and -25.35 ‰ for PCE, TCE and *cis*-DCE were measured in duplicates after every 10 samples and at the end of each measurement run. At the beginning of each measurement sequence, additional five standards were included.

Stable Chlorine Isotope Analysis. Chlorine isotopes of PCE were analyzed using an Agilent Technologies 7890B GC coupled to an Agilent Technologies 5977A MSD and a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). The gas chromatograph was equipped with a Rtx-VMS fused silica column (60 m x 0.25 mm, 1.4 μm film; Restek, Bellefonte, PA) and operated with a constant flow of 1 mL/min helium as carrier gas. Sample enrichment was conducted at 40 °C for 20 min with SPME technique using a StableFlex-Fiber covered with 85 μm Carboxen/Polydimethylsiloxan (Supelco). The samples were desorbed in a Split/Splitless injector for 2 min at 260 °C with a split ratio of 1:10. For each sample, analysis was conducted in quintuplicates which were bracketed by five replicates per concentration-adjusted external standards (EIL-2 and Tü-Inhouse). Bulk chlorine isotope ratios were determined as previously described by Sakaguchi-Söder et al. using the modified multiple ion method after Jin et al.[42,43] A two-point calibration curve with external PCE standards ($\delta^{37}Cl_{EIL-2}$ = -2.52 ‰[44]; $\delta^{37}Cl_{Tü-Inhouse}$ = 10.4 ‰ (Buchner et al., in prep.)) was used to calculate δ^{37}Cl isotope values relative to the SMOC scale as recommended in previous studies (s. SI for a brief description of $\delta^{37}Cl_{Tü-Inhouse}$).[45,46]

Determination of Isotope Enrichment Factors (ε) and Dual Isotope Slopes. Isotope enrichment factors for carbon and chlorine were determined according to the Rayleigh equation using the double logarithmic Rayleigh plot without forcing the regression line through zero as stated by Scott et al.[47]:

$$ln\left(\frac{R(t)}{R(0)}\right) = \varepsilon * ln\left(f(t)_{PCE}\right) \quad (1)$$

R(t) and *R(0)* denote the isotope ratios at different sampling points and the initial isotope ratio at the start of the experiment, respectively; $f(t)_{PCE}$ is the remaining fraction of PCE at sampling point *t*. Fractions of PCE and less chlorinated ethenes were calculated based on the timepoint-wise mass balance correction method described by Buchner et al.[48] to account for

the cumulative mass removal due to repetitive sampling:

$$f_{PCE} = \frac{n(t)_{PCE}}{n(t)_{PCE} + \sum_{i=1}^{k} n(t)_i} \tag{2}$$

where $n(t)_{PCE}$ and $n(t)_i$ represent amounts of PCE and less chlorinated dehalogenation products (TCE and/or *cis*-DCE and *trans*-DCE) at the same time point.

Dual isotope slopes ($\Lambda_{C/Cl}$) and associated standard errors (SE) were calculated for the individual experiments by regressing measured $\delta^{13}C$ and $\delta^{37}Cl$ data using the York method.[49] Resulting slopes were checked against each other for statistical differences using the *z*-score test. A two-tailed hypothesis test was applied to choose the appropriate *p*-value.[49] Slopes were considered to be statistically different if the corresponding *p*-value were below the significance level of 0.05.

PCE-RdhA Amino Acid Sequence Comparison. To analyze the amino acid sequence identity of the catalytic subunit of the investigated $RdhA_{TCE}$ and $RdhA_{cDCE}$ enzymes, sequences of *D. hafniense* strain Y51 and PCE-S (both $RdhA_{cDCE}$) were obtained from the UniProt database. The amino acid sequence of $RdhA_{TCE}$ of *D. dehalogenans* strain PCE1 was extracted from its annotated genome sequence available on the Joint Genome Institute website. The gene with the ID number 0038 was previously annotated as *prdA*-gene encoding the PCE-to-TCE transforming subunit PrdA.[36] An amino acid identity matrix was constructed using the Clustal Omega web tool for multiple sequence alignment for protein sequences.[50]

2.4 Results and Discussion

Table 2.1. Overview of *Desulfitobacterium* spp. strains investigated in this study, their respective PCE-RdhA and corresponding nomenclature in the text.

Strain	**Enzyme**	**Label**[a]	**Stable product**	**Ref**
Desulfitobacterium dehalogenans PCE1	PrdA	$RdhA_{TCE}$	TCE	35,51
Desulfitobacterium sp. Viet1	putative PrdA[b]		TCE	13,52
Desulfitobacterium hafniense Y51	PceA	$RdhA_{cDCE}$	*cis*-DCE	33,39
Desulfitobacterium hafniense PCE-S	PceA		*cis*-DCE	34,53
Desulfitobacterium hafniense PCP-1	CrdA	$RdhA_{co}$	TCE, *cis*-DCE, *trans*-DCE	37,54

[a] refers to the enzyme notation used in this study.

[b] inferred from TCE as stable product of PCE dehalogenation observed in the stated reference.

Stable Products of PCE Dehalogenation and Dual Element Isotope Fractionation

The magnitude of carbon and chlorine isotope fractionation (ε_C, ε_{Cl}) during reductive dehalogenation of PCE was determined for three different types of PCE-transforming enzymes (PCE-RdhAs) using living cells of different *Desulfitobacterium* (*Dsb*) strains. Table 2.1 provides an overview of the characteristics of the investigated strains. Dehalogenation profiles of PCE for the tested strains are provided in the SI (Figure A1 to A4).

All strains completely dehalogenated PCE within the monitored time frame of the experiments. Stable PCE transformation products differed depending on the catalyzing PCE-RdhA with TCE for $RdhA_{TCE}$-carrying strains, *cis*-DCE for $RdhA_{cDCE}$-carrying strains and mainly TCE with small amounts of *cis*- and *trans*-DCE for $RdhA_{co}$-carrying strain PCP-1.

For PCE-to-TCE transformation by $RdhA_{TCE}$-carrying strains PCE1 and Viet1 carbon isotope enrichment factors (ε_C values) of -19.7 ± 0.5 ‰ and -19.0 ± 0.9 ‰ and chlorine isotope enrichment factors (ε_{Cl} values) of -6.3 ± 0.3 ‰ and -5.0 ± 0.1 ‰ were observed. A substantially smaller isotope effect was measured for PCE-to-*cis*-DCE transformation by $RdhA_{cDCE}$-carrying strains Y51 and PCE-S with ε_C values of -5.8 ± 0.3 ‰ and -5.0 ± 0.3 ‰ and ε_{Cl} values of -2.2 ± 0.2 ‰ and -1.9 ± 0.2 ‰. Cometabolic transformation by $RdhA_{co}$-carrying strain PCP-1 showed an intermediate isotope fractionation (ε_C = -9.8 ± 0.4 ‰ and ε_{Cl} = -2.9 ± 0.4 ‰). The observed ε_C values are in the range of -0.4 to -19.0 ‰ of published values for microbial PCE transformation.[11–13] Isotope enrichment factors of $RdhA_{cDCE}$-carrying strains (strains Y51 and PCE-S) agree with the published ε_C of strain PCE-S (-5.2 ± 0.5 ‰)[11] and are similar to ε_C and ε_{Cl} values reported for a *Desulfitobacterium*-dominated consortium dehalogenating PCE to *cis*-DCE (ε_C = -5.6 ± 0.7 ‰ and ε_{Cl} = -2.0 ± 0.5 ‰).[55] Measured ε values of $RdhA_{TCE}$-carrying strains (strains PCE1 and Viet1) agree with carbon and chlorine isotope enrichment factors determined for abiotic reductive dehalogenation of PCE by vitamin B_{12} (ε_C = -16.6 ± 2.7 ‰ to -17.0 ± 1.2 ‰; ε_{Cl} = -4.0 ± 0.4 ‰ to -4.2 ± 0.4 ‰).[21]

Based on our results, carbon and chlorine isotope fractionation during metabolic PCE transformation by *Dsb* can be grouped into (i) a strong isotope effect for $RdhA_{TCE}$-carrying strains and (ii) an approximately four times smaller isotope effect for strains carrying $RdhA_{cDCE}$. A similar pattern was previously observed for carbon isotope fractionation by two different *Sulfurospirillum*-consortia where a fivefold stronger isotope effect was measured for the consortium transforming PCE to TCE (ε_C = -3.6 ± 0.2 ‰) compared to the consortium catalyzing dehalogenation of PCE to *cis*-DCE (ε_C = -0.7 ± 0.1 ‰) which was attributed to different reaction mechanisms.[32]

Application of the Dual Isotope Approach to Decipher a Potential Correlation of ε Values with Different Reaction Mechanisms

For $RdhA_{TCE}$-carrying strain PCE1 a dual isotope slope ($\Lambda_{C/Cl}$) of 3.07 ± 0.03 was obtained matching the $\Lambda_{C/Cl}$ of 3.6 ± 0.1 published for strain Viet1, which harbors a putative $RdhA_{TCE}$ (Table 2.2).[13] For $RdhA_{cDCE}$-carrying strains Y51 and PCE-S, $\Lambda_{C/Cl}$ values of 2.4 ± 0.1 and 2.6 ± 0.1 were determined. Cometabolic PCE transformation by $RdhA_{co}$-carrying

strain PCP-1 resulted in a $\Lambda_{C/Cl}$ value of 3.3 ± 0.1. Based on *z*-score tests ($\alpha = 0.05$)[49], all $\Lambda_{C/Cl}$ values are significantly different from each other except for the comparison of $RdhA_{cDCE}$-carrying strains (SI Table A1) which may indicate different reaction mechanisms of the tested PCE-RdhAs. However, statistically different Λ values measured for microbial transformation reactions cannot be attributed to different reaction mechanisms based on *z*-score test results exclusively. Depending on the character of the rate-determining step within multistep reactions, varying Λ values may be determined for the same reaction mechanism due to changes in reaction conditions or enzyme properties.[4] $\Lambda_{C/Cl}$ values differed by ten for reductive dehalogenation of TCE by addition-elimination (high pH) and addition-protonation (low pH) mechanisms.[21] For oxidation and reductive dehalogenation, $\Lambda_{C/Cl}$ values differed by about 20 and 12 for *cis*-DCE and VC, respectively.[9] Thus, the similar $\Lambda_{C/Cl}$ values (maximum deviation of 1.2) determined in this study suggest that PCE transformation proceeds via the same reaction mechanism in *Dsb* strains irrespective of their catalyzing PCE-RdhA and the resulting transformation product. This is supported by the published range of $\Lambda_{C/Cl}$ determined for enzyme catalyzed PCE transformation in lab scale studies which show consistent values of 2.2 to 2.9 across different PCE-RdhAs which dechlorinate PCE either into TCE or *cis*-DCE (Data published by Wiegert et al.[55] were re-evaluated for the $\Lambda_{C/Cl}$ value according to the York method resulting in a $\Lambda_{C/Cl}$ value of 2.9 ± 0.3. This corresponds to the inverse calculation of the published $\Lambda_{Cl/C}$ value).[31,32,55]

Table 2.2. Summary of isotope enrichment factors and dual isotope slopes for reductive dehalogenation of PCE measured for different strains of the *Desulfitobacterium* genus, their stable products from PCE dehalogenation and their respective PCE reductive dehalogenase enzyme.

***Desulfitobacterium* spp. strain**	**Enzyme**	**Stable product**	**ε_C ± CI[(b)] (‰)**	**ε_{Cl} ± CI[(b)] (‰)**	**$\Lambda_{C/Cl}$ ± SE[(c)]**
PCE1	$RdhA_{TCE}$	TCE	-19.7 ± 0.5	-6.3 ± 0.3	3.07 ± 0.03 (n=15)
Viet1[(a)]			-19.0 ± 0.9	-5.0 ± 0.1	3.6 ± 0.1 (n=16)
Y51	$RdhA_{cDCE}$	*cis*-DCE	-5.8 ± 0.3	-2.2 ± 0.2	2.4 ± 0.1 (n=12)
PCE-S			-5.0 ± 0.3	-1.9 ± 0.2	2.6 ± 0.1 (n=9)
PCP-1	$RdhA_{co}$	TCE, *cis*-DCE, *trans*-DCE	-9.8 ± 0.4	-2.9 ± 0.4	3.3 ± 0.1 (n=13)

[(a)] Data from Cretnik et al.[13] We used the raw isotope data from the SI to calculate $\Lambda_{C/Cl}$ according to the York method.

[(b)] 95 % confidence interval (CI) for the linear regression in the double logarithmic Rayleigh plots.

[(c)] Standard error (SE) for the York regression calculated after Ojeda et al.[49]

The observed $\Lambda_{C/Cl}$ correspond to the recent results of Heckel et al. who determined $\Lambda_{C/Cl}$ values of 3.9 ± 0.4 to 4.2 ± 0.3 for abiotic PCE dehalogenation by vitamin B_{12} and postulated an addition-elimination mechanism.[21] In contrast, the outer-sphere SET ($\Lambda_{C/Cl} = \infty$) shows negligible chlorine isotope fractionation and can be excluded as an underlying reaction mechanism for microbial PCE dehalogenation.[28] It can therefore be concluded that the tested PCE-RdhAs of *Dsb* strains catalyze PCE dehalogenation via an addition-elimination mechanism irrespective of the transformation product or whether the process is metabolic or cometabolic. Consequently, the determined variability of single element ε values for *Dsb* strains differing in their PCE-RdhA cannot be attributed to different reaction mechanisms.

Masking Effects as Determining Factors for the Magnitude of PCE ε Values

Assuming the same reaction mechanism for different PCE-RdhAs of *Dsb*, the varying ε values can be attributed to masking effects which arise from rate-limiting steps preceding C-Cl bond cleavage. Since the strong isotope effect of $RdhA_{TCE}$-carrying strains (e.g. ε_C = -19.0 to -19.7 ‰) can be assumed to be least affected by masking effects, PCE isotope fractionation of $RdhA_{cDCE}$-carrying strains is most affected by masking followed by cometabolic PCE transformation by $RdhA_{co}$-carrying strain PCP-1.

Masking can act upon microbial isotope fractionation at different instances: mass transfer limitation of the substrate and during the enzymatic multistep reaction due to rate-limitation by reaction steps preceding chemical bond cleavage. Limited mass transfer towards the cell and into the cell leads to substrate bioavailability restrictions at the catalyzing enzymes. Thus, mass transfer becomes rate-limiting and masks the enzyme intrinsic ε value.[4,56] Decreasing bulk substrate concentrations during the course of transformation can therefore mask isotope fractionation as observed for microbial toluene degradation. The decrease in toluene concentration was accompanied with a decrease in slope in the Rayleigh plot reflecting the impact of masking on isotope fractionation.[4,57] Since microbial cell walls and membranes were reported to act as transport barriers during PCE transformation[11,29,30], they can be assumed to enhance PCE mass transfer limitation towards the enzyme with decreasing PCE concentration. However, in our experiments no decrease in slopes in the Rayleigh plots was detected which indicates no bottleneck in PCE bioavailability for the catalyzing enzymes.[4]

Desulfitobacterium was recently classified as monoderm, Gram-positive genus without a second outer membrane.[38] Also for Gram-negative staining strains Y51 and PCP-1 no second outer membrane–the characteristic feature of Gram-negative cell walls–was observed in ultrathin section electron micrographs.[34,54] The similar cell wall architecture of investigated *Dsb* strains allows to exclude mass transfer limitation to be responsible for the variable ε values. The reported different Gram staining of $RdhA_{cDCE}$-containing strains points to a thinner peptidoglycan layer of Gram-negative staining strain Y51 compared to Gram-positive staining strain PCE-S.[34,39,40] However, the almost identical ε values (ε_C = -5.8 ± -0.3 ‰ and -5.0 ± 0.3 ‰) showed that potential structural differences in Gram-positive cell walls did not affect PCE mass transfer and observable isotope fractionation.

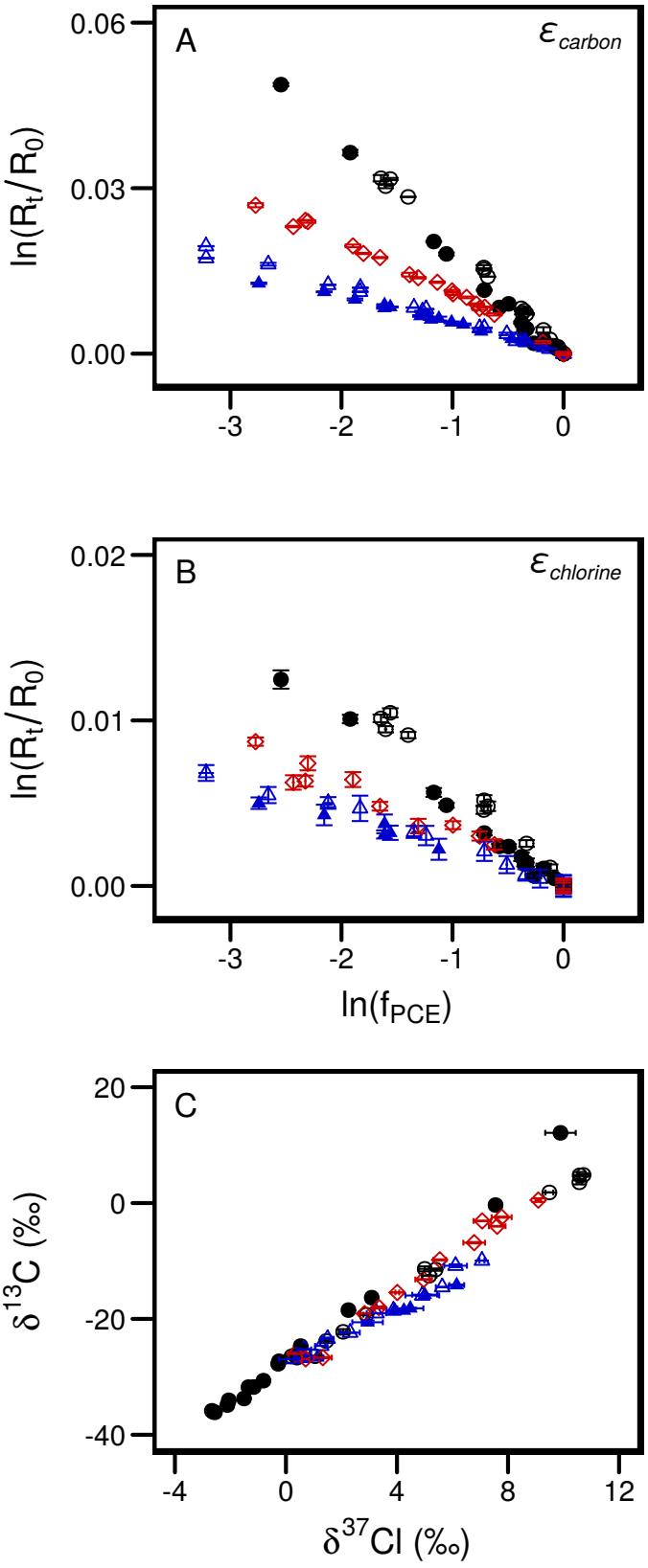

Figure 2.1. Carbon and chlorine isotope fractionation by *Desulfitobacterium* spp. strains PCE1 (○), Viet1 (●), Y51 (△), PCE-S (▲), PCP-1 (◊) during PCE dehalogenation. Data for strain Viet1 were extracted from the SI of Cretnik et al. (2014).

(A) Double logarithmic Rayleigh plot for carbon isotope fractionation. Y-error bars represent standard deviation calculated from measurement replicates of $\delta^{13}C$ values (n=2) via Gaussian error propagation, x-error bars represent standard deviation of PCE fractions (n=2) calculated via Gaussian error propagation.

(B) Double logarithmic Rayleigh plot for chlorine isotope fractionation. Y-error bars represent standard deviation calculated from measurement replicates of $\delta^{37}Cl$ values (n=3-5) via Gaussian error propagation, x-error bars represent standard deviation of PCE fractions (n=2) calculated via Gaussian error propagation.

(C) Dual isotope plot ($\delta^{13}C$ vs $\delta^{37}Cl$). Error bars represent standard deviation of measurement replicates (n=2 for $\delta^{13}C$, n=3-5 for $\delta^{37}Cl$).

Further masking effects for microbial PCE isotope fractionation were reported for a cytoplasmic location of the catalytically active PCE-RdhA. This was attributed to additional transport limitation across the cytoplasmic membrane.[30] Earlier studies proposed a cytoplasmic localization of PCE-RdhAs of strains PCE1 and PCE-S and a periplasmic localization for strain Y51.[33,35,53] However, the presence of a twin-arginine translocalisation (TAT) signal sequence (responsible for protein transport into the periplasm) in PCE-RdhA amino acid sequences of these strains allows to conclude a periplasmic localization of catalytically active PCE-RdhAs in all of these strains (for TAT sequence comparison see Chart A1 in the SI).[25,58,59] Therefore masking effects by mass transfer limitation towards and into the cells can be excluded to cause the variable ε values of *Dsb* strains with different PCE-RdhAs.

In enzyme catalyzed PCE dehalogenation, C-Cl bond cleavage is preceded by reaction steps such as diffusion of PCE to the active site within the enzymatic substrate channel and formation of the enzyme-PCE complex.[18,31] During multistep reactions, the intrinsic isotope effect of C-Cl bond cleavage is reflected in measured ε values only if the bond-cleaving step is the rate-determining step in the overall reaction. Thus, if preceding steps are associated with lower rate-constants than the bond-cleaving step, they become rate-limiting and mask the observed ε value compared to the intrinsic ε value of bond cleavage.[4,14] For abiotic PCE dehalogenation by vitamin B_{12} it is assumed that no rate-limiting steps precede the bond-cleaving step and ε values reflect the reaction intrinsic kinetic isotope effect.[21] The consistent ε and $\Lambda_{C/Cl}$ values of $RdhA_{TCE}$-carrying strains and vitamin B_{12} indicate that for $RdhA_{TCE}$ C-Cl bond cleavage is the rate-limiting step in the multistep reaction and consequently no masking by preceding steps occurs. Thus, ε values of $RdhA_{TCE}$ reflect the reaction intrinsic isotope effect. In contrast, lower ε values of $RdhA_{cDCE}$- and $RdhA_{co}$-carrying *Dsb* strains indicate masking by rate-limitation of reaction steps prior to bond-cleavage. Such masking effects within the multistep reaction of $RdhA_{cDCE}$ enzymes are supported by lower PCE ε values reported for the $RdhA_{cDCE}$ of *Sulfurospirillum multivorans* compared to corrinoid mediated abiotic dehalogenation. Based on significantly lower dehalogenation rates for the enzyme catalyzed reaction, it was concluded that steps preceding the enzymatic bond cleavage constituted bottlenecks reducing the overall reaction rate as well as the observable isotope fractionation.[31] The preceding rate-limiting steps were further hypothesized to be associated with small isotope effects themselves, which led to the measured differences in $\Lambda_{C/Cl}$ values between enzymatic (2.2 to 2.8) and abiotic PCE dehalogenation (4.6 to 7.0).[31] Accordingly, the observed deviation in $\Lambda_{C/Cl}$ values between $RdhA_{cDCE}$ (2.4 to 2.6) and $RdhA_{TCE}$/$RdhA_{co}$ (3.07 to 3.6) of *Dsb* strains may be explained by isotope effects of the rate-limiting step in $RdhA_{cDCE}$ preceding bond cleavage or an overlain isotope effect of multiple steps including bond cleavage. Similar conclusions were made by Renpenning et al. and Lihl et al. explaining the deviation between $\Lambda_{C/Cl}$ values of 0.7 and 3.6 measured for enzyme catalyzed PCE dehalogenation by small but non-negligible isotope effects of a preceding step.[22,31] Further on, modulation of intrinsic kinetic isotope effects by reaction steps aside from bond cleavage was also proposed by Heckel et al. for enzymatic dehalogenation of chlorinated ethenes.[21] An in-depth discussion on the effect of equilibrium and kinetic isotope

effects of multiple reaction steps on observable ε and $\Lambda_{C/Cl}$ values is provided by Elsner.[4] The consistent ε values determined for the same type of PCE-RdhA ($RdhA_{cDCE}$- or $RdhA_{TCE}$-carrying strains) suggest that differences in rate-limiting steps are related to the properties of the enzymes, such as the amino acid sequence. Based on amino acid identity analysis, protein sequences of the catalytic subunit of $RdhA_{cDCE}$-carrying strains (strains Y51 and PCE-S) are 97 % identical, whereas sequences between $RdhA_{TCE}$ (strain PCE1) and $RdhA_{cDCE}$ (strains Y51 and PCE-S) are significantly different with identities of 34 and 35 %, respectively. The primary protein structure (amino acid sequence) determines the spatial structure of the holoenzyme and amino acid identities therefore indicate structural variability of different enzymes.[60] Recent studies on RdhA biochemistry, RdhA protein structure and chlorinated ethene isotope fractionation indicate that rate-limitation by reaction steps preceding bond cleavage may be associated with characteristic structural properties of single parts of RdhA enzymes. For example, a hydrophobic channel in $RdhA_{cDCE}$ of *Sulfurospirillum multivorans* was suggested to limit mass transfer of the hydrophobic PCE to the active site of this enzyme. Consequently, the isotope effect of bond cleavage is masked resulting in the small isotope effect of this enzyme (ε_C = -1.4 ± 0.1 ‰).[18,31,61] Activation energies (E_A) and rate-constants for enzyme catalyzed reactions are influenced by the structure of an enzyme, especially the shape of the active center and the binding site.[60] Thus, differences in rate-limiting steps between $RdhA_{TCE}$ and $RdhA_{cDCE}$ may also be linked to structural differences of the active site. On this basis, two different scenarios emerge explaining the observed rate-limitation in $RdhA_{cDCE}$ (and $RdhA_{co}$) using $RdhA_{TCE}$ as reference system.

(i) In the first scenario, rate constants of bond cleavage are higher (i.e. lower E_A) for $RdhA_{cDCE}$ compared to $RdhA_{TCE}$ due to a more beneficial structure of the active site resulting in comparatively faster bond conversion (s. Figure 2.2, scenario 1). Assuming that rate constants of preceding steps, e.g. diffusion of PCE in the substrate channel, are comparable in both types of enzymes, those would become rate-limiting in $RdhA_{cDCE}$ and mask the intrinsic isotope effect of C-Cl bond cleavage. In contrast, bond cleavage is rate-limiting in $RdhA_{TCE}$ and the intrinsic isotope effect is completely depicted in the ε value. However, differences in rate-limiting steps may not be related to structural differences of the active sites of $RdhA_{TCE}$ and $RdhA_{cDCE}$. Kunze et al. reported highly similar amino acid sequences of cofactor-binding domains (i.e. active sites) of two different RdhAs of *Dsb*[62], while Renpenning et al. inferred that carbon isotope fractionation is not affected by structural differences of corrinoid cofactors.[31] (ii) Thus, on the assumption of similar rate-constants for the bond-converting step in the different PCE-RdhAs, at least one preceding step represents a bottleneck in the multistep reaction of $RdhA_{cDCE}$ (s. Figure 2.2, scenario 2). For two different RdhAs of *Dsb*, the strongest variations between amino acid sequences were found in parts of the enzymes forming the substrate channels.[62] Thus, different amino acids in the substrate channels of $RdhA_{cDCE}$ and $RdhA_{TCE}$ may lead to different diffusion rates of PCE towards the active site. For example, a higher content of hydrophobic amino acid residues in the substrate channel of $RdhA_{cDCE}$ may hamper the diffusion of PCE due to more hydrophobic interactions compared to $RdhA_{TCE}$. This step may

then become rate-limiting and mask the intrinsic isotope effect of C-Cl bond cleavage. However, based on our results, the significant masking effect for RdhA$_{cDCE}$ cannot be unambiguously attributed to one of the scenarios. A combination of both scenarios is also possible.

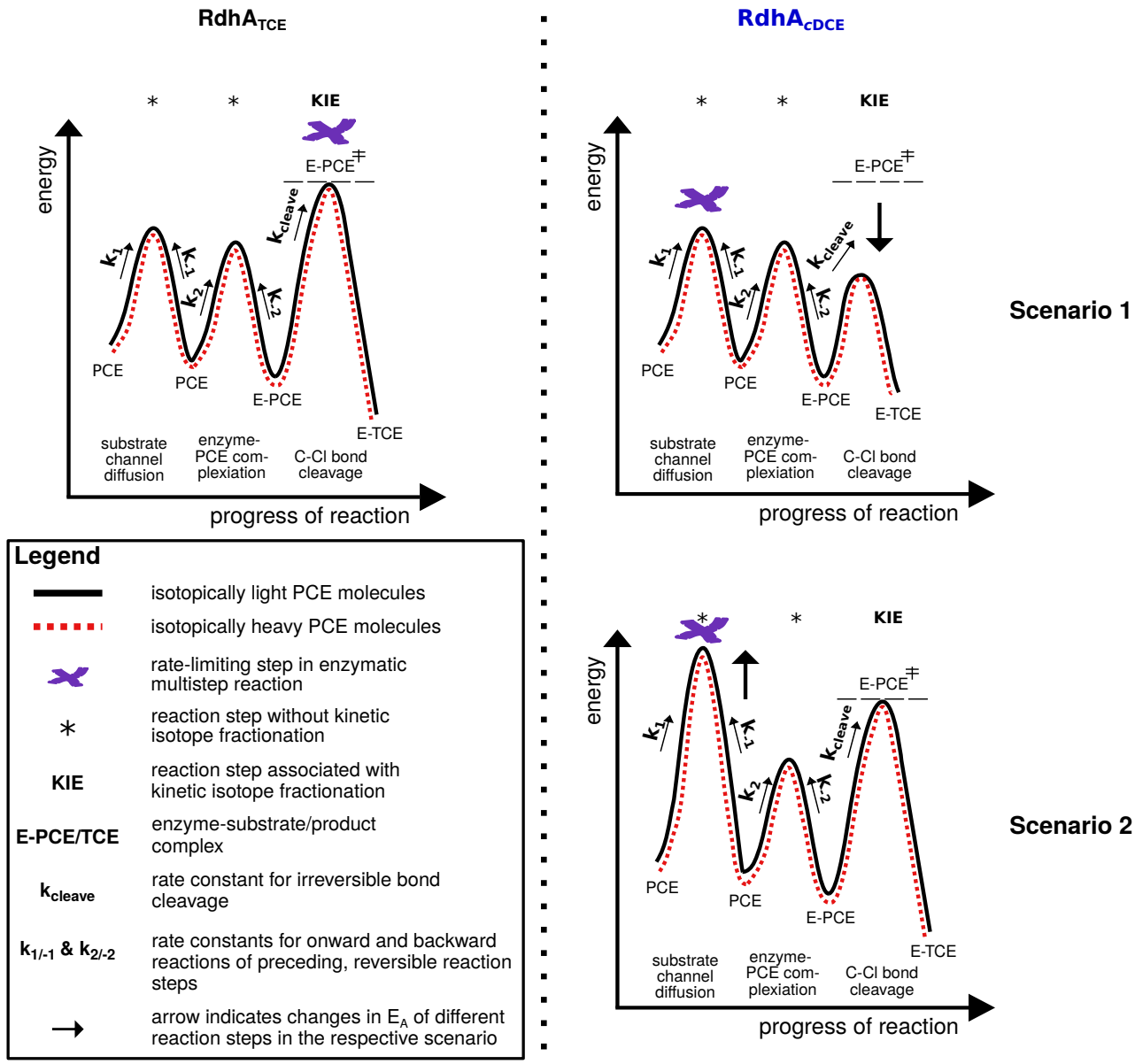

Figure 2.2. Hypothetical scenarios of masking due to different rate constants of single steps within the enzymatic multistep reactions leading to measured variations of ε values for RdhA$_{TCE}$ and RdhA$_{cDCE}$.

In summary, our results provide evidence that the rate-determining reaction step for PCE-to-TCE transformation by RdhA$_{TCE}$ enzymes of *Dsb* strains is given by C-Cl bond cleavage, whereas for the RdhA$_{cDCE}$ enzymes transforming PCE to *cis*-DCE and RdhA$_{co}$ transforming PCE cometabolically, rate-limitation arises from reaction steps preceding the actual C-Cl bond cleavage. Thus, the varying PCE ε values of *Dsb* are caused by different rate-determining steps within the enzymatic multistep reaction which becomes evident by (i) the dual isotope slopes

showing that the different PCE-RdhAs of *Dsb* catalyze PCE dehalogenation via the same reaction mechanism and (ii) the preclusion of masking effects due to PCE mass transfer limitation.

Implications for the Variability of PCE Isotope Enrichment Factors and Their Use in CSIA

Carbon CSIA has been successfully applied to prove and monitor *in situ* transformation of chlorinated ethenes.[5–7] However, the broad variability of ε_C values for microbial PCE dehalogenation[11–13] hampers generalized and reliable quantification schemes for contaminated field sites. This study demonstrates the applicability of dual isotope analysis to discern the contribution of masking effects and different reaction mechanisms to varying ε values determined for *Desulfitobacterium* (*Dsb*) strains with different PCE-RdhAs. Consistent dual isotope slopes of *Dsb* strains with distinct PCE-RdhAs and vitamin B_{12} from a previous study[21] provide evidence for an addition-elimination mechanism for PCE dehalogenation by *Dsb* irrespective of the catalyzing enzyme or its transformation product. This contrasts previous results of *Sulfurospirillum* spp. where different reaction mechanisms have been inferred to cause varying ε values and transformation products.[32] Since virtually all $\Lambda_{C/Cl}$ values determined for enzyme-catalyzed PCE dehalogenation in lab scale studies range from 2.2 to 3.6 and thus are consistent with the addition-elimination mechanism of vitamin B_{12}, it seems likely that microbial PCE dehalogenation proceeds via this reaction mechanism throughout different genera of OHRB and different PCE-RdhAs. An impact of mass transfer limitation on ε values was not observed in this study suggesting that for bacteria without a second outer membrane (i.e. Gram-positive) the reported variations of PCE ε values originate from different magnitudes of masking within the enzymatic multistep reaction of the different PCE-RdhAs. Future studies may aim at combining *in silico* structural modeling of PCE-RdhAs, CSIA and the application of directed mutagenesis to identify potential structural determinants within the holoenzymes for differences in rate-limiting steps. This would help to decipher the structural basis of masking during enzyme-catalyzed PCE isotope fractionation.

For contaminated field site management, the consistency of $\Lambda_{C/Cl}$ and simultaneous differences in ε values and transformation products for PCE-RdhAs of both *Dsb* and other genera of OHRB does not allow to narrow the range of applicable ε values based on $\Lambda_{C/Cl}$ from field isotope data as proposed previously.[32] Thus, determination of site specific ε values seems indispensable for quantification of *in situ* chlorinated ethene transformation using CSIA. However, the robust $\Lambda_{C/Cl}$ strengthens the applicability of CSIA as forensic tool for source identification. Carbon and chlorine isotope ratios of PCE undergoing transformation from a source zone should be constrained by slopes ranging from 2 to 3. PCE carbon and chlorine isotope ratios outside this range would indicate additional PCE sources at the field site.

2.5 References

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3

Effect of Different Enzymatic Phenotypes of Desulfitobacterium dehalogenans Strain PCE1 on Tetrachloroethene Isotope Fractionation

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Daniel Buchner	2.	40 %	0 %	40 %	20 %
Sebastian Behrens	3.	0 %	0 %	0 %	10 %
Stefan Haderlein	4.	10 %	0 %	0 %	10 %

Title of paper:
Effect of Different Enzymatic Phenotypes of *Desulfitobacterium dehalogenans* Strain PCE1 on Tetrachloroethene Isotope Fractionation

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3.1 Abstract

Variable isotope enrichment factors (ε values) were reported for single pure cultures of organohalide-respiring bacteria (OHRB) carrying multiple reductive dehalogenases genes (*rdhA*). The magnitude of ε values and the prevailing reaction mechanism are enzyme specific. Because transcription levels of different *rdhA*-genes can vary depending on environmental conditions, consequent changes in cellular abundances of different reductive dehalogenases enzymes (RdhA) may affect measured ε values of pure cultures. This study investigated the variability of tetrachloroethene (PCE) isotope fractionation of *Desulfitobacterium dehalogenans* strain PCE1 after expression of different RdhAs that are typically not produced upon exposure to PCE. To this end, strain PCE1 was initially exposed to 2,4,6-trichlorophenol (TCP) or 3-chloro-4-hydroxyphenylacetate (OHPA) to enforce chloroaromatic RdhA expression. Carbon and chlorine isotope fractionation was monitored during subsequent PCE transformation and compared with a PCE reference experiment. All test series (TS) showed reproducible ε values (ε_C = -17.9 ± 1.1 ‰ to -19.2 ± 0.7 ‰; ε_{Cl} = -5.1 ± 0.8 ‰ to -6.4 ± 0.4 ‰) and identical dual isotope slopes (3.2 ± 0.1 to 3.3 ± 0.1). This suggests that the presence of different RdhAs did neither affect the observable isotope fractionation nor the reaction mechanism. Significant lag-phases of TS_{OHPA} and TS_{TCP} after PCE addition allowed to attribute the reproducible ε values to minor PCE dechlorination rates of chloroaromatic RdhAs and predominant catalysis of PCE transformation by the designated PCE-RdhA after *de novo* synthesis. These results indicate that OHRB carrying a specialized, chlorinated ethene transforming RdhA among multiple other RdhAs, catalyze chlorinated ethene dehalogenation predominantly by the specialized RdhA. Thus, it can be expected that during reductive dehalogenation of chlorinated ethenes, ε and $\Lambda_{C/Cl}$ values of living cells of OHRB are not affected by changing cellular abundances of different RdhAs.

3.2 Introduction

Extensive usage of chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) in industrial processes have resulted in their frequent occurrence as groundwater contaminants. Their toxic and suspected carcinogenic properties demand remediation of contaminated field sites to accomplish human and environmental health criteria.[1–4] Bioremediation takes advantage of microbially mediated *in situ* contaminant transformation and is considered as a more cost-effective approach compared to *ex situ* techniques such as pump and treat.[2] Key microorganisms for successful bioremediation are organohalide-respiring bacteria (OHRB) which catalyze dechlorination of chlorinated ethenes into innocuous ethene by reductive dehalogenases enzymes (RdhA).[5]

In situ chlorinated ethene transformation can be monitored using compound specific isotope analysis (CSIA).[6,7] In addition, CSIA allows to quantify the magnitude of *in situ* contaminant transformation and to elucidate the underlying reaction mechanisms.[8,9] CSIA makes use of kinetic isotope fractionation during compound transformation which results from faster reaction

rates of light isotopes (e.g. ^{12}C) compared to heavy isotopes (e.g. ^{13}C). This causes a gradual enrichment of heavy isotopes in the non-degraded substrate fraction leading to shifts in the observable isotopic signature.[8] The isotope enrichment factor (ε) relates the change in the isotopic signature to the causative compound transformation according to the Rayleigh equation and can be applied to calculate the extent of *in situ* contaminant transformation based on measured isotope values.[9,10] For microbial chlorinated ethene transformation, highly variable ε_C values were reported for different enrichment cultures or pure isolates ranging from -0.4 to -19.0 ‰ for PCE[11–16] and -2.5 to -18.9 ‰ for TCE.[17–21] Notably, for single pure cultures harboring multiple RdhAs, variable ε_C values were reported for the same chlorinated ethene, e.g. -5.1 and -8.4 ‰ for 1,1-dichloroethene transformation by *Dehalococcoides mccartyi* strain BAV1 or -9.6 to -14.4 ‰ for TCE transformation by *D. mccartyi* strain 195.[12,19,21,22] In contrast, ε values of OHRB carrying only one RdhA were stable even under changing physiological conditions.[23] The variability of ε_C values hampers the implementation of CSIA to quantify *in situ* chlorinated ethene transformation, as application of inappropriate ε values leads to incorrect estimates.[9] Therefore, deciphering the factors causing variable chlorinated ethene ε values of single pure cultures in a first step can provide a profound knowledge base to assess the varying ε values of microbial communities which is a prerequirement for reliable application of CSIA at contaminated sites.

The magnitude of ε is determined by the manner of bond cleavage in the (bio)chemical reaction and thus depends on the underlying reaction mechanism.[10] However, ε values of the same reaction mechanism can be diminished by rate-determining steps preceding bond cleavage (i.e. masking) such as mass transfer limitation or slow reaction steps in the enzymatic multistep reaction.[10] Dual isotope analysis (e.g. $^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) offers the opportunity to assign the variability of ε values to either different reaction mechanisms or the impact of masking. Masking effects cancel out when isotope data of two elements is considered relative to each other in a dual isotope plot (e.g. $\delta^{13}C$ vs $\delta^{37}Cl$) and do not affect resulting dual isotope slopes ($\Lambda_{C/Cl}$) from linear regression.[8,10] For microbial chlorinated ethene transformation, different reaction mechanisms were shown to be associated with significant different $\Lambda_{C/Cl}$ values.[24–27] For example, recent studies reported three to four times higher $\Lambda_{C/Cl}$ values for TCE transformation by the addition-protonation mechanism compared to the addition-elimination mechanism.[25,26] Thus, different $\Lambda_{C/Cl}$ values indicate the impact of different reaction mechanisms on varying ε values, whereas similar $\Lambda_{C/Cl}$ values reflect the impact of masking effects. Based on dual isotope analysis of TCE transformation by different OHRB, RdhA enzyme architecture was recently proposed to determine the prevailing reaction mechanism and corresponding ε values.[26] In addition, slow steps in the enzymatic multistep reaction such as diffusion in the enzyme core and enzyme-substrate association may additionally modulate the mechanism intrinsic isotope effect of chlorinated ethene transformation.[25,28] Thus, ε values are fundamentally enzyme specific. On the level of individual OHRB harboring multiple RdhAs, variable ε values of single chlorinated ethenes may therefore result from transformation by different RdhAs varying in their inherent reaction mechanisms and/or the type of rate-limiting step in the enzymatic

multistep reaction.

RdhAs are membrane-associated, corrinoid-bearing enzymes which are encoded in the genomes of OHRB within *rdh*-gene operons.[5] The number of *rdhA*-genes encoding the catalytically active enzyme varies between 1 to 7 for metabolically versatile OHRB (e.g. *Sulfurospirillum* or *Desulfitobacterium*) and 5 to 38 for obligate OHRB which are restricted to organohalide respiration (e.g. *Dehalococcoides* or *Dehalobacter*).[5,29] *De novo* synthesis of RdhAs is induced in the presence of organohalides by transcriptional regulators of the NosR- and NirI-type, CRP/FNR-type or MarR-type (*rdhC*, *rdhK* and *rdhR*)[30–33] resulting in up-regulation of *rdhA*-gene transcription and an increased RdhA enzyme amount per cell.[23,34–36] Most RdhAs show high selectivity for single substrates that specifically induce their gene expression, but dechlorinate also structurally related organohalides.[37,38] This can result in overlapping substrate spectra of different RdhAs in a single OHRB[38,39] suggesting that simultaneous transformation of single organohalides by various RdhAs is likely to occur after initiation of *de novo* synthesis by an appropriate substrate. Simultaneous transcription of multiple *rdhA*-genes upon exposure to a single organohalide has been reported for pure cultures of obligate and versatile OHRB.[38,40,41] However, relative transcription levels of co-transcribed RdhAs differed depending on the provided organohalide. Transcriptional analysis of all 17 putative *rdhA*-genes of *Dehalococcoides mccartyi* strain 195 revealed significant differences in relative *rdhA*-gene transcript levels for the provision of PCE, TCE or 2,3-DCP.[40] Similar results were obtained for relative transcription levels of six different *rdhA*-genes of *Desulfitobacterium hafniense* strain DCB-2 upon exposure to three different dichlorophenols (DCPs). At this, the spectrum of dechlorinated organohalides was highly similar in subsequent protein crude extract experiments irrespective of the transcribed RdhAs, however, dechlorination proceeded at different rates.[38] This indicates that the enzymatic phenotype of an OHRB carrying multiple *rdhA*-genes can vary with respect to the expressed RdhAs depending on the present organohalides. Further on, these results suggest that single organohalides can be degraded by various RdhAs of the same OHRB albeit at different rates. For pure cultures of OHRB harboring multiple *rdhA*-genes, observable ε values may therefore reflect the average of the intrinsic ε values of different RdhAs contributing to transformation. Thus, changes in the relative amount of different RdhAs may cause the variable ε_C values reported for a single chlorinated ethene degraded by the same strain. The main objective of this study was to evaluate (i) whether chlorinated ethene isotope fractionation differs upon presence of different RdhAs in the cells and (ii) if potential changes in ε values can be related to different reaction mechanisms or masking effects of the contributing RdhAs. To this end, we monitored PCE carbon and chlorine isotope fractionation of living cells of the versatile OHRB *Desulfitobacterium dehalogenans* strain PCE1 after exposure to different chloroaromatic compounds. This OHRB was chosen since it derives growth supporting energy from reductive dehalogenation of PCE, tri- and dichlorophenols as well as 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA) and carries six putative *rdhA*-genes in its genome. PCE and 3-Cl-4-OHPA transformation was assigned to the *prdA*- and *cprA*-gene, respectively, whereas no specific substrate was assigned to the other four putative *rdhA*-genes.[42,43] Thus, exposure to 3-Cl-4-

OHPA, 2,4,6-trichlorophenol and PCE prior to PCE transformation allowed us to compare the ε values and dual isotope slopes of at least two different enzymatic phenotypes of strain PCE1.

3.3 Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) with the highest available level of purity.

Cultivation of *Desulfitobacterium dehalogenans* strain PCE1 and Experimental Setups

Desulfitobacterium dehalogenans strain PCE1 (No. 10344) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Cells of strain PCE1 which had not been exposed to organohalides before were routinely grown in 250 mL serum bottles containing 200 mL of an anoxic, liquid medium as described by Buchner et al.[23] and 50 mL headspace of an N_2/CO_2 gas mixture (80:20). 1 mM pyruvate and fumarate were supplied as electron donor and acceptor from filter-sterilized (0.22 μM), anoxic stock solutions. From this culture, precultures of the individual test series were prepared by inoculating 1 % (v/v) of cell suspension onto fresh liquid medium containing 1 mM pyruvate as electron donor and either 1 mM 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA) or 100 μM 2,4,6-trichlorophenol (TCP) from autoclaved stock solutions or 200 μM neat PCE as electron acceptor. After complete dechlorination, 1 % (v/v) preculture was used as inoculum for the individual experiments comprising three living-replicates and one abiotic, cell-free control each. The preculture grown on PCE was degassed with N_2/CO_2 (80:20) for 1 h to avoid a carryover of volatile transformation products, which was confirmed via GC-MS measurements. For test series in which cells were exposed to PCE (TS_{PCE}) and to TCP prior to PCE transformation (TS_{TCP}), 560 mL serum bottles filled with a final liquid volume of 500 mL liquid were used. For the test series in which cells were exposed to 3-Cl-4-OHPA prior to PCE transformation (TS_{OHPA}), 250 mL serum bottles were utilized containing a final liquid volume of 100 mL. In all test series, 1 mM pyruvate was used as electron donor. For TS_{PCE}, 200 μM PCE was supplied as electron acceptor and dissolved prior to inoculation. TS_{OHPA} and TS_{TCP} contained 1 mM 3-Cl-4-OHPA and 100 μM TCP as electron acceptors, respectively. A total of four 3-Cl-4-OHPA transformation events were conducted in TS_{OHPA} by re-supplying 1 mM 3-Cl-4-OHPA and 1 mM pyruvate after qualitative detection of complete dechlorination in HPLC-UV/VIS chromatograms (Figure B4 to B6 in the SI). After complete conversion of chloroaromatic compounds, PCE was dissolved in fresh liquid medium yielding a final concentration of 200 μM in the respective test series. This spike solution was transferred into TS_{TCP} and TS_{OHPA} in an anoxic glovebox (O_2 <1 ppm) with N_2 atmosphere. PCE spike solutions contained additional pyruvate yielding a final concentration of 1 mM.

Sample Extraction and Analytical Methods

Sampling. All liquid volumes were extracted using gas-tight glass syringes (Innovative Laborsysteme GmbH, Stützerbach, Germany).

For chloroaromatic concentration analysis, 1 mL was extracted and transferred into a 1.5 mL-Eppendorf tube, which was centrifuged for 10 min at 10,000 × g and room temperature. Samples for HPLC measurements were prepared by transferring 200 μL supernatant into a 1.5 mL screw neck HPLC vial to which 799 μL ultrapure water (Merck Millipore, Burlington, MA) and 1 μL pure H_3PO_4 (85 %) were added beforehand. HPLC vials were closed with PTFE-lined screw caps and vortexed for 15 s at maximum speed prior to analysis.

Samples for chlorinated ethene concentration analysis were prepared by dividing 1 mL liquid equally into two 10 mL crimp glass vials which were previously filled with 4.5 mL ultrapure water (Merck Millipore, Burlington, MA) and 100 μL of 1 M H_3PO_4 (85 %). The latter was added to stop microbial activity. Sample vials were sealed with aluminium crimp caps containing silicone/PTFE septa and directly measured.

For carbon and chlorine isotope measurements, 4 mL were extracted and distributed in portions of 1 mL into 1.5 mL screw neck HPLC vials to which 50 μL of 10 M NaOH were added to stop microbial activity. Vials were closed with PTFE-lined screw caps, frozen and stored upside down at -20 °C for later isotope analysis.

For molecular biological analysis of TS_{PCE} and TS_{TCP}, 20 mL were extracted and divided into portions of 5 mL in Eppendorf tubes for subsequent DNA and RNA analysis. Samples were immediately stored on ice to prevent RNA degradation. Cell pellets were obtained by centrifuging the samples for 10 min at 10,000 rpm and 4 °C. The supernatant was discarded and two sample replicates were stored at -80 °C, whereas the other were stored at -20 °C. During the course of the experiments, sample volumes were decreased to 2 mL per replicate yielding a total volume of 8 mL per sampling point.

Chloroaromatic Concentrations. Concentration analyses of chloroaromatics comprised quantification of 2,4,6-TCP; 2,4,-dichlorophenol (2,4-DCP) and 4-monochlorophenol (4-MCP) for TS_{TCP} and qualitative monitoring of 3-Cl-4-OHPA plus its transformation product 4-OHPA for TS_{OHPA}. Measurements were conducted with a HPLC-UV/VIS system (Bischoff Analysentechnik und -geräte GmbH, Leonberg, Germany) comprising a LC-CaDI 22-14 interface, a DAD-Detector, two binary pumps, a degasser and AL 3110 injector. 50 μL sample were injected for both test series. All chloroaromatic compounds were detected at a wavelength of 200 nm. Chlorophenols were measured with a Prontosil Eurobond C18 column (150 mm x 4 mm, 5 μm particle size; Bischoff, Leonberg, Germany) isothermally at 30 °C. Separation of chlorophenols was achieved using 95 % H_2O / 5 % acetonitrile / 0.1 % H_3PO_4 (85 %) as eluent A and pure acetonitrile as eluent B with a constant flow of 0.8 mL/min and the following gradient: 2.5 min held at 35 % B, increase to 80 % B in 0.5 min, held at 80 % for 2 min, increase to 95 % B in 0.5 min, held for 3.5 min followed by a decrease to 35 % B in 0.5 min, which was held for 5.5 min. Quantification of chlorophenol concentrations was enabled by measuring substance specific standards at the start and the end of each analysis sequence. Phenylacetates were measured

with a Prontosil 120-3-C18-AQ column (150 mm x 3 mm, 3 μm particle size; Bischoff, Leonberg, Germany) isothermally at 30 °C. Separation was achieved using an isocratic flow of 95 % H_2O / 5 % acetonitrile / 0.1 % H_3PO_4 (85 %) (A) and pure acetonitrile (B) with at a ratio 80(A)/20(B) and a constant flow of 0.5 mL/min.

Chlorinated Ethene Concentrations. Analysis of chlorinated ethenes was performed in duplicates on a GC-MS system (Agilent Technologies 7890A GC coupled to an Agilent Technologies 5975VL MS Detector, Santa Clara, CA) via static headspace injection of 500 μL using a Gerstel MultiPurpose Sampler MPS (Mülheim an der Ruhr, Germany). Injections were done in split mode 1:10 with a Split/Splitless injector operated at 240 °C. Chromatographic separation of *cis*-DCE, *trans*-DCE, TCE and PCE was achieved using a Rtx-VMS fused silica column (60 m x 0.25 mm, 1.4 μm film; Restek, Bellefonte, PA) applying the following temperature program: 40 °C held for 1 min, 30 °C/min to 110 °C and afterwards 25 °C/min up to a final temperature of 200 °C which was held for 2 min. Helium was used as carrier gas with a constant flow of 1 mL/min. For each measurement run, calibration curves were analyzed at the beginning and the end of the sequence.

Stable Carbon Isotope Analysis. Stable carbon isotopes of PCE, TCE and *cis*-DCE were determined using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) comprising a Trace GC Ultra (Thermo Finnigan, San Jose, CA) coupled to a Delta Plus XP IRMS detector (ThermoFinnigan) via a GC Combustion III interface (ThermoFinnigan). For analysis, duplicate samples of selected time points (frozen in 1.5 mL HPLC vials) were diluted with ultrapure water to a total volume of 3 mL in glass screw vials and sealed with magnetic screw caps containing a silicone/PTFE septum. Dilution was adjusted to obtain peak amplitudes of 2000 to 4000 mV for precise $\delta^{13}C$ determination. Based on current instrumental performance this corresponded to a PCE concentration of 200 μg/L. Samples were enriched and extracted using solid phase microextraction (SPME) via a StableFlex-Fiber covered with 85 μm Carboxen/Polydimethylsiloxan (Supelco, Bellefonte, PA) and a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). Enrichment was done at 40 °C for 20 min after which the sample was desorbed at 270 °C for 1 min in an OPTIC 3 PTV injector (GL Sciences, Eindhoven, Netherlands). For the chromatographic separation of PCE, TCE and *cis*-DCE, a Rtx-VMS capillary column (60 m x 32 mm; 1.8 μm film) and the following temperature program were applied: 40 °C held for 4 min, 8 °C/min to 180 °C, afterwards 20 °C/min up to 200 °C which was held for 2 min. Helium was used as carrier gas with an initial flow of 2 mL/min for 2 min during desorption and 1.5 mL/min for the rest of the measurement run. Compound specific standards with known carbon isotope signatures ($\delta^{13}C$) of -27.35 ‰, -26.68 ‰ and -25.35 ‰ for PCE, TCE and *cis*-DCE were measured in duplicates after every 10 samples and at the end of each measurement run. At the beginning of each measurement sequence, additional five standards were included.

Stable Chlorine Isotope Analysis. Chlorine isotopes of PCE were analyzed using an Agilent Technologies 7890B GC coupled to an Agilent Technologies 5977A MSD and a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). The gas chromatograph was equipped

with a Rtx-VMS fused silica column (60 m x 0.25 mm, 1.4 μm film; Restek, Bellefonte, PA) and operated with a constant flow of 1 mL/min helium as carrier gas. Sample enrichment was conducted at 40 °C for 20 min with SPME technique using a StableFlex-Fiber covered with 85 μm Carboxen/Polydimethylsiloxan (Supelco). The samples were desorbed in a Split/Splitless injector for 2 min at 260 °C with a split ratio of 1:10. For each sample, analysis was conducted in quintuplicates which were bracketed by five replicates per concentration-adjusted external standards (EIL-2 and Tü-Inhouse). Bulk chlorine isotope ratios were determined as previously described by Sakaguchi-Söder et al. using the modified multiple ion method after Jin et al.[44,45] A two-point calibration curve with external PCE standards ($\delta^{37}Cl_{EIL-2}$ = -2.52 ‰[46]; $\delta^{37}Cl_{Tü-Inhouse}$ = 10.4 ‰ (Buchner et al., in prep.)) was used to calculate δ^{37}Cl isotope values relative to the SMOC scale as recommended in previous studies (s. SI of chapter 2 for a brief description of $\delta^{37}Cl_{Tü-Inhouse}$).[47,48]

Determination of Isotope Enrichment Factors (ε) and Dual Isotope Slopes. Isotope enrichment factors for carbon and chlorine were determined according to the Rayleigh equation using the double logarithmic Rayleigh plot without forcing the regression line through zero as stated by Scott et al.[49]:

$$ln\left(\frac{R(t)}{R(0)}\right) = \varepsilon * ln\left(f(t)_{PCE}\right) \quad (1)$$

R(t) and *R(0)* denote the isotope ratios at different sampling points and the initial isotope ratio at the start of the experiment, respectively; *f(t)$_{PCE}$* is the remaining fraction of PCE at sampling point *t*. Fractions of PCE and less chlorinated ethenes were calculated based on the timepoint-wise mass balance correction method described by Buchner et al.[50] to account for the cumulative mass removal due to repetitive sampling:

$$f_{PCE} = \frac{n(t)_{PCE}}{n(t)_{PCE} + \sum_{i=1}^{k} n(t)_i} \quad (2)$$

where *n(t)$_{PCE}$* and *n(t)$_i$* represent amounts of PCE and less chlorinated dehalogenation products (TCE and/or *cis*-DCE and *trans*-DCE) at the same time point.

Dual isotope slopes ($\Lambda_{C/Cl}$) and associated standard errors (SE) were calculated for the individual experiments by regressing measured δ^{13}C and δ^{37}Cl data using the York method.[51] Resulting slopes were checked against each other for statistical differences using the *z*-score test. A two-tailed hypothesis test was applied to choose the appropriate *p*-value.[51] Slopes were considered to be statistically different if the corresponding *p*-value were below the significance level of 0.05.

Molecular Biological Analysis

Cell abundances of *D. dehalogenans* strain PCE1 were determined using a quantitative PCR (qPCR) assay targeting the *prdA*-gene which encodes the PCE-transforming PrdA enzyme.[42] Based on genome analysis, one copy of the *prdA*-gene is present per genome and measured gene copy numbers therefore equal the total amount of strain PCE1 cells.[42]

DNA Extraction. Genomic DNA was extracted from living-replicates using the DNeasy® UltraClean® Microbial Kit (Qiagen GmbH, Hilden, Germany) following the manufacturers instruction. To increase DNA yields, samples were kept in a water bath at 70 °C for 10 min prior to bead treatment.

Quantification of prdA-gene. For each sample, the qPCR assay was performed in triplicates in 10 μL reaction volume containing 1 μL of template DNA, 250 nM of forward (5‘ -*CTGGTCTTGGAGAGTTGGGC*- 3‘) and reverse (5‘ -*TCTGCGGCTCCAAAACTGAT*- 3‘) primer and 5 μL Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc. Hercules, CA). Amplification and detection were conducted using an iCycler (Bio-Rad Laboratories, Inc. Hercules, CA) with an iQ™5 qPCR detection system and Optical System software (Bio-Rad Laboratories, Inc. Hercules, CA) with the following temperature settings: initial 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 15 s. Specificity of product formation was ensured at the end of each assay run by melt curve analysis using the following temperature program: 1 min at 95 °C, 1 min at 55 °C and stepwise increase by 0.5 °C every 10 s up to 95 °C. Specific amplification of the desired *prdA*-gene fragment was additionally checked by performing gel electrophoresis after each qPCR analysis. Quantification of *prdA*-gene copies was enabled by simultaneous analysis of plasmid standards containing a *prdA*-fragment. The plasmid standard was synthesized *de novo* by MWG Eurofins GmbH (Ebersberg, Germany) according to the annotated genome sequence of *D. dehalogenans* strain PCE1 available at IMG Genome database (ID: 2512875014).

Error of Molecular Biological Analysis. Errors of measured *prdA*-gene abundances reflect the percentage standard deviation of each sample accounting for introduced biases during sample extraction, removal of the supernatant, DNA extraction and sample preparation for the qPCR assay. The error was determined by processing five replicate samples extracted from a pure culture of strain PCE1 grown on pyruvate and PCE. DNA concentrations were measured after extraction using the Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) and statistically tested for outliers using the two-sided Grubbs test. Since no outliers were detected, *prdA*-gene abundances were determined for the five replicates using the qPCR assay. Measured gene abundances (3 wells per sample * 5 samples = 15 measurement replicates) were tested again for outliers using two-sided Grubbs test. No outliers were detected and samples were corrected for the elution volume and extraction volume. For measured gene abundances, the average and standard deviation were calculated using the individual 15 replicates. Based on this, the percentage standard deviation was calculated which amounted to 21 %.

3.4 Results and Discussion

Chlorinated Ethene Transformation Profiles, Growth of Strain PCE1 and Generated Enzymatic Phenotypes

After initial exposure to either 2,4,6-TCP (TS_{TCP}) or 3-Cl-4-OHPA (TS_{OHPA}) to induce expression of different RdhAs, cells of strain PCE1 were exposed to PCE. In a reference experiment, strain PCE 1 was exclusively exposed to PCE (TS_{PCE}).

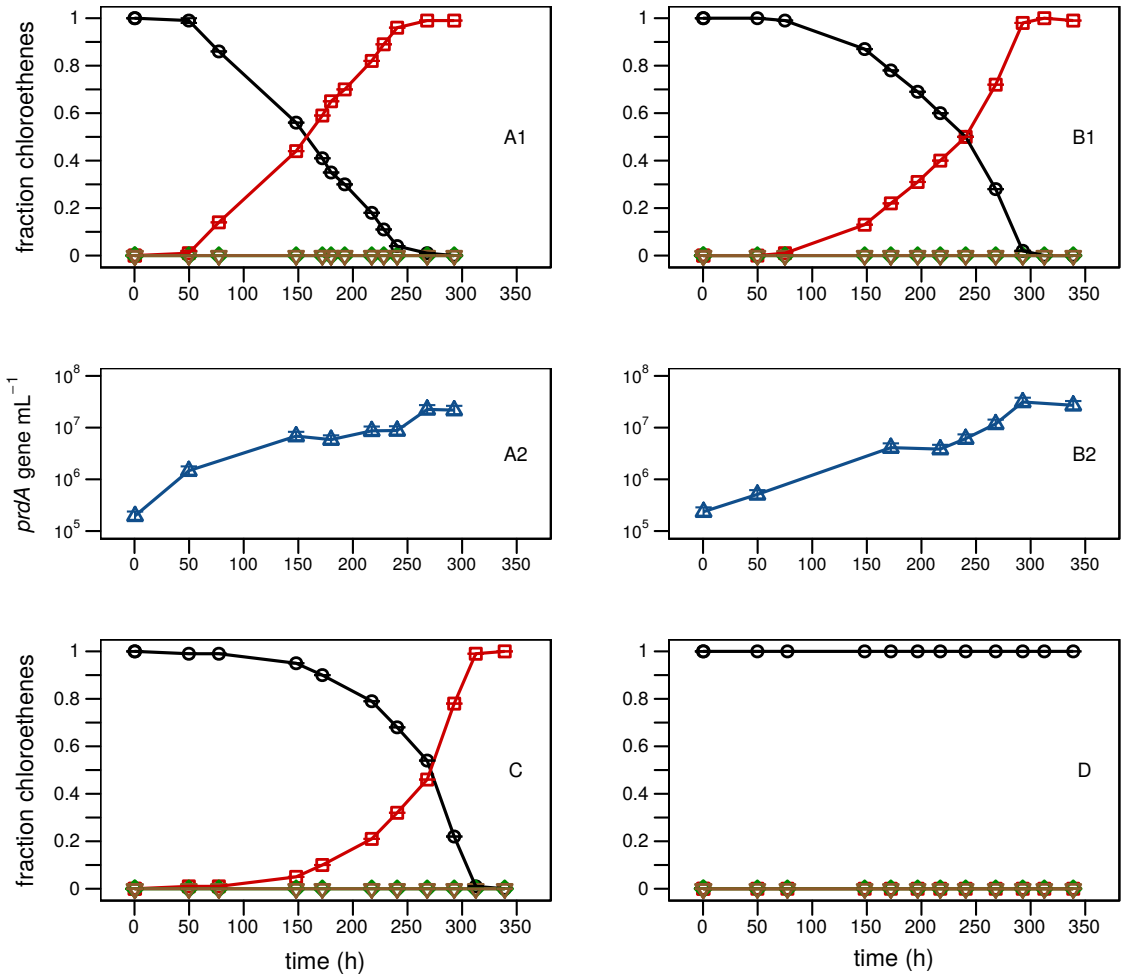

Figure 3.1. Chlorinated ethene transformation profiles and growth of *Desulfitobacterium dehalogenans* strain PCE1 in the PCE reference experiment (TS_{PCE}). (A1, B1, C, D) Fractions of PCE (circle), TCE (square), *cis*-DCE (diamond) and *trans-DCE* (inverted triangle) over time in MC1 (A1), MC2 (B1), MC3 (C) and the control (D). Error bars represent standard deviation of GC-MS concentration measurements (n=2). (A2, B2) Cell numbers of strain PCE1 carrying the *prdA*-gene (triangle) versus time in MC1 and MC2. Error bars represent the percentage standard deviation of measured cell numbers introduced during single steps of molcular biological analysis (s. Materials and Methods for details).

TS_{PCE}. Following inoculation, PCE transformation occurred after a lag-phase of 50 h in microcosm 1 (MC1) and after 75 h in MC2 and MC3 (Figure 3.1). Subsequently, PCE fraction decreased exponentially, being completely transformed after about 300 h with TCE as end product. For MC1 and MC2, comparable growth curves were measured for cells carrying

the *prdA*-gene (Figure 3.1 A2, B2). After inoculation, an immediate increase in cell numbers from about $2.2*10^5$ cells/mL in both MC to $1.5*10^6$ cells/mL in MC1 and $5.1*10^5$ cells/mL in MC2 was measured in the first 50 h. In this interval, PCE was not significantly transformed. Exponential PCE dechlorination was accompanied with an increase in cell numbers carrying the *prdA*-gene to a maximum of $2.2*10^7$ cells/mL and $3.1*10^7$ cells/mL in MC1 and MC2 after complete dechlorination into TCE. This agrees with the observation that PCE dechlorination is coupled to the generation of growth supporting energy in strain PCE1.[52]

Exposure of strain PCE1 to PCE was shown to induce exclusively *de novo* synthesis of the PrdA enzyme which catalyzes dechlorination of PCE into TCE.[43] Thus, the enzymatic phenotype of strain PCE1 cells is characterized by the sole presence of PrdA in this test series.

The observed lag-phase of PCE dehalogenation suggests *de novo* synthesis of PrdA after inoculation, although PCE adapted cells were transferred. For repetitive cultivation of *D. hafniense* strain Y51 on TCE, each TCE addition was associated with an up-regulation of *pceA*-gene transcription.[23] This indicates that during repeated cultivation with the same organohalide, OHRB synthesize the specific RdhA *de novo* upon each organohalide addition. Thus, the absence of PCE dechlorination in the first 50 to 75 h after inoculation can be explained by *de novo* synthesis of PrdA and the low amount of inoculated preculture (1 % (v/v)). The immediate increase in cell numbers in this interval may resulted from fermentative growth of strain PCE1 with pyruvate[52] which was provided as electron donor for PCE transformation.

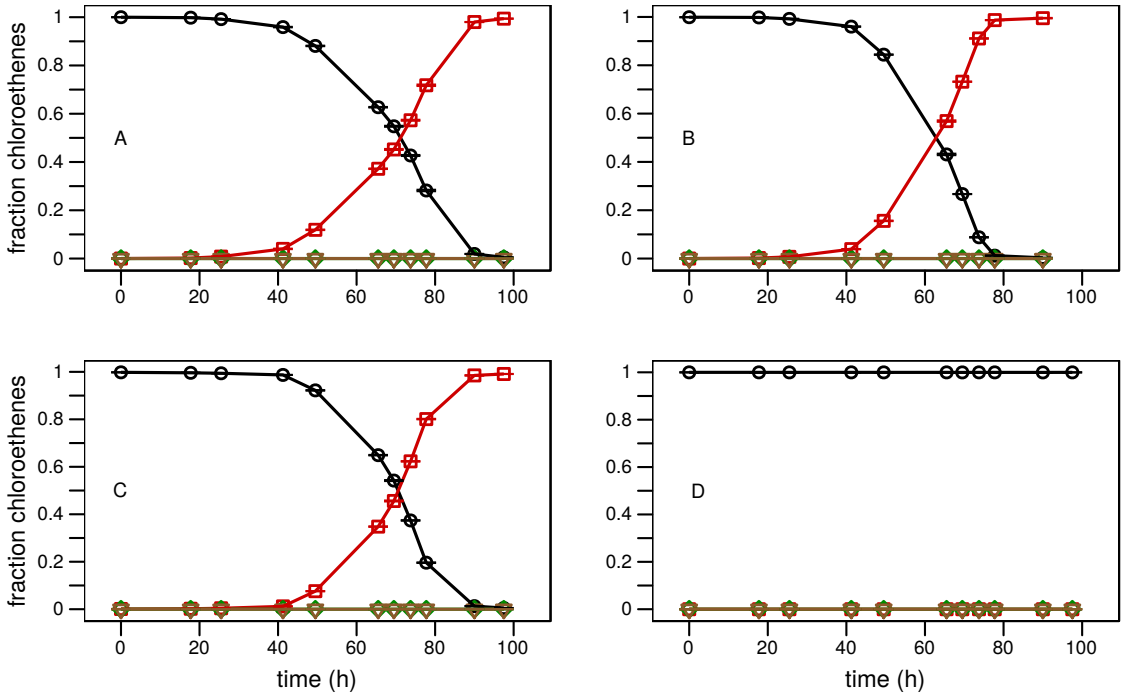

Figure 3.2. Chlorinated ethene transformation profiles after exposure of *Desulfitobacterium dehalogenans* strain PCE1 to 3-chloro-4-hydroxyphenylacetate in TS_{OHPA}. Shown are fractions of PCE (circle), TCE (square), *cis-DCE* (diamond) and *trans*-DCE (inverted triangle) over time in MC1 (A), MC2 (B), MC3 (C) and the control (D). Error bars represent standard deviation of GC-MS concentration measurements (n=2).

TS_{OHPA}. Cells of strain PCE1 were initially grown in medium containing 3-Cl-4-OHPA (1 mM). After a total of four complete 3-Cl-4-OHPA transformation events, 200 μM PCE were added to the cultures. Following a lag-phase of 50 h in all microcosm, complete PCE transformation into TCE was observed after a total run time of 80 to 90 h (Figure 3.2).

Exposure of strain PCE1 towards 3-Cl-4-OHPA was reported to specifically induce expression of the CprA enzyme catalyzing reductive dehalogenation of 3-Cl-4-OHPA into 4-OHPA.[43] This agrees with observations on other *Desulfitobacterium* strains carrying the *cprA*-gene/CprA enzyme. In the closely related *D. dehalogenans* strain JW/IU-DC1, 3-Cl-4-OHPA specifically induces transcription of CprA which was subsequently purified from protein crude extracts.[32,33,42,53] In addition, exposure of *Desulfitobacterium* sp. strain Viet1 to 3-Cl-4-OHPA triggered transcription of CprA, while transcription of PrdA was not observed.[54] For *Desulfitobacterium* strains carrying multiple *rdhA*-genes but lacking the particular *cprA*-gene, no dehalogenation of 3-Cl-4-OHPA has been reported.[55] These results suggest that 3-Cl-4-OHPA is an appropriate substrate for CprA exclusively and does not up-regulate transcription of other RdhAs in strain PCE1. The generated enzymatic phenotype of strain PCE1 is therefore presumably characterized by the exclusive presence of CprA prior to PCE transformation.

TS_{TCP}. PCE dechlorination by strain PCE1 was monitored after prior complete transformation of 2,4,6-TCP into 4-monochlorophenol (4-MCP). 2,4,6-TCP transformation was detected after a lag-phase of 50 h in all MC (Figure 3.3). In MC1, exponential 2,4,6-TCP transformation started after 50 h and was completed after 120 h (Figure 3.3 C). 2,4-dichlorophenol (2,4-DCP) was the predominant transformation product accumulating up to a fraction of 80 %. In the presence of 2,4,6-TCP, a minor fraction of 12 % of the end product 4-MCP was observed. 2,4-DCP dechlorination into 4-MCP started after 120 h and was completed after 170 h. In MC2 and MC3, 2,4,6-TCP decreased to a fraction of about 90 % after 170 h, which was accompanied with an increase in 2,4-DCP fraction to 10 % (Figure 3.3 A1, B1). Subsequently, 2,4,6-TCP fraction decreased exponentially until complete transformation after 200 h. 2,4,6-TCP was initially dechlorinated to 2,4-DCP which accumulated up to a fraction of 75 %. In the presence of 2,4,6-TCP, no 4-MCP was detected. 2,4-DCP dechlorination occurred after 190 h and was completely transformed to 4-MCP after about 220 h.

Following the addition of PCE, a direct decrease in PCE fraction was measured in MC3. MC2 showed also a direct but very minor decrease of 8 % in PCE fraction in the first 120 h after PCE addition. In MC1, PCE dechlorination was preceded by a lag-phase of 150 h. All MC showed complete PCE transformation after 210 to 250 h.

Cell growth of strain PCE1 during 2,4,6-TCP and PCE transformation was similar in MC2 and MC3 (Figure 3.3 A2, B2). In MC2, cell numbers showed an immediate increase by a factor of 50 from $5.2*10^4$ cells/mL to $2.8*10^6$ cells/mL in the first 100 h after inoculation onto 2,4,6-TCP. In this interval, 5 % of 2,4,6-TCP were dechlorinated into 2,4-DCP. Subsequently, during increasing 2,4,6-TCP transformation, cell numbers decreased to $7.5*10^5$ cells/mL and showed only a minor increase to $2.1*10^6$ cells/mL when 2,4-DCP transformation started after 190 h. During transformation of 2,4-DCP and PCE (8 % of PCE transformed) cell numbers

increased by a factor of 4 to $8.2*10^6$ cells/mL. Cell numbers remained stationary during further PCE transformation until the end of the experiment. In MC3, cell numbers increased by factor of 10 from $9.2*10^4$ cells/mL to $9.1*10^5$ cells/mL within 50 h after inoculation onto 2,4,6-TCP. In this interval, 2,4,6-TCP was not dechlorinated. The onset of 2,4,6-TCP transformation was accompanied with stagnating cell numbers until exponential transformation of 2,4,6-TCP and 2,4-DCP started and cell numbers continuously increased to $4.2*10^6$ cells/mL during transformation into 4-MCP. After addition of PCE, cells numbers showed a constant slight increase from $7.8*10^6$ cells/mL to a maximum of $1.4*10^7$ cells/mL during PCE transformation. Calculating the average of maximum cell numbers that were reached during PCE transformation in MC2 and MC3 of TS_{TCP} yields $1.2*10^7 \pm 2.5*10^6$ cells/mL which is 2.3 times smaller than the value calculated for TS_{PCE} ($2.8*10^7 \pm 5.7*10^6$ cells/mL).

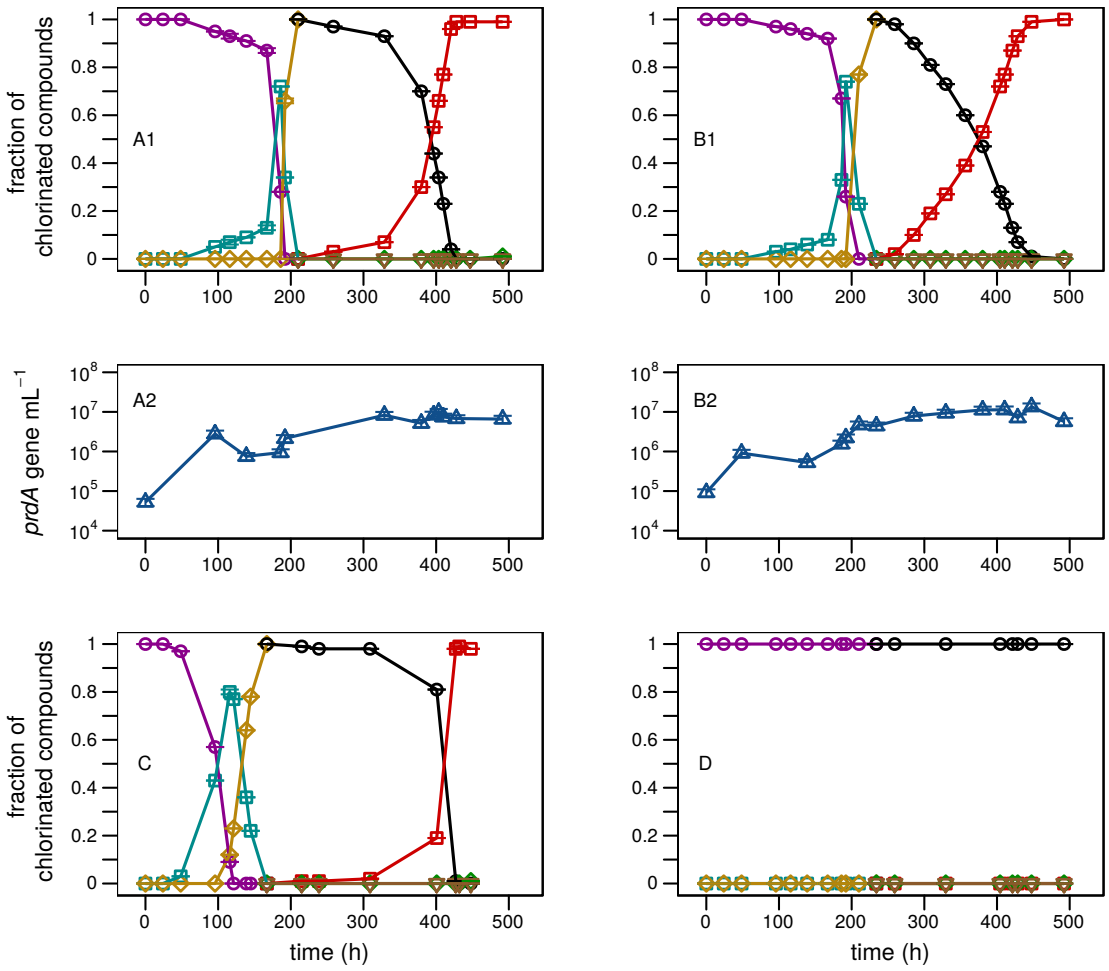

Figure 3.3. Chlorophenol and chlorinated ethene transformation profiles as well as growth of *Desulfitobacterium dehalogenans* strain PCE1 in TS_{TCP}. (A1, B1, C, D) Fractions of 2,4,6-TCP (purple cirlce), 2,4-DCP (teal square), 4-MCP (golden diamond), PCE (black circle), TCE (red square), *cis*-DCE (green diamond) and *trans*-DCE (brown inverted triangle) over time in MC2 (A1), MC3 (B1), MC1 (C) and control (D). Error bars represent standard deviation of concentration measurements (n=2). (A2, B2) Cell numbers of strain PCE1 carrying the *prdA*-gene (blue triangles) versus time in MC2 and MC3. Error bars represent the percentage standard deviation of measured cell numbers determined via error quantification (s. Materials and Methods for details).

Within 50 h after cell transfer, 2,4,6-TCP was not significantly dechlorinated, but cell numbers increased immediately. This supports the hypothesis of pyruvate fermentation as the initial metabolic process for generation of growth supporting energy after inoculation, which agrees with growth data of TS_{PCE}. Increasing cell numbers from 140 to 230 h during exponential transformation of 2,4,6-TCP and 2,4-DCP suggest that chlorophenol dechlorination was coupled to the formation of growth supporting energy after *de novo* synthesis of the appropriate RdhA enzyme. Stagnation of growth with the onset of 2,4,6-TCP transformation and lower maximum average cell numbers compared to TS_{PCE} can be explained by an elevated toxicity of 2,4-DCP and 4-MCP for microorganisms.[56] Formation of toxic 2,4-DCP and 4-MCP from 2,4,6-TCP transformation may caused a higher energy demand for cell homeostasis in TS_{TCP} resulting in less growth. This is supported by reported changes in fatty acid composition of cell membranes of Gram-positive and Gram-negative bacteria upon exposure to 2,4-DCP and 4-MCP. Changes in fatty acid composition correlated with decreasing growth rates and were proposed to consume energy equivalents indicating growth inhibiting effects of 2,4-DCP and 4-MCP.[57,58]

No RdhA has been specifically attributed to 2,4,6-TCP dechlorination by strain PCE1. Crude protein extracts from cells of strain PCE1 grown on PCE did not show dechlorination for a total of seven different tested chlorophenols.[43] This indicates that chlorophenols are inappropriate substrates for the PrdA enzyme. In addition, PrdA expression is tightly regulated by the presence of PCE in *Desulfitobacterium* sp. strain KBC1, and no upregulation was observed with other chlorinated compounds such as TCE or 3-Cl-4-OHPA.[54] These results suggest that *de novo* synthesis of PrdA by strain PCE1 in the presence of 2,4,6-TCP is an unlikely scenario. Microbial 3-Cl-4-OHPA transformation depends on the presence of the *cprA*-gene in the genome of the single strains.[43,53–55] In contrast, dechlorination of 2,4,6-TCP into 4-MCP is a common feature of *Desulfitobacterium* strains and is also catalyzed by multiple strains lacking the *cprA*-gene (see Futagami & Furukawa for a recent compendium).[59] This suggests that CprA is not the responsible RdhA for 2,4,6-TCP transformation in strain PCE1 and is therefore not expressed upon exposure to 2,4,6-TCP. The putative RdhA_0781 of strain PCE1 shows an amino acid sequence identity of 50 % with CprA3 of strain PCP-1[42] which catalyzes dechlorination of pentachlorophenol (PCP) and several tetra- (TeCP) and trichlorophenols, but not di- and monochlorophenols.[41,42,60] Dechlorination of 2,3,5,6-TeCP was observed for strain PCE1[61] suggesting that RdhA_0781 may catalyze dechlorination of 2,4,6-TCP. However, inferring overlapping RdhA substrate spectra based on amino acid similarity has proved difficult[62] and RdhA_0781 was hypothesized to not participate in dechlorination since it lacks necessary accessory genes.[42] Thus, based on our results and available evidence from literature, we cannot assign 2,4,6-TCP dechlorination to a certain putative RdhA of strain PCE1.

In *D. hafniense* strain PCP-1, stepwise dechlorination of 2,4,6-TCP into 2,4-DCP and further into 4-MCP is catalyzed by two different RdhAs that are sequentially expressed in the presence of 2,4,6-TCP and the produced 2,4-DCP.[41] The authors attributed the observed accumulation of 2,4-DCP up to a fraction of 90 % to consecutive *de novo* synthesis of the necessary RdhAs by *D. hafniense* strain PCP-1 after exposure to 2,4,6-TCP.[41] In contrast, for RdhA enzymes catalyzing

both dechlorination steps of PCE to *cis*-DCE, minor accumulation of about 20 to 25 % of TCE as intermediate was observed.[63,64] This indicates that RdhA enzymes catalyzing multiple dechlorination steps of the initial substrate produce only minor amounts of the intermediate. Based on these results, the accumulation of 2,4-DCP of up to 80 % in TS_{TCP} suggests that dechlorination of 2,4,6-TCP into 4-MCP by strain PCE1 is also catalyzed by two different RdhAs. In a previous study, the specific enzyme activity for 2,4-DCP dechlorination in protein crude extracts of 3-Cl-4-OHPA grown cells of strain PCE1 amounted to 41 % compared to 3-Cl-4-OHPA.[43] For the purified CprA enzyme of *D. dehalogenans* strain JW/IU-DC1T, a specific enzyme activity for 2,4-DCP dechlorination of 35 % relative to 3-Cl-4-OHPA was reported.[53] In addition, from three tested dichlorophenols, only 2,4-DCP induced the transcription of the 3-Cl-4-OHPA dechlorinating CprA in *D. hafniense* strain DCB-2 (denoted as RdhA6).[38] This suggests that 2,4-DCP triggers *de novo* synthesis of CprA in *cprA*-carrying strains and displays an appropriate substrate for this enzyme. 2,4-DCP dechlorination of strain PCE1 may therefore be associated with *de novo* synthesis and activity of CprA. In TS_{TCP}, the generated enzymatic phenotype of strain PCE1 prior to PCE addition can therefore be assumed to comprise two different RdhAs: an unknown RdhA produced during 2,4,6-TCP transformation ($RdhA_{2,4,6-TCP}$) and presumably CprA produced during 2,4-DCP transformation.

Table 3.1. RdhAs expressed prior to PCE transformation by different enzymatic phenotypes of *Desulfitobacterium dehalogenans* strain PCE1, carbon and chlorine isotope fractionation (ε) and corresponding dual isotope slopes ($\Lambda_{C/Cl}$) for PCE dehalogenation by the different enzymatic phenotypes.

Test series	**Initial substrate**	**RdhA expressed prior to PCE addition**	$\varepsilon_C \pm$ **CI**[(a)] (‰)	$\varepsilon_{Cl} \pm$ **CI**[(a)] (‰)	$\Lambda_{C/Cl} \pm$ **SE**[(b)]
TS_{PCE}	PCE	PrdA	-17.9 ± 1.1	-5.1 ± 0.8	3.2 ± 0.1 (n=9)
TS_{OHPA}	3-Cl-4-OHPA	CprA	-19.2 ± 0.7	-5.8 ± 0.4	3.3 ± 0.1 (n=9)
TS_{TCP}	2,4,6-TCP	$RdhA_{2,4,6-TCP}$, CprA	-18.4 ± 0.8	-6.4 ± 0.4	3.2 ± 0.1 (n=9)

[(a)] 95 % confidence interval (CI) for the linear regression in the double logarithmic Rayleigh plots.

[(b)] Standard error (SE) for the York regression calculated after Ojeda et al.[51]

PCE Isotope Fractionation of Different Enzymatic Phenotypes of Strain PCE1

Carbon and chlorine isotope fractionation (ε_C, ε_{Cl}) was monitored for PCE dechlorination of three different enzymatic phenotypes of strain PCE1 (Table 3.1). For all test series, highly similar ε values were determined for carbon (TS_{PCE} = -17.9 ± 1.1 ‰; TS_{OHPA} = -19.2 ± 0.7 ‰; TS_{TCP}= -18.4 ± 0.8 ‰) and chlorine (TS_{PCE} = -5.1 ± 0.8 ‰; TS_{OHPA} = -5.8 ± 0.4 ‰; TS_{TCP} = -6.4 ± 0.4 ‰). The ε values measured in this study fall within the range of published ε values for microbial PCE transformation ranging from -0.4 to -19.0 ‰ for carbon and -0.4 to -5.0 ‰ for chlorine and show high similarity with abiotic reductive dehalogenation of PCE by vitamin B_{12} (ε_C = -16.6 ± -17.0 ‰; ε_{Cl} = -4.0 to -4.2 ‰).[11–15,25,28] Dual isotope slopes resembled

the high similarity of measured ε values and were almost identical with $\Lambda_{C/Cl}$ values of 3.2 ± 0.1, 3.3 ± 0.1 and 3.2 ± 0.1 for TS_{PCE}, TS_{OHPA} and TS_{TCP}. The $\Lambda_{C/Cl}$ values are in line with published dual isotopes slopes for microbial PCE transformation (0.7 to 3.6) and agree with PCE dechlorination by vitamin B_{12} (3.9 to 4.2) and *Desulfitobacterium* sp. strain Viet1 (3.6).[13,14,25,28]

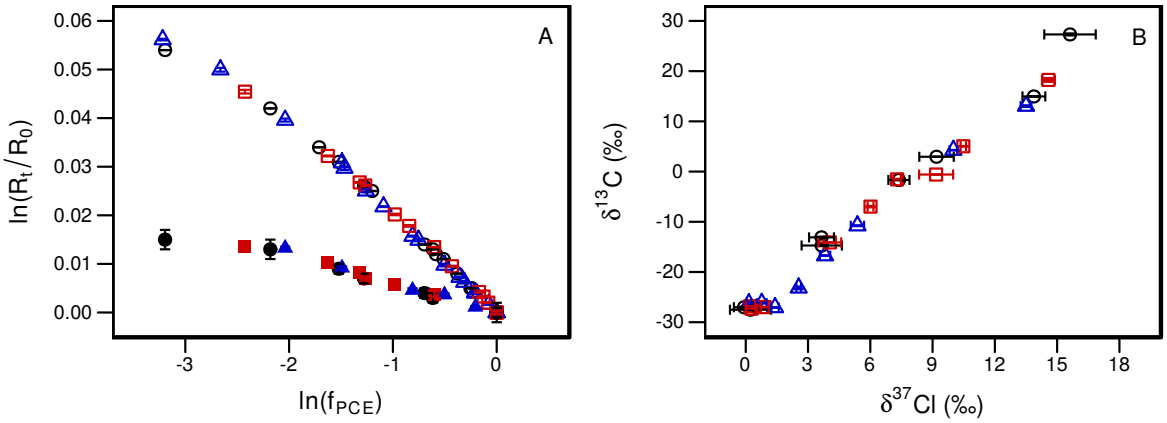

Figure 3.4. Carbon and chlorine isotope fractionation of PCE by different enzymatic phenotypes of *Desulfitobacterium dehalogenans* strain PCE1 after prior exposure to PCE (circle); 2,4,6-TCP (triangle) and 3-Cl-4-OHPA (squares). (A) Double logarithmic Rayleigh plot for carbon (open symbols) and chlorine (closed symbols) isotope fractionation. Y-error bars represent standard deviation of isotope measurement replicates (n=2 for carbon, n=5 for chlorine) via Gaussian error propagation, x-error bars represent standard deviation of PCE fractions (n=2) calculated via gaussian error propagation. (B) Dual isotope plot for the different enzymatic phentotypes of *Desulfitobacterium dehalogenans* strain PCE1. Error bars represent standard deviation of isotope measurement replicates (n=2 for carbon, n=5 for chlorine).

Contribution of Chloroaromatic RdhAs to PCE Transformation and Isotope Fractionation by Strain PCE1

Different reaction mechanisms of chlorinated ethene dehalogenation were previously reported to result in different $\Lambda_{C/Cl}$ values.[24–26] In addition, $\Lambda_{C/Cl}$ values of 3.6 to 4.2 for PCE dehalogenation were attributed to an addition-elimination reaction mechanism in previous studies.[25,26] Based on these results, the highly similar $\Lambda_{C/Cl}$ values determined in this study (3.2 to 3.3) suggest that PCE transformation by strain PCE1 proceeded via an addition-elimination mechanism for all generated enzymatic phenotypes. This indicates that either PCE dechlorination was primarily catalyzed by the PrdA enzyme or that PCE transformation by CprA and $RdhA_{2,4,6-TCP}$ of strain PCE1 proceeds also via the addition-elimination mechanism. For cometabolic PCE transformation by a chloroaromatic transforming RdhA of *D. hafniense* strain PCP-1, a $\Lambda_{C/Cl}$ value of 3.3 was reported.[64] This suggests that RdhAs catalyze PCE dechlorination via the addition-elimination mechanism regardless of whether PCE is a designated substrate (e.g. strain PCE1) or degraded within a cometabolic process (e.g. strain PCP-1). Therefore, a potential contribution of CprA or $RdhA_{2,4,6-TCP}$ to PCE transformation in TS_{OHPA} and TS_{TCP} cannot be excluded based on $\Lambda_{C/Cl}$ values.

The results of this study and previous studies indicate that ε values for metabolic PCE transformation by *Desulfitobacterium* strains cluster in the range of -5.0 to -5.8 ‰ and -17.9 to -19.7 ‰ for carbon and -1.9 to 2.2 ‰ and -5.0 to -6.3 ‰ for chlorine.[13,64] In contrast, ε values of -9.8 ‰ and -2.9 ‰ were determined for carbon and chlorine during PCE transformation by the chloroaromatic RdhA of strain PCP-1.[64] This suggests that PCE ε values differ between designated PCE-RdhAs and RdhAs transforming chloroaromatics. The highly similar ε values of the different test series therefore imply that PCE transformation was primarily catalyzed by the designated PCE-RdhA enzyme (PrdA) of strain PCE1 irrespective of the enzymatic phenotype. These results are in line with measured concentration profiles which reflect a predominant catalysis of PCE transformation by PrdA. For repetitive provision of TCE to cultures of *D. hafniense* strain Y51, immediate and continuously faster transformation after the re-supply of TCE was reported.[23] This suggest that organohalide-respiring bacteria (OHRB) show immediate dehalogenation if the cells contain an appropriate RdhA that can metabolize the provided organohalide. However, pronounced lag-phases of PCE dechlorination were measured for TS_{OHPA} (50 h) and MC1 and MC2 of TS_{TCP} (120 to 150 h) despite prior repetition of four 3-Cl-4-OHPA transformation events in TS_{OHPA} and the presence of $>2*10^6$ cells/mL in MC2 of TS_{TCP} when PCE was supplied. This indicates that PCE dechlorination is not catalyzed by CprA and $RdhA_{2,4,6-TCP}$ or only at minor rates. For PCE dechlorination by CprA in protein crude extracts of strain PCE1, a specific enzyme activity of 0.17 nkat/mg protein was reported.[43] This agrees with specific enzyme activities of 0 to 0.25 nkat/mg protein measured for PCE dechlorination by three different chloroaromatic RdhAs in protein crude extracts of *D. hafniense* strain DCB-2.[38] In contrast, a specific enzyme activity of 4.7 nkat/mg protein was measured for PCE transformation by PrdA of strain PCE1 in protein crude extracts.[43] Since the specific enzyme activity describes the catalysis rate of an enzyme for a reaction with a specific substrate, PCE transformation rates by PrdA are faster by a factor of 19 to 27 compared to chloroaromatic RdhAs. Comparatively low PCE transformation rates of chloroaromatic RdhAs are supported by the interval of 30 d required for complete PCE dechlorination by the RdhA of *D. hafniense* strain PCP-1[64], whereas 6 d were required in TS_{PCE}. The much higher PCE dechlorination rate of PrdA compared to chloroaromatic RdhAs allows to conclude that although PrdA needs to be *de novo* synthesized in TS_{OHPA} and TS_{TCP}, a potential contribution of CprA and $RdhA_{2,4,6-TCP}$ to PCE transformation is exceeded by PrdA at early stages of *de novo* synthesis with low amounts of catalytically active enzyme. This resulted in the primary catalysis of PCE dechlorination by PrdA in TS_{OHPA} and TS_{TCP} which agrees with the observed lag-phases in most of the microcosms and the high similarity of measured ε values with TS_{PCE}. The direct albeit low PCE transformation in MC2 and MC3 of TS_{TCP} resulted presumably from higher initial cell numbers ($3.3*10^6 \pm 7.3*10^5$ cells/mL) compared to TS_{PCE} ($2.2*10^5 \pm 4.6*10^4$ cells/mL) during PCE addition which could *de novo* synthesize the PrdA enzyme. This led to relatively higher production of catalytically active PrdA per time unit in TS_{TCP} and a consequent early onset of PCE dechlorination. The individual ε_C values of MC3 (-19.1 ± 0.7 ‰) and MC2 (-17.6 ± 1.8 ‰) resemble PCE isotope fractionation by PrdA in TS_{PCE} (-17.9

± 1.1 ‰) which provides another line of evidence to exclude significant PCE dechlorination by CprA and $RdhA_{2,4,6-TCP}$ in these MC. The faster complete PCE dechlorination (80 to 90 h) in TS_{OHPA} compared to TS_{PCE} (about 300 h) can also be explained by higher cell numbers and higher PrdA *de novo* synthesis per time unit in TS_{OHPA}. Although no growth data is available for this experiment, higher cell numbers compared to TS_{PCE} can be assumed based on the repetition of 3-Cl-4-OHPA transformation (4x) and the observation that cell numbers of strain PCE1 increased concomitantly with multiple 3-Cl-4-OHPA additions in a previous study.[52] Thus, our results indicate that chloroaromatic RdhAs of strain PCE1 did not significantly contribute to PCE dehalogenation. However, based on the reported occurrence of PCE dechlorination by chloroaromatic RdhAs[38,43,65], we cannot completely exclude minor contributions of CprA and $RdhA_{2,4,6-TCP}$ in TS_{TCP} and TS_{OHPA}. This applies particularly for the initial interval after PCE addition in TS_{TCP}.

In conclusion, the presence of CprA and $RdhA_{2,4,6-TCP}$ in active cells of strain PCE1 did not affect observable PCE isotope fractionation. This can result from two scenarios. (1) PCE dechlorination rates of CprA and $RdhA_{2,4,6-TCP}$ are significantly lower compared to PrdA. Therefore, observable PCE isotope fractionation is dominated by the enzyme intrinsic isotope fractionation of PrdA. For enzymatic phenotypes containing $RdhA_{2,4,6-TCP}$ and/or CprA, the observed minor PCE dechlorination activity directly after PCE addition implies low PCE dechlorination rates and contributions to PCE transformation of these enzymes. The effect of potential minor contributions of CprA and $RdhA_{2,4,6-TCP}$ on the observable PCE ε value can be illustrated assuming an intrinsic PCE ε value of -9.8 ‰ for these enzymes which was previously reported for a chloroaromatic RdhA.[64] Calculating the observable ε value for a constant contribution of 10 % by CprA/$RdhA_{2,4,6-TCP}$ and 90 % by PrdA (-17.9 ± 1.1 ‰) according to equation 3[66] results in an ε value of -17.1 ‰ which is in the range of the calculated uncertainty for the ε value of PrdA.

$$\varepsilon_{Observable}\ (‰) = (-9.8\ ‰ * 0.1) + (-17.9\ ‰ * 0.9) \tag{3}$$

Based on the observed minor PCE dechlorination in TS_{TCP} and TS_{OHPA} after PCE provision, a theoretical contribution of 10 % may significantly overestimate the real contribution of CprA and $RdhA_{2,4,6-TCP}$. This suggests that potential contributions of CprA and $RdhA_{2,4,6-TCP}$ would not substantially affect observable PCE ε values.

(2) CprA, $RdhA_{2,4,6-TCP}$ and PrdA have similar enzyme intrinsic ε values for PCE dechlorination. However, reported ε values of *D. hafniense* strain PCP-1 point to significant different PCE ε values of designated PCE-RdhAs and chloroaromatic RdhAs. In addition, ε values differ even between designated PCE-RdhAs.[64] To further substantiate this hypothesis, the intrinsic ε values and specific enzyme activities of CprA and $RdhA_{2,4,6-TCP}$ for PCE transformation could be determined in protein crude extracts using cells of strain PCE1 grown on 3-Cl-4-OHPA and 2,4,6-TCP. Furthermore, analysis of *rdhA*-gene transcripts of strain PCE1 during dechlorination of 2,4,6-TCP to 2,4-DCP may allow to identify the responsible RdhA for 2,4,6-TCP transformation. Analyzing *rdhA*-transcripts after addition of 2,4-DCP as the ini-

tial substrate would allow to confirm that CprA catalyzes dechlorination of 2,4-DCP into 4-MCP.

Implications for Chlorinated Ethene ε Values of OHRB Carrying Multiple RdhAs
The capability of OHRB to catalyze chlorinated ethene transformation via reductive dehalogenases enzymes (RdhA) makes them key players for bioremediation of contaminated sites.[5] Although CSIA of carbon allows to reliably prove and monitor *in situ* biodegradation,[6,7] quantification of chlorinated ethene transformation is impeded by the high variability of published ε_C values for OHRB.[9] Variable ε_C values of the same substrate have been reported for single pure cultures harboring multiple *rdhA*-genes and relative transcription levels of *rdhA*-genes were shown to differ upon different environmental stimuli. Because ε values are enzyme specific, potential effects due to changing cellular abundances of the different RdhAs on isotope fractionation need to be investigated. In this study, dual element isotope analysis of carbon and chlorine revealed consistent PCE isotope fractionation (ε values and $\Lambda_{C/Cl}$ values) of different enzymatic phenotypes of the same OHRB with regard to the expressed RdhAs. Our results suggest that due to comparatively low chlorinated ethene dechlorination rates of non-specialized RdhAs, significant dechlorination by other than the specialized RdhA is not expected for living cells of OHRB. Thus, chlorinated ethene isotope fractionation is likely not affected by the expression and presence of multiple RdhAs. Therefore, changes in the expression level of different RdhAs during chlorinated ethene transformation are presumably not responsible for the varying ε values of OHRB pure cultures harboring multiple *rdhA*-genes. To substantiate our findings, future studies may focus on characterization of the substrate spectra and specific activities of putative RdhAs which are co-transcribed with the specialized RdhA during chlorinated ethene transformation. However, recent studies on proteome analysis of OHRB indicate that only few RdhAs are translated, although multiple *rdhA*-genes are transcribed.[29] Thus, simultaneous quantification of *rdhA*-gene transcripts, cellular RdhA enzyme content and chlorinated ethene isotope fractionation under changing environmental conditions may allow to exclude an effect of varying RdhA abundances on isotope fractionation.
Re-evaluation of experimental raw data with the stepwise or mass balance correction method proposed by Buchner et al.[50] could further clarify whether the variability of reported ε values of pure cultures arise from different evaluation methods which were deployed to account for the volatility of chlorinated ethenes and mass removal during sampling.

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4

Assessing Tetrachloroethene Dehalogenation Dynamics in Mixed Cultures of Organohalide-Respiring Bacteria by Analysis of Dual Element Isotope Fractionation (C, Cl) and Functional Gene Abundances

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4.1 Abstract

Quantification of *in situ* contaminant transformation using compound specific isotope analysis (CSIA) relies on a constant and appropriate isotope enrichment factor (ε). Reported carbon ε values (ε_C) for chlorinated ethene transformation can differ considerably among organohalide-respiring bacteria (OHRB) and multiple OHRB are typically present at contaminated sites. The observed ε value of such microbial communities is, therefore, a lumped parameter affected by the chlorinated ethene dehalogenation activity of each contributing strain. This study investigated the variability of tetrachloroethene (PCE) isotope fractionation due to different abundances of two OHRB in a binary mixed culture. To this end, *Desulfitobacterium dehalogenans* strain PCE1 and *D. hafniense* strain Y51 were deployed because they show significant different intrinsic ε_C values for PCE (strain Y51 = -5.8 ‰, strain PCE1 = -19.7 ‰). Different initial Y51:PCE1 cell ratios resulted in ε_C values resembling those of the pure cultures (ε_C = -5.6 and -18.3 ‰) as well as intermediate ε_C values (-13.2 ± 0.6 ‰). Recurrent exposure of one of these binary mixed cultures (ε_C = -5.6 ‰) to PCE or trichloroethene resulted in highly variable PCE isotope fractionation with ε_C values ranging from -7.3 to -17.7 ‰ (± 0.6 ‰). Starvation adversely affected the physiological activity of both strains leading to the loss of dehalogenation activity of strain Y51. Dual isotope analysis and consistent ε_C values in multiple pure culture experiments allowed to exclude changes in reaction mechanisms or masking effects to cause varying ε_C values. Thus, variable ε_C values resulted from different dehalogenation activities of the single strains in the mixed cultures. However, ε_C values were not correlated with cell ratios of the strains (e.g. Y51:PCE = 1:20 showed an ε_C value of -7.3 ‰). This suggests that strain specific dehalogenation rates were determined by physiological and enzymatic properties of the strains such as enzyme amount per cell or adaption to substrate limiting conditions rather than by the cell abundance. Our results demonstrate that chlorinated ethene isotope fractionation of microbial communities can vary because environmental conditions affect dehalogenation activities of single OHRB. This emphasizes the need for determination of site specific ε values and the development of reliable methods to monitor physiological activities of OHRB in microbial communities.

4.2 Introduction

Organohalide-respiring bacteria (OHRB) are key microorganisms for bioremediation of chlorinated ethene contaminated sites because they dehalogenate chlorinated ethenes (e.g. PCE, TCE) into innocuous ethene.[1,2]

OHRB are found in diverse microbial taxa. They can be grouped as metabolically versatile (e.g. *Desulfuromonas*, *Desulfitobacterium*) and obligate OHRB which are metabolically restricted to organohalides as terminal electron acceptor (e.g. *Dehalococcoides*, *Dehalobacter*).[3,4] Enzymes catalyzing chlorinated ethene transformation belong to the group of reductive dehalogenases (RdhA) and are encoded in the genome of OHRB within *rdh*-gene operons.[3] Chlorinated ethene RdhAs (CE-RdhAs) of versatile OHRB dehalogenate PCE into TCE or *cis*-DCE. Obligate

OHRB are capable of complete dehalogenation of PCE into ethene and carry CE-RdhAs catalyzing one or multiple dechlorination steps of chlorinated ethenes.[1,5]

The application of quantitative real-time PCR (qPCR) targeting either 16S rRNA-genes or specific CE-*rdhA*-genes of different OHRB has become a routine approach to quantify and monitor OHRB populations at contaminated sites and in laboratory experiments.[6–13] In laboratory experiments, dehalogenation activities can be tracked by concentration measurements and correlated with changing CE-*rdhA*-gene abundances if other metabolic pathways can be excluded. However, also inactive cells harbor 16S rRNA- and CE-*rdhA*-genes (functional genes) and versatile OHRB can generate growth supporting energy using multiple different electron acceptors at contaminated sites. In addition, chlorinated ethene concentrations can be biased in the field by processes aside from transformation including dilution or sorption.[14] At contaminated sites, measured functional gene abundances and chlorinated ethene concentrations are therefore inconclusive to track actual dehalogenation activities.[6]

To prove and monitor *in situ* reductive dehalogenation in the field, stable isotope ratios of chlorinated ethenes ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) can be measured using compound specific isotope analysis (CSIA).[14–16] Reaction rates are typically faster for molecules containing a light isotope (e.g. ^{12}C) at the reactive position compared to molecules containing a heavy isotope (e.g. ^{13}C). This causes an enrichment of the heavy isotope in the residual substrate pool which is denoted as isotope fractionation.[14] Other processes than transformation typically show minor isotope fractionation. An enrichment of heavy isotopes within time series or downstream from a source zone is therefore indicative for *in situ* microbial transformation.[14] The magnitude of isotope fractionation is quantified by the isotope enrichment factor (ε) according to the Rayleigh equation.[14] Isotope enrichment factors are typically derived from laboratory experiments and can be applied to estimate the magnitude of chlorinated ethene transformation in the field. However, the application of inappropriate ε values was shown to result in wrong estimates.[17]

Highly variably carbon ε values (ε_C) were reported for chlorinated ethene dehalogenation by different pure cultures of OHRB and microbial enrichment cultures. Measured ε_C values cover a range of -0.4 to -19.7 ‰ for PCE, -2.5 to -18.9 ‰ for TCE and -14.1 to -29.7 ‰ for *cis*-DCE (s. also chapter 2).[18–27] In addition, for a single microbial enrichment culture different ε values were reported for transformation of TCE (-2.5 to -13.8 ‰) and *cis*-DCE (-14.1 to -20.4 ‰).[22,23,27] Reliable application of CSIA to quantify *in situ* transformation demands a detailed assessment of the underlying causes for variable ε values. Previous studies investigated the effect of different cultivation conditions on chlorinated ethene ε values in pure cultures and enrichment cultures where a single OHRB catalyzed dehalogenation (Buchner et al., in prep.).[25,26] For both, measured ε values were robust under different conditions such as repeated chlorinated ethene addition, different electron donor and acceptor concentrations or micronutrient availability (Buchner et al., in prep.).[25,26] However, in the field typically multiple OHRB with different RdhAs coexists including strains of *Dehalococcoides*, *Dehalobacter*, *Desulfitobacterium* and others.[7,13,28–33] Thus, likely multiple OHRB contribute to chlorinated ethene dehalogenation.[30–33] It was hypothesized that dechlorination of PCE into TCE and *cis*-DCE is

catalyzed by multiple OHRB strains of *Desulfitobacterium*, *Dehalobacter*, *Geobacter* and/or *Dehalococcoides*, while dehalogentation of *cis*-DCE to ethene is generally attributed to strains of *Dehalococcoides*.[30–33] For concomitant compound transformation by multiple strains, the measured ε value is a lumped parameter affected by the specific PCE dehalogenation activity (i.e. contribution) and ε value of each contributing strain.[34] Thus, variable chlorinated ethene isotope fractionation of microbial communities and enrichment cultures may arise from changes in strain specific dehalogenation activities upon different growth and cultivation conditions.
OHRB population dynamics and dehalogenation activities during chlorinated ethene transformation have been assessed in previous studies based on 16S rRNA- and CE-*rdhA*-gene analysis. Addition of different electron donors and chlorinated ethenes resulted in changing abundances of single OHRB strains.[13,32,35,36] This suggests that environmental conditions exert selective pressure on single OHRB strains in the microbial community.[13] Potential effects of changing microbial community structures (i.e. OHRB strain abundances) on chlorinated ethene isotope fractionation have rarely been investigated. Constant ε_C values were measured for TCE, *cis*-DCE and VC for different cultivation conditions of an enrichment culture containing multiple *Dehalococcoides* strains. However, this was attributed to non-overlapping dechlorination activities of the single OHRB and their CE-*rdhA*-genes.[37] In contrast, a shift of 8 ‰ between PCE ε_C values and a change in the predominantly dechlorinating OHRB was reported for a microbial enrichment culture when the electron donor was changed from formate to H_2.[35] These results emphasize the need to investigate the effect of changing strain abundances and environmental conditions on ε values during simultaneous dehalogenation by multiple OHRB.
This study evaluated the variability of PCE isotope fractionation due to changing strain abundances during simultaneous dehalogenation by two OHRB with different intrinsic ε values. In addition, the effect of changing cultivation conditions on isotope fractionation, single strain abundances and their dehalogenation activity was assessed. To this end, we measured PCE isotope fractionation in various PCE transformation experiments with binary mixed cultures of *Desulfitobacterium hafniense* strain Y51 and *D. dehalogenans* strain PCE1. As the strain intrinsic ε_C values for PCE differ considerably (strain Y51: ε_C = -5.8 ‰, strain PCE1: ε_C = -19.7 ‰)[38], mixed cultures of these strains can serve as a suitable model system to evaluate potential changes in strain abundances on their dehalogenating activities and mixed culture ε_C values. We monitored PCE-*rdhA*-gene abundances of the strains to track mixed culture compositions during PCE transformation. Initial inoculation volumes of the strains were altered to simulate different microbial community structures with regard to single strain abundances. Continuous PCE transformation experiments were conducted with a single mixed culture to investigate potential effects of changing cultivation conditions (electron acceptor, starvation) on PCE isotope fractionation. Finally, a potential correlation between PCE-*rdhA*-gene abundances and dehalogenation activities of the single strains was investigated.

4.3 Materials and Methods

Chemicals

Chemicals were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany) or Merck KGaA (Darmstadt, Germany) with the highest available level of purity.

Cultivation of *Desulfitobacterium* spp. and Experimental Setups

Desulfitobacterium dehalogenans strain PCE1 (No. 10344) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *D. hafniense* strain Y51 was kindly provided by Prof. Furukawa of the Department of Food and Bioscience, Beppu University (Japan).

All dehalogenation experiments were conducted in 560 mL serum bottles sealed with butyl rubber stoppers. Serum bottles contained 500 mL of an anoxic, sodium carbonate buffered medium as described by Buchner et al.[26] and 60 mL headspace flushed with N_2/CO_2 (80:20) gas mixture. Precultures of the binary mixed cultures experiments (MC) were cultivated accordingly in 250 mL serum flasks containing 200 mL liquid medium.

Two MC set-ups with an initially low cell content were prepared by adding 200 μM PCE and 1 mM pyruvate as electron acceptor and donor. Serum bottles were placed on a horizontal shaker (140 rpm, 48 h) to dissolve the PCE. For preparation of a MC with cells that were not adapted to PCE dehalogenation (MC_{NA}), 2 % (v/v) of *Desulfitobacterium dehalogenans* strain PCE1 and *D. hafniense* strain Y51 were inoculated from precultures grown with 1 mM pyruvate and 1 mM fumarate as electron donor and acceptor. For preparation of a MC with PCE adapted cells (MC_{PA}), 2 % (v/v) of strain PCE1 and 0.5 % (v/v) of strain Y51 were added from precultures grown with 1 mM pyruvate and 200 μM PCE. Precultures of MC_{PA} were degassed with N_2/CO_2 (80:20) for 90 min prior to inoculation to remove PCE transformation products and screened for residual chlorinated ethenes via GC-MS. Both setups comprised three living replicates and one cell-free control.

Two MC setups with an initially high cell content were prepared by direct cultivation of strain PCE1 (MC_{PCE1}) or strain Y51 (MC_{Y51}) in the experimental flasks with 1 mM pyruvate and 200 μM PCE. In parallel, precultures of the respective other strain were prepared according to MC_{PA}. After complete PCE transformation, precultures and experimental flasks were degassed with N_2/CO_2 (80:20) for 90 min and removal of chlorinated ethenes was confirmed via GC-MS measurements. Experiments were started in an anaerobic glove box (O_2 <1 ppm) by adding 8 % (v/v) strain Y51 preculture to MC_{PCE1} and 8 % (v/v) strain PCE1 preculture to MC_{Y51}. PCE and pyruvate were added pre-dissolved in 250 mL fresh medium as spike solution yielding a final concentration of 200 μM PCE and 1 mM pyruvate. Both setups comprised two living-replicates each and a shared cell-free control.

MC_{Y51} was continuously cultivated within eight monitored respike experiments (RSP) to investigate an effect of changing cultivation conditions in PCE isotope fractionation. All RSP experiments were conducted with PCE (200 μM) as electron acceptor, except for RSP-III in

which 200 μM trichloroethene (TCE) were supplied as electron acceptor. After MC_{Y51} through RSP-II, PCE was continuously supplied to the culture and selected dehalogenation events were monitored (s. Table 4.2). For non-monitored PCE additions, 200 μM of neat PCE were added and dissolved by placing the cultures on a rotary shaker at 140 rpm. Monitored RSP experiments were initiated in the glovebox by adding PCE spike solutions according to MC_{Y51}/MC_{PCE1}. For RSP-III, initial TCE transformation was monitored and followed by 3 additional non-monitored additions of TCE (each 200 μM). In RSP-IV, 200 μM PCE were provided as electron acceptor and dehalogenation was monitored. Between RSP-IV and RSP-V, two non-monitored additions of PCE (200 μM) were conducted and dehalogenation was monitored in RSP-V for an additional PCE addition. Subsequently, cultures were starved without addition of degradable substrates and stored at 15 °C for 3 months. For the following RSP-VI experiment, cell suspension of one microcosm of RSP-V was split into two portions of 10 mL (RSP-VI-LCC) and two portions of 150 mL (RSP-VI-HCC). Cell suspensions were added to 240 mL (RSP-VI-LCC) or 100 mL (RSP-VI-HCC) fresh medium and spike solutions were added to start the experiment. For RSP-VII, PCE-adapted cells of strain Y51 were added to RSP-VI-HCC from a freshly grown preculture. The preculture was cultivated with PCE as electron acceptor according to MC experiments. Microcosms of RSP-VI-HCC and strain Y51 preculture were degassed with N_2/CO_2 as described above. 2 % (v/v) of preculture were added to the microcosms which were stored for 4 d at 20 °C after inoculation. Thereafter, the experiment was initiated by addition of spike solutions.

Each chlorinated ethene addition was accompanied with the addition of 1 mM pyruvate as electron donor. After each complete monitored or non-monitored PCE/TCE transformation, cultures were degassed with N_2/CO_2 and screened for residual chlorinated ethenes by GC-MS. Experimental flasks were individually maintained through RSP-III. Thereafter, both living-replicates were pooled in a sterilized 1 L-glass bottle after monitored degradation experiments and equally divided into sterilized 560 mL-serum flasks prior to the following monitored experiment. Cultures were stored in the dark and storage temperatures were adjusted depending on degradation kinetics but never exceeded 33 °C or were below 15 °C. The control bottle was treated the same way as living replicates, but non-monitored PCE/TCE additions were omitted.

Sample Extraction and Analytical Methods

Sampling. Liquid samples were extracted using gas-tight glass syringes (Innovative Laborsysteme GmbH, Stützerbach, Germany).

Samples for chlorinated ethene concentration analysis were prepared by dividing 1 mL liquid equally into two 10 mL crimp glass vials which were previously filled with 4.5 mL ultrapure water (Merck Millipore, Burlington, MA) and 100 μL 1 M H_3PO_4 (85 %). The latter was added to stop microbial activity. Sample vials were sealed with aluminium crimp caps containing silicone/PTFE septa and directly measured. For carbon and chlorine isotope measurements, 4 mL were extracted and distributed in portions of 1 mL into 1.5 mL-screw neck HPLC vials to which 50 μL of 10 M NaOH were added to stop microbial activity. Vials were closed with

PTFE-lined screw caps, frozen and stored upside down at -20 °C for later isotope analysis. For molecular biological analysis, 4 mL were extracted and divided into portions of 2 mL in Eppendorf tubes for subsequent quantification of strain PCE1 and strain Y51 cell numbers. Samples were immediately stored on ice. Cell pellets were obtained by centrifuging the samples for 10 min at 10,000 rpm and 4 °C. The supernatant was discarded and samples were stored at -20 °C.

Chlorinated Ethene Concentrations. Analysis of chlorinated ethenes was performed in duplicates on a GC-MS system (Agilent Technologies 7890A GC coupled to an Agilent Technologies 5975VL MS Detector, Santa Clara, CA) via static headspace injection of 500 μL using a Gerstel MultiPurpose Sampler MPS (Mülheim an der Ruhr, Germany). Injections were done in split mode 1:10 with a Split/Splitless injector operated at 240 °C. Chromatographic separation of *cis*-DCE, *trans*-DCE, TCE and PCE was achieved using an Rtx-VMS fused silica column (60 m x 0.25 mm, 1.4 μm film; Restek, Bellefonte, PA) applying the following temperature program: 40 °C held for 1 min, 30 °C/min to 110 °C and afterwards 25 °C/min up to a final temperature of 200 °C which was held for 2 min. Helium was used as carrier gas with a constant flow of 1 mL/min. For each measurement run, calibration curves were analyzed at the beginning and the end of the sequence.

Stable Carbon Isotope Analysis. Stable carbon isotopes of PCE, TCE and *cis*-DCE were determined using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) comprising a Trace GC Ultra (Thermo Finnigan, San Jose, CA) coupled to a Delta Plus XP IRMS detector (ThermoFinnigan) via a GC Combustion III interface (ThermoFinnigan). For analysis, duplicate samples of selected time points (frozen in 1.5 mL HPLC vials) were diluted with ultrapure water to a total volume of 3 mL in glass screw vials and sealed with magnetic screw caps containing a silicone/PTFE septum. Dilution was adjusted to obtain peak amplitudes of 2000 to 4000 mV for precise δ^{13}C determination. Based on current instrumental performance this corresponded to a PCE concentration of 200 μg/L. Samples were enriched and extracted using solid phase microextraction (SPME) via a StableFlex-Fiber covered with 85 μm Carboxen/Polydimethylsiloxan (Supelco, Bellefonte, PA) and a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). Enrichment was done at 40 °C for 20 min after which the sample was desorbed at 270 °C for 1 min in an OPTIC 3 PTV injector (GL Sciences, Eindhoven, Netherlands). For the chromatographic separation of PCE, TCE and *cis*-DCE, an Rtx-VMS capillary column (60 m x 32 mm; 1.8 μm film) and the following temperature program were applied: 40 °C held for 4 min, 8 °C/min to 180 °C, afterwards 20 °C/min up to 200 °C which was held for 2 min. Helium was used as carrier gas with an initial flow of 2 mL/min for 2 min during desorption and 1.5 mL/min for the rest of the measurement run. Compound specific standards with known carbon isotope signatures (δ^{13}C) of -27.35 ‰, -26.68 ‰ and -25.35 ‰ for PCE, TCE and *cis*-DCE were measured in duplicates after every 10 samples and at the end of each measurement run. At the beginning of each measurement sequence, additional five standards were included.

Stable Chlorine Isotope Analysis. Chlorine isotopes of PCE were analyzed for MC_{Y51}, RSP-I, RSP-II and RSP-VII. Chlorine isotopes of TCE were analyzed for RSP-III. Analyses were conducted using an Agilent Technologies 7890B GC coupled to an Agilent Technologies 5977A MSD and a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). The gas chromatograph was equipped with an Rtx-VMS fused silica column (60 m x 0.25 mm, 1.4 μm film; Restek, Bellefonte, PA) and operated with a constant flow of 1 mL/min helium as carrier gas. Sample enrichment was conducted at 40 °C for 20 min with SPME technique using a StableFlex-Fiber covered with 85 μm Carboxen/Polydimethylsiloxan (Supelco). The samples were desorbed in a Split/Splitless injector for 2 min at 260 °C with a split ratio of 1:10. For each sample, analysis was conducted in quintuplicates which were bracketed by five replicates per concentration-adjusted external standards (EIL-2 and Tü-Inhouse). Bulk chlorine isotope ratios were determined as previously described by Sakaguchi-Söder et al. using the modified multiple ion method after Jin et al.[39,40] A two-point calibration curve with external standards (PCE: $\delta^{37}Cl_{EIL-2}$ = -2.52 ‰[41]; $\delta^{37}Cl_{Tü-Inhouse}$ = 10.4 ‰ (Buchner et al., in prep.), TCE: $\delta^{37}Cl_{EIL-2}$ = -2.7 ‰[41], $\delta^{37}Cl_{EIL-1}$ = 3.05 ‰[41]) was used to calculate δ^{37}Cl isotope values relative to the SMOC scale as recommended in previous studies (s. SI of chapter 2 for a brief description of $\delta^{37}Cl_{Tü-Inhouse}$).[42,43]

Determination of Isotope Enrichment Factors (ε) and Dual Isotope Slopes. Isotope enrichment factors for carbon and chlorine were determined according to the Rayleigh equation using the double logarithmic Rayleigh plot without forcing the regression line through zero as stated by Scott et al.[44]:

$$ln\left(\frac{R(t)}{R(0)}\right) = \varepsilon * ln\left(f(t)_{PCE/TCE}\right) \quad (1)$$

R(t) and *R(0)* denote the isotope ratios at different sampling points and the initial isotope ratio at the start of the experiment, respectively; $f(t)_{PCE/TCE}$ is the remaining fraction of PCE or TCE at sampling point *t*. Fractions of PCE and less chlorinated ethenes were calculated based on the timepoint-wise mass balance correction method described by Buchner et al.[45] to account for the cumulative mass removal due to repetitive sampling:

$$f_{PCE} = \frac{n(t)_{PCE/TCE}}{n(t)_{PCE/TCE} + \sum_{i=1}^{k} n(t)_i} \quad (2)$$

where $n(t)_{PCE/TCE}$ and $n(t)_i$ represent amounts of PCE or TCE and less chlorinated dehalogenation products (TCE and/or *cis*-DCE) at the same time point.

Dual isotope slopes ($\Lambda_{C/Cl}$) and associated standard errors (SE) were calculated for the individual experiments by regressing measured δ^{13}C and δ^{37}Cl data using the York method.[46]

Molecular Biological Analysis

Cell abundances of *D. dehalogenans* strain PCE1 and *D. hafniense* strain Y51 were determined using quantitative real-time PCR (qPCR) assays. For strain PCE1, copy numbers of the *prdA*-gene encoding the PCE transforming PrdA enzyme were quantified.[47] For strain Y51,

copy numbers of the *pceA*-gene encoding the PCE transforming PceA enzyme were quantified.[48] Based on genome analysis, one copy of the genes is present per genome of the individual strains and measured gene copy numbers therefore equal the total amount of strain PCE1 and strain Y51 cells.[47,49]

DNA Extraction. Genomic DNA was extracted from living-replicates using the DNeasy® UltraClean® Microbial Kit (Qiagen GmbH, Hilden, Germany) following the manufacturers instruction. To increase DNA yields, samples were kept in a water bath at 70 °C for 10 min prior to bead treatment.

Quantification of prdA- and pceA-genes. For each sample, the qPCR assay was performed in triplicates in 10 μL reaction volume containing 1 μL of template DNA, 250 nM of forward (*prdA*: 5‘ -*CTGGTCTTGGAGAGTTGGGC*- 3‘; *pceA*: 5‘ -*GCCGGCGTTCAAGGCCTCAT*- 3’) and reverse (*prdA*: 5‘ -*TCTGCGGCTCCAAAACTGAT*- 3‘; *pceA*: 5‘ -*GGGGAAAGACCTGCCCACGC*- 3’) primer and 5 μL Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc. Hercules, CA). Amplification and detection were conducted using an iCycler (Bio-Rad Laboratories, Inc. Hercules, CA) with an iQ™5 qPCR detection system and Optical System software (Bio-Rad Laboratories, Inc. Hercules, CA). Temperature settings for *prdA* were initial 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 15 s. Temperature settings for *pceA* were initial 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 65 °C for 15 s. Specificity of product formation was ensured at the end of each assay run by melt curve analysis using the following temperature program: 1 min at 95 °C, 1 min at 55 °C and stepwise increase by 0.5 °C every 10 s up to 95 °C. Specific amplification of the desired gene fragments was additionally checked by performing gel electrophoresis after each qPCR analysis. Quantification of *prdA*- and *pceA*-gene copies was enabled by simultaneous analysis of plasmid standards containing either a target gene fragment (*prdA*) or the complete target gene (*pceA*). The *prdA* plasmid standard was synthesized *de novo* by MWG Eurofins GmbH (Ebersberg, Germany) according to the annotated genome sequence of *D. dehalogenans* strain PCE1 available at IMG Genome database (ID: 2512875014). Plasmid standards for the *pceA* gene were prepared as described by Buchner et al.[26] For comparability of total cell numbers, gene abundances of the respike experiments were corrected for dilution due to the addition of spike solutions.

Error of Molecular Biological Analysis. Errors of measured gene abundances reflect the percentage standard deviation of each sample accounting for introduced biases during sample extraction, removal of the supernatant, DNA extraction and sample preparation for the qPCR assay. The error was determined by processing five replicate samples extracted from a pure culture of strain PCE1 for the *prdA*-gene and strain Y51 for the *pceA*-gene which were both grown on pyruvate and PCE. DNA concentrations were measured after extraction using the Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) and statistically tested for outliers using the two-sided Grubbs test. Since no outliers were detected, gene abundances were determined for the five replicates of each strain using the qPCR assay. Measured gene abundances (3 wells per sample * 5 samples = 15 measurement replicates each) were tested again for outliers using two-sided Grubbs test. No outliers were detected and

samples were corrected for the elution volume and extraction volume. For measured gene abundances, the average and standard deviation were calculated using the individual 15 replicates. Based on this, the percentage standard deviation was calculated which amounted to 21 % for the *prdA*-gene and 18 % for the *pceA*-gene. Primer specificity and potential cross-amplification of gene fragments from both strains were tested by analyzing two replicate samples comprising either *pceA* primer and genomic DNA of strain PCE1 or *prdA* primer and genomic DNA of strain Y51. No amplification or bands in subsequent gel electrophoresis analysis were detected.
Calculation of Cell Fractions. Cell fractions of the individual strains were calculated according to:

$$f_{Y51}(t) = \frac{\frac{cells_{Y51-M1}(t) \; + \; cells_{Y51-M2}(t)}{2}}{\frac{cells_{Y51-M1}(t) \; + \; cells_{Y51-M2}(t)}{2} + \frac{cells_{PCE1-M1}(t) \; + \; cells_{PCE1-M2}(t)}{2}} \tag{3}$$

and

$$f_{PCE1}(t) = \frac{\frac{cells_{PCE1-M1}(t) \; + \; cells_{PCE1-M2}(t)}{2}}{\frac{cells_{Y51-M1}(t) \; + \; cells_{Y51-M2}(t)}{2} + \frac{cells_{PCE1-M1}(t) \; + \; cells_{PCE1-M2}(t)}{2}} \tag{4}$$

where $cells_{strain-M}$ correspond to the cell number of the respective strain in the individual microcosm at time point t. Since measured cell numbers of each microcosm are associated with an error, the error of averaged strain fractions at time point t was calculated via Gaussian error propagation by:

$$f_{error}(t) = \sqrt{er_{Y51-M1}(t) \; + \; er_{Y51-M2}(t) \; + \; er_{PCE1-M1}(t) \; + \; er_{PCE1-M2}(t)} \tag{5}$$

where $er_{strain-M}$ corresponds to the calculated error term for the respective microcosm at time point t. Detailed calculations of the individual error terms can be found in the SI of this chapter.

4.4 Results and Discussion

To investigate the variability of PCE isotope fractionation during simultaneous transformation by *Desulfitobacterium hafniense* strain Y51 and *D. dehalogenans* strain PCE1, four binary mixed culture experiments (MC) were conducted with different inoculum sizes (i.e. initial cell numbers) and ratios of the strains (Table 4.1). Two MC setups were inoculated with a low amount of cell numbers of both strains (inoculum size: 0.5 to 2 % (v/v)). For MC_{NA} (PCE1:Y51 ratio 1:1), precultures were previously not adapted to PCE dehalogenation, while PCE adapted precultures were inoculated in MC_{PA} (PCE1:Y51 ratio 4:1). In addition, two setups were prepared with high amounts of inoculated cell numbers of PCE adapted precultures (inoculum size: 8 and 40 % (v/v)). Strain PCE1 was predominantly inoculated in MC_{PCE1} (PCE1:Y51 ratio 5:1), while strain Y51 was predominantly added to MC_{Y51} (PCE1:Y51 ratio 1:5). The variability of ε values upon changing cultivation conditions was investigated by repeated addition of PCE and TCE to MC_{Y51} and monitoring of selected dehalogenation events. These experiments

are denoted as respike experiments (RSP). Details about the cultivation conditions and the experimental setup of each RSP experiment are provided in Materials and Methods section and summarized in Table 4.2.

Table 4.1. PCE dehalogenation experiments with binary mixed cultures (MC) of *Desulfitobacterium hafniense* strain Y51 and *Desulfitobacterium dehalogenans* strain PCE1, inoculum sizes and ratios, preculture conditions and duration of the experiments.

Experiment	**Inoculum size strain PCE1 (% v/v)**	**Inoculum size strain Y51 (% v/v)**	**Inoculated strain ratio (PCE1:Y51)**	**Duration (d)**	**Preculture condition**
MC_{NA}	2	2	1:1	17	not adapted to PCE dehalogenation
MC_{PA}	2	0.5	4:1	3	adapted to PCE dehalogenation
MC_{Y51}	8	40	1:5	<1	adapted to PCE dehalogenation
MC_{PCE1}	40	8	5:1	1	adapted to PCE dehalogenation

Chlorinated Ethene Transformation and Observable Isotope Fractionation

Concentrations of chlorinated ethenes and stable carbon isotope fractionation (ε_C) were measured for 12 individual dehalogenation experiments. Carbon ε values were determined for 11 PCE transformation experiments and one TCE transformation experiment. Furthermore, chlorine isotope fractionation (ε_{Cl}) and dual isotope slopes ($\delta^{13}C$ vs $\delta^{37}Cl \approx \Lambda_{C/Cl}$) were determined for selected PCE and TCE transformation experiments.

Dehalogenation of Chlorinated Ethenes. In all MC experiments, PCE was completely transformed into *cis*-DCE as end product with TCE as intermediate (s. SI Figure C1 to C4). The maximum TCE fraction was 80 % in MC_{NA}, while lower TCE fractions of 25 to 35 % were observed in the other experiments. In MC_{NA}, precultures not adapted to PCE dehalogenation showed a lag-phase of 1 d and PCE transformation was completed after 17 d. In setups with PCE adapted precultures (MC_{PA}, MC_{PCE1}, MC_{Y51}), transformation started immediately after PCE addition and was completed within <1 to 3 d.

In all RSP experiments, complete transformation of PCE and TCE into *cis*-DCE was observed except for RSP-VI setups (s. SI Figure C5 to C12). Maximum TCE fractions varied between 25 to 65 % for RSP-I to RSP-V. In RSP-VI setups, PCE was predominantly dehalogenated to TCE as end-product (90 ± 5 %). Dehalogenation without a lag-phase occurred in all RSP experiments after chlorinated ethene addition except for RSP-VI-LCC. In previously starved cultures (RSP-VI-HCC and RSP-VII), dehalogenation paused after a maximum PCE trans-

formation of 35 % and proceeded only after a stagnation phase. When PCE was added in regular intervals (RSP-I, RSP-II, RSP-IV, RSP-V), transformation was completed within 1 d. Starvation phases of 3 weeks (RSP-III) and 3 to 3.5 months (RSP-VI and RSP-VII) resulted in significantly longer time periods for complete chlorinated ethene transformation (RSP-III: up to 13 d for TCE; RSP-VII: up to 65 d for PCE).

Table 4.2. Respike experiments (RSP) with the binary mixed culture MC_{Y51}, provided chlorinated ethenes, corresponding number of total chlorinated ethene (CE) dehalogenation events, duration of the experiments, time in between the experiments and the altered cultivation condition.

Experiment	**Provided CE (no. of transformation event)**	**Duration (d)**	**Time since last monitored dehalogenation event (d)**	**Altered cultivation condition**
RSP-I	PCE (3)	1 (M1)[a] 2 (M2)[a]	22	Repeated PCE addition
RSP-II	PCE (6)	<1	36	Repeated PCE addition
RSP-III	TCE (1)	6 (M1)[a] 13 (M2)[a]	21	Starvation phase of 3 weeks, provided chlorinated ethene
RSP-IV	PCE (7) after 4x TCE	<1	26	Provided chlorinated ethene
RSP-V	PCE (10)	<1	38	Repeated PCE addition
RSP-VI-HCC	PCE (11)	17[b] (117)[c]	95	Starvation phase of 3 months, high cell suspension volume (150 mL of RSP-V)
RSP-VI-LCC	PCE (11)	25[b] (110)[c]	95	Starvation phase of 3 months, low cell suspension volume (10 mL of RSP-V)
RSP-VII	PCE (12)	34 (M1)[a,b] 65 (M2)[a,b] (72)[c]	7	3.5 months starvation during RSP-VI-HCC, Addition of fresh Y51 cells

[(a)] Refers to the single microcosms (M) of the individual experiment that contained cell suspension.

[(b)] Time needed for complete PCE transformation.

[(c)] Total (monitored) running time of the experiment.

The measured concentration profiles of chlorinated ethenes provide a tentative indicator for PCE dehalogenation activities of the single strains. The PceA enzyme of strain Y51 dehalogenates PCE and TCE into *cis*-DCE.[48,50] With PCE as initial substrate, maximum TCE fractions of 20 to 25 % were previously reported for this strain.[38,50] In contrast, the PrdA enzyme of strain PCE1 dehalogenates PCE into TCE leading to accumulation of TCE with PCE as initial substrate.[51,52] Thus, high transient TCE fractions such as 65 to 80 % (RSP-V, MC_{NA}) or accumulation of TCE (RSP-VI setups) suggest that strain PCE1 predominantly catalyzed PCE dehalogenation. Conversely, low transient TCE fractions such as 25 % in RSP-I indicate predominant PCE transformation by strain Y51. Since the PrdA enzyme of strain PCE1 does not significantly dechlorinate TCE[51,52], TCE transformation in RSP-III can be attributed to exclusive dehalogenation activity of strain Y51.

The observed lag-phase of non-adapted cells (MC_{NA}) as well as immediate and fast PCE transformation after prior adaption to PCE (e.g. MC_{Y51}) or during repeated chlorinated ethene addition (RSP-I to RSP-V) agree with previous studies on OHRB.[8,26,53–57] In strain Y51, a protein precursor of PceA is constitutively transcribed and expressed. Catalytically active PceA is produced upon presence of an appropriate substrate such as PCE or TCE.[26,47,58,59] In strain PCE1, PrdA is encoded in the *prd*-gene operon which comprises the transcriptional regulatory protein PrdK.[47,60–63] PrdK was reported to initiate *de novo* synthesis of catalytically active PrdA in *Desulfitobacterium* sp. strain KBC1 exclusively upon exposure to PCE.[60] For MC_{NA}, inoculated precultures were not previously exposed to chlorinated ethenes. Thus, the observed lag-phase in MC_{NA} can be explained by the absence of active PceA and PrdA in transferred cells and the consequent need for RdhA *de novo* synthesis prior to PCE transformation.

Recurrent exposure of OHRB to chlorinated ethenes leads to physiological adaption by immediate upregulation of *rdhA*-gene transcription[8,26,53,55,56] and direct RdhA *de novo* synthesis after chlorinated ethene addition.[26,53] Furthermore, OHRB maintain catalytically active RdhA in their cells up to 6 months after completed chlorinated ethene transformation (Buchner et al., in prep.).[56] Maintenance of catalytically active PCE-RdhA and direct RdhA *de novo* synthesis in adapted OHRB cells explains the immediate and fast PCE dehalogenation ($\leq$1 to 3 d) in pre-adapted MC setups and recurrently spiked RSP experiments.

Starvation phases, however, resulted in long time periods for complete PCE and TCE transformation (RSP-III, RSP-VI setups, RSP-VII). Immediate dehalogenation was observed in most of these experiments but proceeded either at low rates or stagnated after an initially fast transformation. This was previously reported for TCE transformation by strain Y51 pure cultures after a starvation phase (Buchner et al., in prep.). Immediate chlorinated ethene transformation can be explained by the presence of active PCE-RdhAs in resting cells mediating the transfer of stored electrons to the newly provided PCE and TCE. Decreasing dehalogenation rates can be attributed to slow restoration of the cellular electron pool by electron donor oxidation (Buchner et al., in prep.). This suggests an initially dormant physiological state of the cells in the mixed cultures after starvation.

Table 4.3. Isotope enrichment factors and dual isotope slopes measured for *Desulfitobacterium hafniense* strain Y51 and *Desulfitobacterium dehalogenans* strain PCE1 in pure cultures, binary mixed culture (MC) experiments and respike (RSP) experiments of MC_{Y51}. Adjusted R^2 were ≥0.98 for carbon isotope enrichment factors and ≥0.97 for chlorine isotope enrichment factors (except for RSP-VII). See text for additional information on isotope fractionation in RSP-VII.

Experiment	$\varepsilon_C \pm$ **CI**[a] (‰)	$\varepsilon_{Cl} \pm$ **CI**[a] (‰)	$\Lambda_{C/Cl} \pm$ SE[b]
Pure Cultures			
Dsb strain Y51 pure[c]	-5.8 ± 0.3	-2.2 ± 0.2	2.4 ± 0.1
Dsb strain PCE1 pure[c]	-19.7 ± 0.5	-6.3 ± 0.3	3.07 ± 0.03
MC Experiments			
MC_{NA}	-18.3 ± 0.5	–	–
MC_{PA}	-12.7 ± 0.7	–	–
MC_{PCE1}	-13.7 ± 0.5	–	–
MC_{Y51}	-5.6 ± 0.6	-2.7 ± 0.2	2.1 ± 0.1
RSP Experiments			
RSP-I	-7.3 ± 0.3	-2.6 ± 0.4	3.0 ± 0.1
RSP-II	-17.4 ± 0.7	-5.6 ± 0.6	3.13 ± 0.04
RSP-IV	-7.3 ± 0.2	–	–
RSP-V	-18.0 ± 0.6	–	–
RSP-VI-HCC	-17.9 ± 0.8	–	–
RSP-VI-LCC	-18.8 ± 1.1	–	–
RSP-VII (pooled)	-16.7 ± 1.2	-5.5 ± 0.4	3.05 ± 0.02
RSP-VII (>65 %)	-7.5 ± 0.9	-2.9 ± 1.4	2.9 ± 0.6
RSP-VII (<65 %)	-18.0 ± 0.9	-5.8 ± 0.2	3.08 ± 0.03
RSP-III (TCE)	-8.0 ± 0.2	-2.9 ± 0.1	2.8 ± 0.1

[a] 95 % confidence interval (CI) for the linear regression in the double logarithmic Rayleigh plots.

[b] Standard error (SE) for the York regression calculated after Ojeda et al.[46]

[c] Data from Büsing et al.[38]

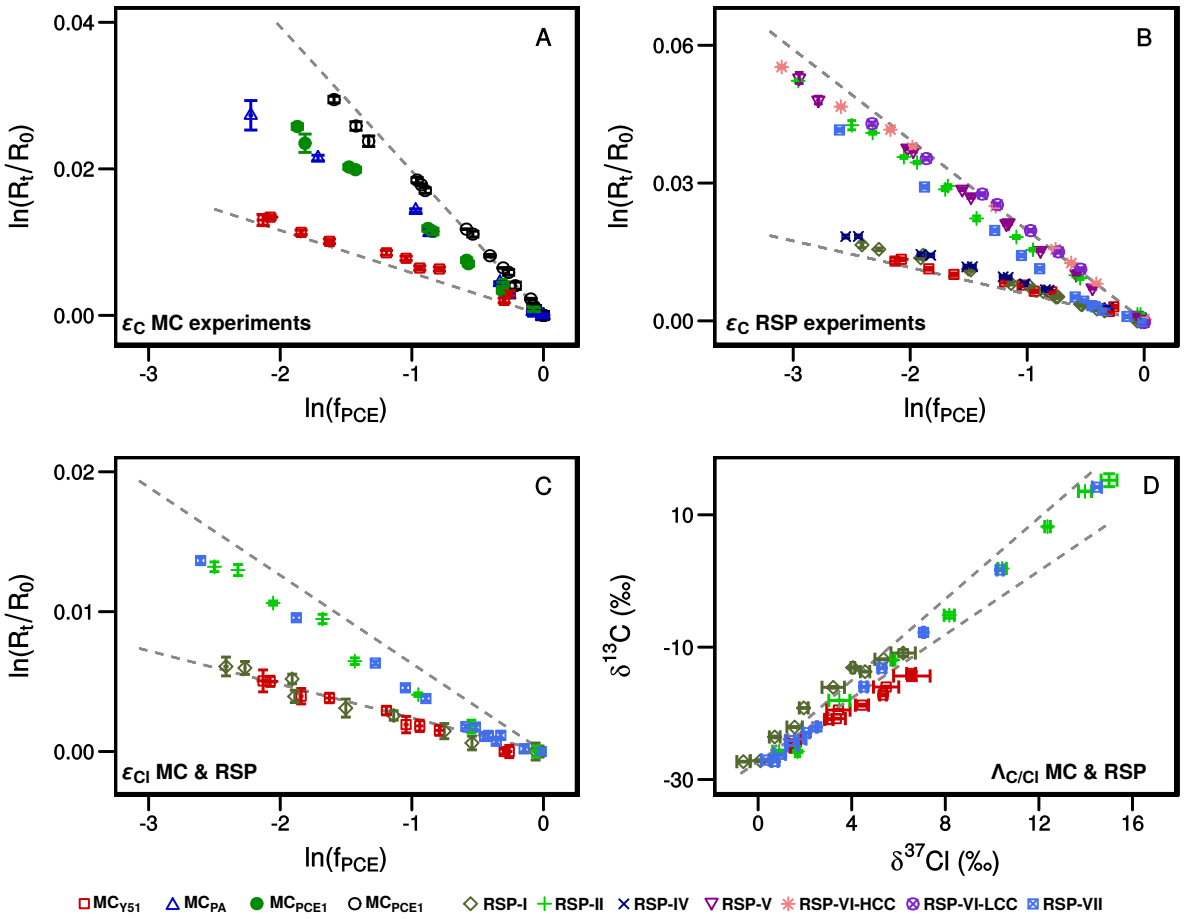

Figure 4.1. Carbon and chlorine isotope fractionation of PCE in binary mixed culture (MC) experiments and respike (RSP) experiments of MC_{Y51}. (A) Double logarithmic Rayleigh plot for carbon isotope fractionation in MC experiments. (B) Double logarithmic Rayleigh plot for carbon isotope fractionation in RSP experiments. Y-error bars of (A) and (B) represent standard deviation calculated from measurement replicates of $\delta^{13}C$ values (n=2) via Gaussian error propagation. (C) Double logarithmic Rayleigh plot for chlorine isotope fractionation in MC and RSP experiments. Y-error bars represent standard deviation calculated from measurement replicates of $\delta^{37}Cl$ values (n=3-5) via Gaussian error propagation. X-error bars of (A), (B) and (C) represent standard deviation of PCE fractions (n=2) calculated via Gaussian error propagation. (D) Dual isotope plot ($\delta^{13}C$ vs $\delta^{37}Cl$) of MC and RSP experiments. Error bars represent standard deviation of measurement replicates (n=2 for $\delta^{13}C$, n=3-5 for $\delta^{37}Cl$). Dashed lines in (A), (B), (C), (D) represent PCE isotope fractionation of *Desulfitobacterium hafniense* strain Y51 and *Desulfitobacterium dehalogenans* strain PCE1 in pure culture after Büsing et al.[38]

PCE Isotope Fractionation. ε and $\Lambda_{C/Cl}$ values of PCE were highly variably for the different MC and RSP experiments, but always within the range given by the pure cultures of strain Y51 and strain PCE1 (ε_C = -5.8 to -19.7 ‰; ε_{Cl} = -2.2 to -6.3 ‰; $\Lambda_{C/Cl}$ = 2.4 to 3.07).[38] *PCE Isotope Fractionation in MC Experiments.* For MC_{NA}, the ε_C value of -18.3 ± 0.5 ‰ agrees with the ε_C value determined for strain PCE1 in pure culture. Observable isotope fractionation in MC_{PA} and MC_{PCE1} was similar with ε_C values of -12.7 ± 0.7 ‰ and -13.7 ± 0.5 ‰ reflecting an intermediary ε_C value of the two pure cultures. In MC_{Y51}, the ε_C value of -5.6 ± 0.6 ‰ resembled the strain Y51 pure culture. The measured ε_{Cl} (-2.7 ± 0.2 ‰) and $\Lambda_{C/Cl}$ values (2.1 ± 0.1) of MC_{Y51} also reflect PCE isotope fractionation of strain Y51 in pure

culture.

PCE Isotope Fractionation in RSP Experiments. Repeated PCE addition to MC_{Y51} was accompanied by a gradual shift of isotope fractionation towards strain PCE1 pure culture. ε_C values for RSP-I and RSP-II were -7.3 ± 0.3 ‰ and -17.4 ± 0.7 ‰, respectively. The shift was also reflected in ε_{Cl} and $\Lambda_{C/Cl}$ values of RSP-I (ε_{Cl} = -2.6 ± 0.4 ‰, $\Lambda_{C/Cl}$ = 3.0 ± 0.1) and RSP-II (ε_{Cl} = -5.6 ± 0.6 ‰, $\Lambda_{C/Cl}$ = 3.13 ± 0.04). In RSP-IV, with a history of four sequential TCE additions, a shift in PCE isotope fractionation occurred towards the pure culture of strain Y51 with an ε_C value of -7.3 ± 0.2 ‰. Following two PCE additions, the measured ε_C value of -18.0 ± 0.6 ‰ in RSP-V shifted towards the pure culture of strain PCE1. After a three-month starvation phase, PCE ε_C values of both RSP-VI setups remained unchanged with -18.8 ± 1.1 ‰ for RSP-VI-LCC and -17.9 ± 0.8 ‰ for RSP-VI-HCC. The inoculation of fresh PCE adapted cells of strain Y51 to RSP-VI-HCC in RSP-VII resulted in a slightly more positive ε_C value of -16.7 ± 1.2 ‰. The ε_{Cl} value of -5.5 ± 0.4 ‰ and the $\Lambda_{C/Cl}$ value of 3.05 ± 0.02 were similar to PCE isotope fractionation of strain PCE1 in pure culture. In RSP-VII, the double logarithmic Rayleigh plot for carbon showed an increased slope at a PCE fraction of about 65 % ($\ln f_{PCE}$ = -0.4). Thus, ε and $\Lambda_{C/Cl}$ values were determined for the range of 100 to 65 % and 65 to 0 % residual PCE (Figure 4.2 C, 4.2 D). These two ε values differed significantly and approximated the pure culture of strain Y51 for the initial PCE transformation (ε_C = -7.5 ± 0.9 ‰; ε_{Cl} = -2.9 ± 1.4 ‰) and the pure culture of strain PCE1 in the range of <65 % residual PCE (ε_C = -18.0 ± 0.9 ‰; ε_{Cl} = -5.8 ± 0.2 ‰). Corresponding $\Lambda_{C/Cl}$ values, however, were almost identical with 2.9 ± 0.6 (100 to 65 %) and 3.08 ± 0.03 (65 to 0 %).

The observed variability of mixed culture ε values suggests that the PCE dehalogenation activities of the two strains in the mixed culture differed between the various experiments. The PCE dehalogenation activities of each of the two strains can be qualitatively inferred from mixed cultures ε_C values based on the pure culture ε_C values of strain PCE1 (ε_C = -19.7 ‰) and strain Y51 (ε_C = -5.8 ‰). Predominant PCE dehalogenation by strain PCE1 in the mixed cultures likely results in pronounced ε_C values (e.g. -17.4 to -18.8 ‰), while low ε_C values (e.g. -5.6 ± to -7.3 ‰) in the mixed culture may be attributed to predominant PCE transformation by strain Y51. Intermediary values in the mixed culture (e.g. -12.7 ‰) likely reflect similar contributions of both strains to PCE dehalogenation.

TCE Isotope Fractionation. TCE transformation in RSP-III resulted in ε values of -8.0 ± 0.2 ‰ and -2.9 ± 0.1 ‰ for carbon and chlorine and a $\Lambda_{C/Cl}$ value of 2.8 ± 0.1 (Table 4.3, Figure 4.2 A, 4.2 B). These values agree with TCE isotope fractionation reported for strain Y51 pure cultures (ε_C = -8.7 ± 0.2 ‰; ε_{Cl} = -2.7 ± 0.2 ‰; $\Lambda_{C/Cl}$ = 3.2 ± 0.2).[26] Because the PrdA enzyme of strain PCE1 does not significantly dechlorinate TCE[51,52], TCE transformation in RSP-III was exclusively catalyzed by strain Y51. Thus, the highly similar TCE isotope fractionation of strain Y51 in pure cultures and RSP-III suggests that the intrinsic isotope fractionation (i.e. ε values) of strain Y51 remained constant in the mixed cultures.

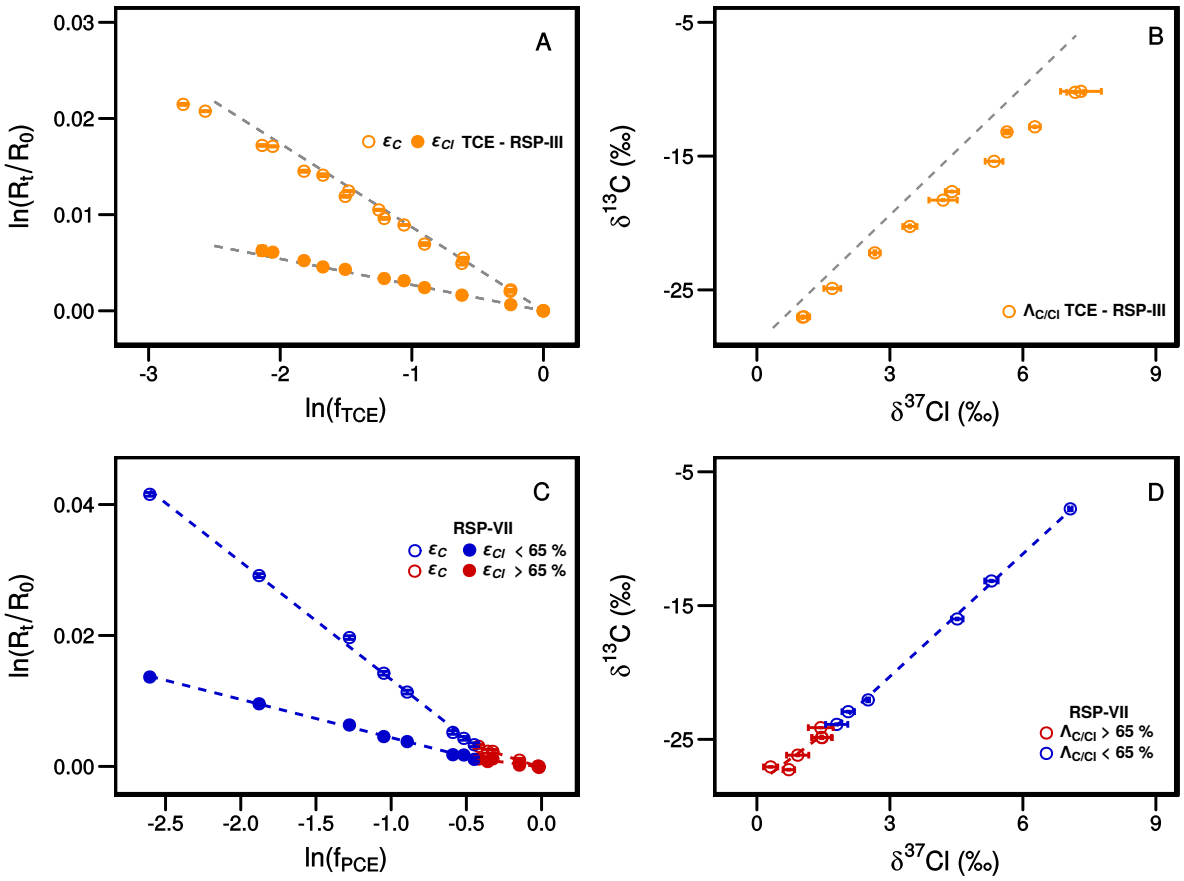

Figure 4.2. Carbon and chlorine isotope fractionation of TCE in RSP-III experiment as well as carbon and chlorine isotope fractionation of PCE in RSP-VII experiment. (A) and (C) Double logarithmic Rayleigh plots for carbon and chlorine isotope fractionation of TCE in RSP-III and PCE in RSP-VII. Y-error bars represent standard deviation calculated from measurement replicates of δ^{13}C values (n=2) and δ^{37}Cl values (n=3-5) via Gaussian error propagation. X-error bars represent standard deviation of TCE and PCE fractions (n=2) calculated via Gaussian error propagation. (B) and (D) Dual isotope plot (δ^{13}C vs δ^{37}Cl) of RSP-III and RSP-VII experiments. Error bars represent standard deviation of measurement replicates (n=2 for δ^{13}C, n=3-5 for δ^{37}Cl). Dashed lines in (A) and (B) represent TCE isotope fractionation of *Desulfitobacterium hafniense* strain Y51 in pure culture after Buchner et al.[26]

Isotope Fractionation as Tool to Decipher Dehalogenation Activities in the Mixed Cultures

For concomitant compound transformation by multiple strains, the apparent ε value depends on the PCE turnover (i.e. contribution) and intrinsic ε value of each contributing strain.[34] Thus, variable mixed culture ε_C values result from different PCE dehalogenation activities of the individual strains provided that pure culture ε values remain constant in mixed cultures. The magnitude of ε values can change depending on the reaction mechanism (i.e. manner of bond cleavage). Furthermore, for the same reaction mechanism, ε values can vary due to masking of rate-limiting steps such as substrate transfer into the cells.[14]

Different reaction mechanisms of microbial chlorinated ethene dehalogenation can be distinguished based on dual isotope slopes (i.e. $\Lambda_{C/Cl}$ values). While similar $\Lambda_{C/Cl}$ values reflect the same reaction mechanism, significantly different $\Lambda_{C/Cl}$ values (e.g. an absolute difference of

10) indicate different reaction mechanisms.[36,64,65] Based on similar $\Lambda_{C/Cl}$ values (2.4, 3.03), the same reaction mechanism of PCE transformation was recently proposed for strain PCE1 and strain Y51 in pure culture.[38] The similar $\Lambda_{C/Cl}$ values measured in mixed (2.1 to 3.1) and pure culture experiments (Table 4.3) thus demonstrate that the reaction mechanism of PCE dehalogenation of both strains was not altered in mixed cultures.

Consistent ε values were observed in multiple pure culture PCE dehalogenation experiments with each strain and masking effects due to e.g. limited PCE mass transfer were not detected.[38] Since we applied the same cultivation conditions (i.e. electron donor and acceptor concentrations) in this study, masking of pure culture isotope fractionation in the mixed cultures can be excluded. Thus, constant strain specific ε values can be assumed for the conducted mixed culture experiments. This is supported by consistent TCE isotope fractionation of strain Y51 in pure cultures and the mixed cultures (RSP-III). Therefore, the observed variability of ε_C values resulted from varying PCE dehalogenation activities of the two strains (i.e. strain specific dehalogenation activities) in the different experiments.

Data of all PCE dehalogenation experiments showed good fits to the Rayleigh model in the double logarithmic plots (adjusted $R^2 \geq 0.98$ for carbon; $R^2 \geq 0.97$ for chlorine) except for RSP-VII. This indicates that isotope fractionation (i.e. ε values) did not change during substrate transformation.[34,66] For simultaneous substrate transformation by two strains with different intrinsic ε values, constant isotope fractionation in mixed culture only occurs if strain specific contributions do not change during transformation.[34] Thus, our results suggest that contributions of strain PCE1 and strain Y51 were constant during PCE transformation in the experiments. In this case, mixed culture ε values (ε_{Mix}) are expressed as the weighted average of strain intrinsic ε values (ε_{Strain}) and their specific contributions (F_{Strain}) at any time point during the experiments[14,34]:

$$\varepsilon_{Mix} \;=\; (\varepsilon_{Y51} * F_{Y51}) \;+\; (\varepsilon_{PCE1} * F_{PCE1}) \tag{6}$$

Rearranging equation 6 allows to calculate percental strain specific contributions in mixed cultures according to equation 7[34,67]:

$$F_{Y51} \;=\; \left(\frac{\varepsilon_{Mix} - \varepsilon_{PCE1}}{\varepsilon_{Y51} - \varepsilon_{PCE1}}\right) * 100\,\% \tag{7}$$

Strain Specific Contributions in MC Experiments. Strain specific contributions to PCE transformation did not depend on inoculum sizes (i.e. initial cell numbers) and inoculum ratios of the strains in MC experiments. In MC_{NA}, both strains were inoculated in 1:1 ratio, but PCE was mainly transformed by strain PCE1 with a contribution of 90 ± 5 % (ε_C = -18.3 ‰). Predominant inoculation of strain Y51 in MC_{Y51} (PCE1:Y51 ratio 1:5) resulted in exclusive PCE dehalogenation by strain Y51 (ε_C = -5.6 ‰). Strain PCE1 and strain Y51 contributed equally to PCE dehalogenation in MC_{PCE1} and MC_{PA} with an average contribution of 53 ± 5 % and 45 ± 5 %, respectively (ε_C = -13.2 ± 0.5 ‰). The high contribution of strain Y51 in MC_{PCE1} and MC_{PA} was unexpected, because strain PCE1 was predominantly inoculated in

these experiments (PCE1:Y51 ratio 5:1 and 4:1). Lower initial cell numbers can be assumed for strains with smaller inoculum sizes. This indicates that the dehalogenation activities and thus PCE turnover of the two strains were not governed by their cell abundances in the mixed culture.

Table 4.4. Calculated contributions of *Desulfitobacterium dehalogenans* strain PCE1 and *D. hafniense* strain Y51 to PCE transformation in mixed culture (MC) experiments and respike (RSP) experiments with MC_{Y51}. Contributions were calculated based on the theoretical framework of van Breukelen.[34] 95 % confidence intervals of strain specific contributions were calculated via error propagation based on the determined confidence intervals of ε_C values (s. SI for detailed equations).

Experiment	$\varepsilon_C \pm$ **CI (‰)**	**Contribution strain Y51 (%)**	**Contribution strain PCE1 (%)**	**CI of contributions (%)**
MC Experiments				
MC_{NA}	-18.3 ± 0.5	10	90	5
MC_{PA}	-12.7 ± 0.7	50	50	5
MC_{PCE1}	-13.7 ± 0.5	43	57	4
MC_{Y51}	-5.6 ± 0.6	100	0	5
RSP Experiments				
RSP-I	-7.3 ± 0.3	89	11	3
RSP-II	-17.4 ± 0.7	17	83	6
RSP-IV	-7.3 ± 0.2	89	11	2
RSP-V	-18.0 ± 0.6	12	88	5
RSP-VI-HCC	-17.9 ± 0.8	13	87	7
RSP-VI-LCC	-18.8 ± 1.1	6	94	9
RSP-VII (pooled)	-16.7 ± 1.2	22	78	9
RSP-VII (>65 %)	-7.5 ± 0.9	88	12	7
RSP-VII (<65 %)	-18.0 ± 0.9	12	88	7

Strain Specific Contributions in RSP Experiments. In RSP experiments, variable ε_C values were caused by systematic shifts of strain specific dehalogenation activities with changing cultivation conditions. PCE dehalogenation activity of strain PCE1 successively increased during recurrent PCE additions to MC_{Y51} from no contribution (s. above) to 11 ± 3 % and 83 ± 6 % in RSP-I and RSP-II, respectively (ε_C = -7.3 and -17.4 ‰). A decrease in PCE dehalogenation activity of strain PCE1 was measured after TCE additions (RSP-III) with a contribution of 11 ± 2 % in RSP-IV (ε_C = -7.3 ‰). PCE dehalogenation activity of strain PCE1 increased again after repeated PCE addition with a contribution of 88 ± 5 % in RSP-V (ε_C = -18.0 ‰). These results suggest that the added chlorinated ethene had major impacts on physiological activities of the

strains. Physiological activity of strain PCE1 was enhanced by repeated PCE addition, while TCE selectively enhanced physiological activity of strain Y51. This demonstrates that the added chlorinated ethene exerts selective pressure on single OHRB strains which agrees with previous studies on microbial enrichment cultures and at contaminated sites.[13,36]

After starvation (RSP-VI), PCE was almost exclusively dehalogenated by strain PCE1 with contributions of 87 ± 7 % and 94 ± 9 % (ε_C = -17.9 and -18.8 ‰). TCE stagnated as predominant transformation product suggesting that physiological activity of strain Y51 was not recovered after the starvation phase. This contrasts with reported immediate and fast TCE dehalogenation of strain Y51 pure cultures after a starvation phase of six months (Buchner et al., in prep.). These results indicate that physiological activity of strain Y51 was adversely affected in the mixed cultures. This is corroborated by the observed dichotomy of PCE ε values in RSP-VII (ε_C = -7.5 vs -18.0 ‰). Initial PCE transformation was predominantly catalyzed by strain Y51 based on a contribution of 88 ± 7 %. Immediate PCE dehalogenation likely resulted from PceA mediated transfer of stored electrons from the freshly added strain Y51 cells. The cellular electron pool of strain Y51 was not restored and dehalogenation stagnated after 35 % PCE transformation indicating a physiological inactive state of strain Y51. Thereafter, PCE transformation was exclusively performed by strain PCE1 based on a calculated contribution of 88 ± 7 % (ε_C = -18.0 ‰). Thus, adverse effects in mixed culture may have impacted physiological activity of strain Y51 in the period between inoculation of strain Y51 and PCE addition (4 d).

Based on PCE isotope fractionation, three major conclusions can be drawn for dehalogenation activities of strain Y51 and strain PCE1 in the mixed cultures: Strain specific dehalogenation activities appeared to be independent from initial cell abundances of the strains. The type of the added chlorinated ethene significantly affected strain specific dehalogenation activities. The physiological activity of strain Y51 was adversely affected by cultivation in mixed cultures and repeated PCE addition.

Correlation Between PCE-*rdhA*-Gene Abundances and Strain Specific Dehalogenation Activity

PCE isotope fractionation clearly demonstrates that dehalogenation activities of the single strains differed in the mixed culture experiments. Therefore, it was investigated whether strain specific activities correlated with PCE-*rdhA*-gene abundances of the strains. For each experiment, PCE-*rdhA*-genes (strain Y51: *pceA*; strain PCE1: *prdA*) were quantified at the start of the experiment, the last point with a $\delta^{13}C$ value for PCE and the last sampling point of the experiment (Figure 4.3, Figure 4.4). Note that measured gene abundances correspond to total cell numbers of each strain, because only one copy of the respective gene is present per genome.[47,49] Strain abundances were converted into fractions of total cell numbers of both strains (equations 3, 4) to allow comparability between the different experiments with varying total cell numbers. Strain fractions are presented as: % strain PCE1 / % strain Y51.

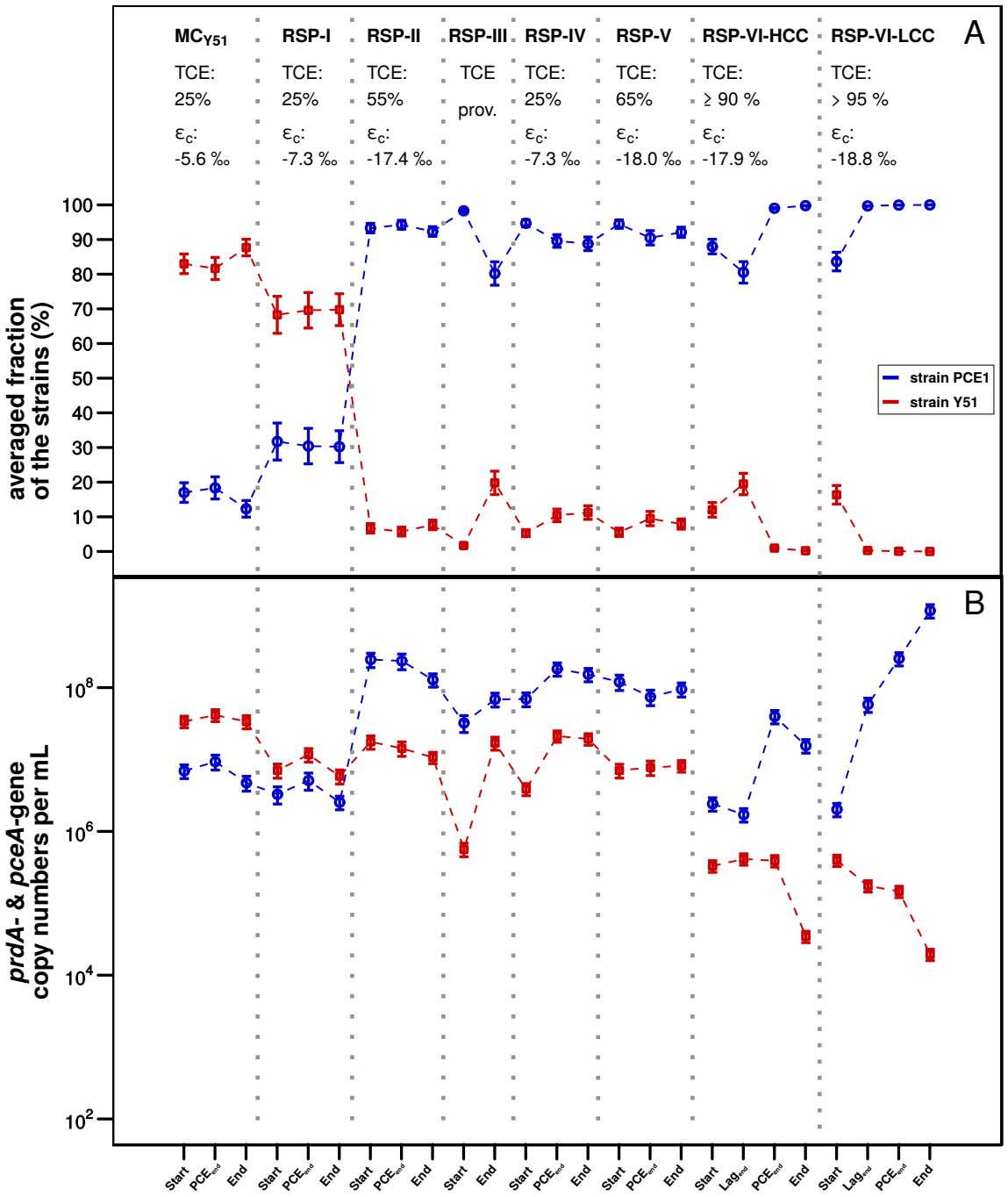

Figure 4.3. Culture composition, produced TCE fractions and measured carbon isotope enrichment factors during the MC_{Y51} experiment and subsequent RSP experiments with this culture. (A) Averaged strain fractions of total cell numbers in living replicates (n=2). (B) Averaged *prdA*- and *pceA*-gene copy numbers of living replicates (n=2). Gene abundances were corrected for dilution due to addition of spike solutions or splitting of the cultures (RSP-VI setups). Error bars represent percentage errors calculated via error propagation from qPCR assays. Equations for calculation of averaged strain fractions and error propagation are provided in Materials and Methods.

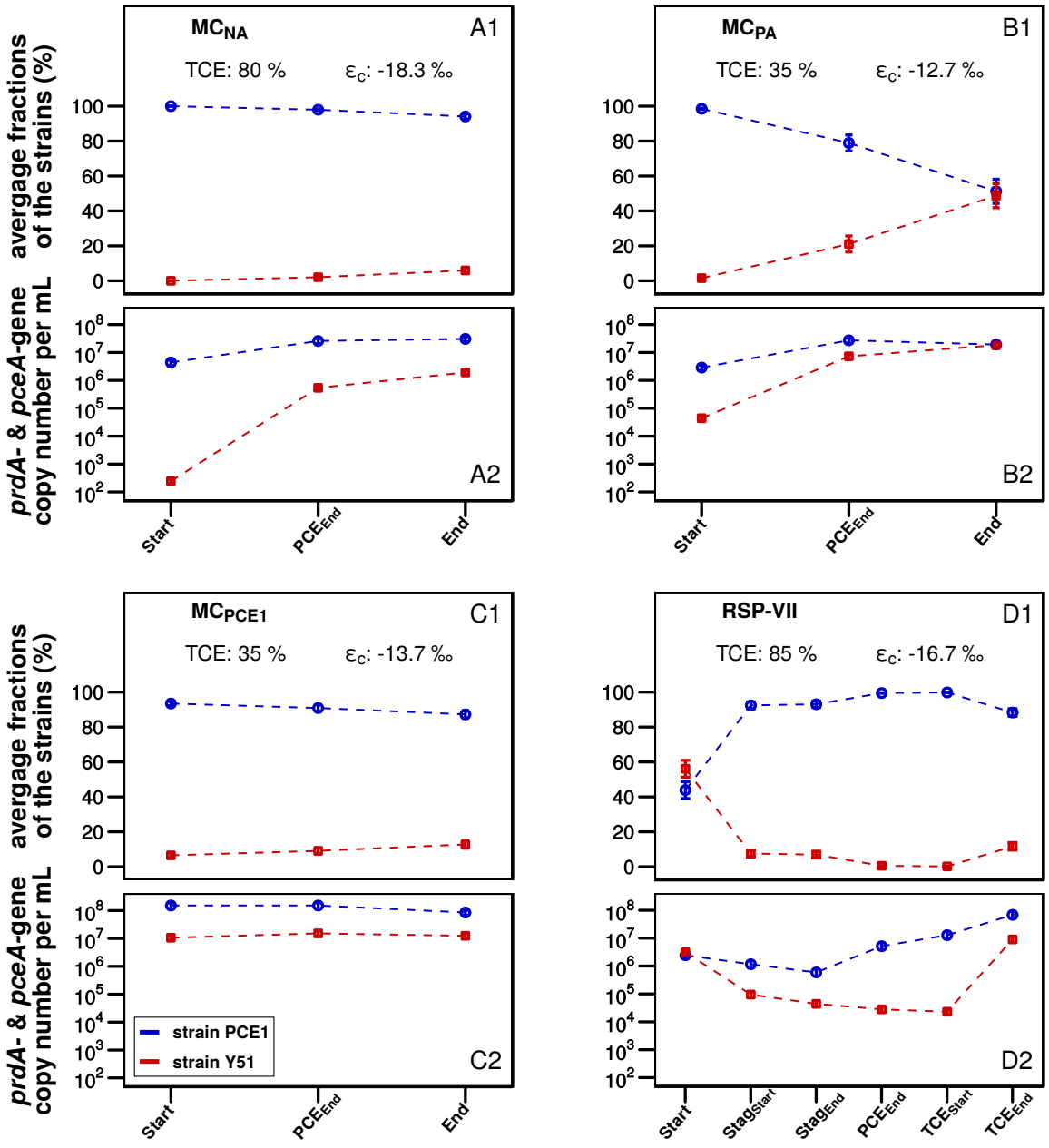

Figure 4.4. Culture composition, produced TCE fractions and measured carbon isotope enrichment factors during MC_{NA} (A), MC_{PA} (B), MC_{PCE1} (C) and RSP-VII (D) experiments. (A1, B1, C1, D1) Averaged strain fractions of total cell numbers in living replicates (n=2). (A2, B2, C2, D2) Averaged *prdA*- and *pceA*-gene copy numbers of living replicates (n=2). Error bars represent percentage errors calculated via error propagation from qPCR assays. Equations for calculation of averaged strain fractions and error propagation are provided in Materials and Methods.

Cell Growth in Mixed Cultures. Minor changes in strain fractions and cell numbers were observed in PCE transformation experiments with high inoculum sizes (≥8 % (v/v) in MC_{Y51}, MC_{PCE1}) and recurrently spiked cultures (RSP-I to RSP-V). For example, in MC_{Y51} initial fractions shifted slightly from initially 17 % strain PCE1 and 83 % strain Y51 (17 / 83) to final fractions of 12 / 88 at the end of the experiment. Significant cell growth (increase in cell numbers by at

least one order of magnitude) and changing strain fractions were only observed during PCE transformation in experiments with low inoculum sizes (≤2 % (v/v) in MC_{NA} and MC_{PA}) or previously starved cells (RSP-VI, RSP-VII). For example, strain fractions shifted from 98 / 2 to 79 / 21 during PCE transformation in MC_{PA} (Figure 4.4 B1). Thus, the strains decoupled cell growth from PCE dehalogenation when cells were adapted to PCE transformation and total cell numbers exceeded 10^7 cells/mL. This agrees with previous reports on cell growth of OHRB in pure or enrichment cultures during chlorinated ethene transformation.[8,26,53–57] Nutrient limitation and accumulation of toxic metabolites can limit microbial cell growth in batch cultures. This, however, can be excluded based on 1:2 dilution of the cultures with fresh medium and degassing of dehalogenation products in each experiment. A quorum-sensing-type regulatory mechanism was proposed to control maximum cell densities and decoupling of growth of a *Dehalococcoides* strain from chlorinated ethene dehalogenation.[57] This may explain constant cell numbers in the mixed cultures with elevated numbers (>10^7 cells/mL) of PCE adapted cells.

The largely constant strain fractions during the experiments are in line with constant contributions of the strains to PCE transformation based on PCE isotope fractionation data. The dependency between the PCE turnover of the strains and their abundances was explored by calculating the abundance based mixed culture ε_C values (ε_{Calc}) taking into account measured strain fractions and the ε_C values of the pure cultures of the strains (Table 4.5). To this end, strain fractions were deployed as strain specific contributions (F_{strain}) in equation 6:

$$\varepsilon_{Calc} \;=\; (\varepsilon_{Y51} * F_{Y51}) \;+\; (\varepsilon_{PCE1} * F_{PCE1}) \tag{6}$$

ε_{Calc} depicts the average of ε_C values calculated with strain fractions for the initial (ε_{Ini}) and the last sampling point with a $\delta^{13}C$ value for PCE (ε_{Fin}). In addition, the deviation between ε_{Calc} and the measured mixed culture ε_C value (ε_{Meas}) was determined.

The results show that strain fractions mostly did not correlate with measured ε values (ε_{Meas}) regardless of whether strain fractions were constant or changed during PCE transformation (Table 4.5). For example, in MC_{Y51} constant strain fractions of 17 / 83 predict an ε_{Calc} of -8.3 ‰, but an ε value of -5.6 ‰ was measured. Thus, strain fractions did not reflect exclusive PCE transformation by strain Y51 as indicated by isotope fractionation. In MC_{PA}, fractions shifted by 19 % from 98 / 2 to 79 / 21 during PCE transformation. Accordingly, predicted strain specific contributions and ε values changed in the course of the experiment from -19.5 to -16.8 ‰ yielding an average ε value of -18.1 ‰. The value significantly underestimates the contribution of 50 % of strain Y51 which was deduced from measured isotope fractionation (ε_{Meas} = -12.7 ‰). In addition, the predicted shifts in strain specific contributions during PCE dehalogenation contrast with constant isotope fractionation in this experiment. This indicates that strain abundances did not determine strain specific PCE dehalogenation activities and contributions in the mixed culture experiments.

Changes in Mixed Culture Composition. A significant shift in mixed culture composition was observed during repeated PCE additions (Figure 4.3 A). Mixed culture composition changed in

the course of MC_{Y51} to RSP-II from Y51-dominated (12 / 88) to PCE1-dominated (93 / 7). Thereafter, mixed culture composition was dominated by strain PCE1 with fractions ≥80 % until the end of RSP-VI. Notably, identical strain fractions were measured before and after repeated TCE addition in RSP-III. Only strain Y51 can dehalogenate TCE in the mixed culture and addition of TCE as selective electron acceptor for this strain was therefore expected to increase its abundance. However, the results show that repeated TCE addition did not cause changes in mixed culture composition.

Table 4.5. Predicted PCE carbon isotope fractionation (ε_{Calc}) based on measured relative strain abundances (fractions) as potential proxy for strain specific dehalogenation activities in selected mixed culture (MC) and respike (RSP) experiments.

Experiment	**Initial strain fractions (%) (PCE1/Y51)**	ε_{Ini} (‰)	**Final strain fractions (%) (PCE1/Y51)**	ε_{Fin} (‰)	ε_{Calc} (‰)	ε_{Meas} (‰)	**Deviation (%)** [a]
MC_{Y51}	17/83	-8.2	18/82	-8.3	-8.3	-5.6	47
MC_{PCE1}	93/7	-18.8	91/9	-18.4	-18.6	-13.7	36
RSP-I	32/68	-10.2	30/70	-10.0	-10.1	-7.3	39
RSP-II	93/7	-18.8	94/6	-18.9	-18.8	-17.4	8
RSP-IV	95/5	-19.0	90/10	-18.3	-18.6	-7.3	155
MC_{PA}	98/2	-19.5	79/21	-16.8	-18.1	-12.7	43

[a] Deviation was calculated using the formula: $\text{dev} = |\frac{\varepsilon_{Meas}-\varepsilon_{Calc}}{\varepsilon_{Meas}}| * 100\,\%$

Cell numbers of both strains followed a similar trend from MC_{Y51} to RSP-V. The shift from Y51- to PCE1-dominated mixed culture composition resulted from relatively lower decreases in cell numbers of strain PCE1 compared to strain Y51 (MC_{Y51} to RSP-I) and relatively higher growth of strain PCE1 (RSP-I to RSP-II). These results suggest that the adaptability of strain PCE1 to thrive in the mixed culture during repeated PCE dehalogenation was higher compared to strain Y51. In addition, strain PCE1 maintained its predominant abundance during TCE addition, although no appropriate electron acceptor for this strain was provided. This may be explained by an efficient generation of metabolic energy by strain PCE1 from fermentation of pyruvate[51] which was added with each chlorinated ethene addition.

The observed shift in mixed culture composition from Y51- to PCE1-dominated was reflected in mixed culture isotope fractionation and single strain abundances. Shifting PCE ε_C values (-5.6 to -17.4 ‰) were accompanied with changing strain fractions from 18 / 82 after MC_{Y51} to 30 / 70 after RSP-I and 92 / 8 after RSP-II. However, strain abundances do not correlate with strain specific dehalogenation activities in these experiments (Table 4.5). Dehalogenation activity of strain Y51 is again underestimated by the predicted ε_{Calc} (-8.3 to -18.8 ‰) compared to measured isotope fractionation (ε_{Meas} = -5.6 to -17.4 ‰). These values deviated up to 47 % revealing a poor applicability of strain abundances as proxy for dehalogenation activities. This

is confirmed by the high deviation of 155 % between ε_{Calc} and ε_{Meas} in RSP-IV after repeated TCE addition. Strain PCE1 was the most abundant strain during this experiment with fractions of 95 / 5 to 90 / 10 corresponding to a predicted ε_{Calc} of -18.6 ‰. However, PCE was almost exclusively dehalogenated by strain Y51 (ε_{Meas} = -7.3 ‰). Similar results were obtained in MC_{PCE1} where strain Y51 constituted less than 10 % of total cell numbers but contributed by almost 50 % to PCE transformation (ε_{Meas} = -13.7 ‰).

These results demonstrate that measured strain abundances generally underestimated PCE dehalogenation activities of strain Y51. This allows to conclude that strain abundances did not determine the dehalogenation activity of the two strains and other physiological factors must have compensated for low cell numbers of strain Y51. PceA and PrdA are the catalysts of PCE transformation suggesting that strain specific dehalogenation activities were governed by a complex interplay between RdhA *de novo* synthesis rates, amount of catalytically active RdhA per cell and RhdA specific properties of the strains. Thus, low abundances of strain Y51 were likely compensated by a higher content of active PCE-RdhA per cell and/or faster *de novo* RdhA synthesis rates compared to strain PCE1. In addition, more beneficial RdhA properties such as high substrate specificity for PCE (i.e. low Michaelis-Menten constant K_M) and high enzymatic dehalogenation rates may have contributed to high dehalogenation activities of this strain. The high contribution of strain Y51 in RSP-IV, may be explained by constant upregulation of PceA synthesis during prior TCE additions.[26] In contrast, PrdA is not expressed upon exposure to TCE.[60] Thus, a higher physiological adaption to recurrent chlorinated ethene addition by strain Y51 compared to strain PCE1 may explain major PCE transformation by strain Y51 despite its low abundance.

Cultivation in Mixed Culture Affects Cell Viability of the Strains. Cell numbers of both strains decreased in the mixed culture during starvation phases (RSP-VI, RSP-VII). For example, cell numbers of strain PCE1 decreased from $1*10^8$ to $2*10^6$ cells/mL between RSP-V and RSP-VI (Figure 4.3). Cell numbers of strain Y51 decreased from $8*10^6$ to $4*10^5$ cells/mL in this interval. This contrasts with previous reports for pure cultures of strain Y51 which showed constant high cell numbers ($3*10^9$ cells/mL) over a starvation phase of 6 months (Buchner et al., in prep.). During PCE transformation in RSP-VI, further decreases in strain Y51 cell numbers from $4*10^5$ to $3*10^4$ cells/mL were measured. Decreases in cell numbers result from cell death and subsequent cell lysis. Thus, gene abundances corroborate the adverse effect of mixed culture cultivation on strain Y51 as indicated by PCE isotope fractionation in RSP-VI and RSP-VII. Decreasing cell numbers of strain PCE1 show that cell viability of this strain was also adversely affected. The reason behind decreasing cell numbers and potential adverse effects due to mixed culture cultivation cannot be explained based on our results. The lack of pyruvate and chlorinated ethenes during starvation are unlikely to affect cell viability (Buchner et al., in prep.). This suggests that the presence of a second strain in the mixed culture negatively impacted the respective other strain. For strain Y51, derogated physiological activity and cell viability may be associated with PCE dehalogenation activity of strain PCE1. This would explain the reproducible decrease in PCE dehalogenation activity of strain Y51 parallel to

increasing PCE dehalogenation activity of strain PCE1 in RSP-I to RSP-V. Decreasing cell numbers and inactivity of strain Y51 during and after PCE dehalogenation by strain PCE1 in RSP-VI may corroborate adverse effects of activity of this strain on cell viability of strain Y51. The results of RSP-VI clearly demonstrate the limitations of functional gene analysis to characterize strain activities in microbial communities. Although strain Y51 constituted 14 % of initial cell numbers in RSP-VI, no dehalogenation activity of this strain was observed. This confirms that actual physiological activity of single strains or enzyme activity cannot be inferred from functional gene abundances, because also inactive or dead cells carry the targeted genes.
In conclusion, this study demonstrated that strain specific dehalogenation activities of OHRB during chlorinated ethene transformation in mixed cultures are not correlated with strain abundances. Cultivation conditions affected strain specific dehalogenation activities which resulted in highly variable PCE isotope fractionation of the same culture. CSIA allowed to deduce dehalogenation activities of the single strains demonstrating that CSIA is an expedient tool to track OHRB activities in microbial communities. Therefore, microbial dehalogenation in complex natural or laboratory systems should be assessed by combined analysis of functional gene abundances and isotope fractionation. Applying this bimodal approach, we demonstrated that changes in PCE isotope fractionation were associated with changing physiological activities of the strains rather than mixed culture composition. Thus, more conclusive biomarkers signifying physiological activities of single strains are needed to assess dehalogenation in microbial communities. Quantification of mRNA transcripts provides a measure of functional gene expression and thus only active cells are quantified.[6,56] In addition, absolute RdhA enzyme quantification may enable prediction of microbial enzyme activities.[63] Therefore, the applicability of *rdhA*-gene transcripts as well as RdhA content as proxies for strain specific activity needs to be evaluated in future studies. This can be achieved by the presented approach combining CSIA and molecular biological analyses to defined mixed cultures of well characterized strains.

Environmental Significance

The feasibility to reliably monitor and quantify *in situ* transformation of chlorinated ethenes (CE) at contaminated sites is a crucial requirement for successful application of bioremediation. For this purpose, quantification of functional genes encoding for CE-RdhA enzymes and CSIA are increasingly applied techniques.[6,16] While functional gene analysis allows to characterize the present microbial community, CSIA can be applied to quantify *in situ* biotransformation with an appropriate isotope enrichment factor. Because multiple different OHRB are typically present at contaminated sites, the underlying factors determining their dehalogenation activities and consequent effects on isotope fractionation need to be investigated. In this study, application of CSIA and PCE-*rdhA*-gene analysis to mixed cultures of two OHRB revealed that in microbial communities dehalogenation activities and substrate turnover of different OHRB are not necessarily governed by their cell abundances. Thus, activities of single OHRB cannot not be reliably assessed in microbial communities by CE-*rdhA*-gene abundances. Our results demonstrate that low *rdhA*-gene abundance may be compensated by strain specific RdhA

properties and physiological properties resulting in high dehalogenation activity of less abundant OHRB. This proves the worth of complement application of CSIA and functional gene analysis in microbial studies to elucidate contaminant transformation in mixed microbial cultures.
Strain specific dehalogenation activities and mixed culture ε_C values varied under changing cultivation conditions suggesting that PCE isotope fractionation of microbial communities is not necessarily constant. Since *rdhA*-gene abundances are not indicative for dehalogenation activities, apparent isotope fractionation of microbial communities cannot be inferred based on *rdhA*-gene analysis. Therefore, specific ε values for contaminated sites should be determined from autochtonous groundwater and sediment in laboratory experiments and periodically re-measured if environmental conditions change (e.g. availability of injected auxiliary substrates). In addition, site specific ε_C values could be determined under different cultivation conditions. This would allow to estimate the variability of isotope fractionation of the present microbial community under changing environmental conditions.
Recently, a method to quantify isotope fractionation in chemostat cultures has been presented.[68] Future studies should therefore attempt to study the effect of changing cultivation conditions on isotope fractionation of mixed cultures in continuous cultures because this allows a more dynamic simulation of changing environmental conditions compared to batch cultures. Combination of CSIA and molecular biological tools quantifying *rdhA*-gene transcription or RdhA enzymes can then be applied to elucidate the effect of changes in microbial physiologies on dehalogenation activities and isotope fractionation.

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5

General Conclusion and Outlook

5.1 General Conclusion

Organohalide-respiring bacteria (OHRB) catalyzing reductive dehalogenation by reductive dehalogenases enzymes (RdhA) are key microorganisms for bioremediation of chlorinated ethene contaminated sites.[1] Successful implementation of microbial *in situ* dehalogenation requires a deep understanding of RdhA transformation mechanisms and techniques to conclusively monitor and quantify OHRB dehalogenation activity. Compound specific isotope analysis (CSIA) combined with microbiological studies provides a versatile tool to achieve these tasks. CSIA relies on the determination of kinetic isotope effects which are expressed as ε values for single elements and Λ values as ratios of dual element isotope effects (dual isotope slopes).[2] During microbial reductive dehalogenation multiple factors can influence the magnitude of measured isotope effects which demand detailed assessment for reliable application of CSIA. The main goal of this work was to investigate potential causes for the published variability of chlorinated ethene isotope fractionation of pure cultures of OHRB and dehalogenating microbial communities. For pure cultures, it was investigated (i) whether RdhAs differing in their inherent properties (transformation products, metabolic vs cometabolic transformation) catalyze reductive dehalogenation by different reaction mechanisms leading to variable isotope fractionation (i.e. ε values) and (ii) if changes in RdhA expression patterns affect isotope fractionation. For microbial communities, (iii) the variability of isotope fractionation during concomitant dehalogenation activity of multiple OHRB strains was investigated. These tasks were addressed by laboratory experiments with pure and binary mixed cultures of organohalide-respiring strains from the *Desulfitobacterium* genus using tetrachloroethene (PCE) and trichloroethene (TCE) as model compounds. In the following the main observations of this work are discussed in terms of pure cultures of OHRB (i & ii) and microbial communities (iii).

(i) Dual isotope slopes ($\Lambda_{C/Cl}$) were determined for PCE dehalogenation in living cell suspensions of Gram-positive *Desulfitobacterium* strains differing in their PCE-transforming RdhA. Consistent $\Lambda_{C/Cl}$ values revealed the same reaction mechanism for all investigated types of RdhAs (PceA, PrdA, CrdA), despite highly variable ε values. Comparison with published PCE isotope effects for vitamin B_{12} enabled to identify the addition-elimination mechanism as the underlying reaction mechanism of the investigated PCE-RdhAs.[3] High accordance of measured ε values between PrdA-carrying strains and vitamin B_{12} provided evidence that C-Cl bond cleavage is the rate-limiting step in the PrdA enzyme. In contrast, much lower ε values of CrdA- and PceA-carrying strains (50 to 70 %) indicated that the isotope effect of bond cleavage is masked in these enzymes by a preceding rate-limiting step in the enzymatic multistep reaction. Thus, variable PCE ε_C values of Gram-positive OHRB presumably arise from different extents of masking within the enzymatic multistep reaction of the catalyzing RdhAs. Strong differences between protein sequences of PrdA- and PceA-enzymes pointed to structural differences in holoenzymes causing the apparent masking effects. However, the effect of differences in protein sequences on enzyme structure and masking of PCE isotope fractionation could not be resolved in detail in this study.

(ii) Carbon and chlorine isotope effects (ε and $\Lambda_{C/Cl}$ values) were consistent for PCE dehalogenation by living cells of *Desulfitobacterium dehalogenans* strain PCE1 with different RdhA expression patterns. Cells that were previously exposed to other organohalides than PCE showed lag-phases before exponential PCE transformation. This indicated *de novo* synthesis of the designated PCE-RdhA (PrdA) and minor PCE dechlorination rates of RdhAs expressed during prior exposure to other organohalides. A 25-fold lower PCE dechlorination rate of one of the previously expressed RdhAs compared to PrdA [4] allowed to conclude that a potential contribution of this RdhA was rapidly surpassed in active cells by *de novo* synthesis of PrdA. These results indicate that OHRB carrying a specialized, chlorinated ethene transforming RdhA among multiple other RdhAs, catalyze chlorinated ethene dehalogenation predominantly by the specialized RdhA. Thus, it can be expected that during reductive dehalogenation of chlorinated ethenes, ε and $\Lambda_{C/Cl}$ values of living cells of OHRB are not affected by changing cellular abundances of different RdhAs.

(iii) PCE dehalogenation experiments were conducted with mixed cultures of *Desulfitobacterium dehalogenans* strain PCE1 and *D. hafniense* strain Y51 which show significantly different ε_C values for PCE in pure culture (strain Y51 = -5.8 ‰, strain PCE1 = -19.7 ‰). Carbon isotope fractionation of PCE (ε_C) varied significantly for different inoculum ratios of the strains and under changing cultivation conditions. Dual isotope analysis ($\Lambda_{C/Cl}$) allowed to exclude changes in PCE-RdhA reaction mechanism in the mixed cultures, while masking effects were excluded based on consistent ε values in multiple pure culture experiments. Thus, varying mixed culture ε_C values reflected different strain specific contributions to PCE transformation (i.e. dehalogenation activities) in the single experiments. This allowed to calculate strain specific contributions based on a Rayleigh equation-based approach for simultaneous isotope fractionation by multiple processes. [5] Cell abundances of the strains showed no correlation with strain specific contributions and mixed culture ε_C values. Theoretical mixed culture ε_C values calculated from strain abundances deviated up to 155 % from measured ε_C values and frequently underestimated contributions of strain Y51. This suggests a compensation of low cell abundances of strain Y51 by RdhA- and strain specific properties such as a higher cellular amount of catalytically active RdhA. These results indicate that dehalogenation activities of single OHRB in microbial communities are not necessarily governed by their numerical abundance. This has important implications for quantification of chlorinated ethene dehalogenation at contaminated sites: Although a sound database of pure culture ε_C values and the related *rdhA*-genes is available, a representative ε_C value for the microbial community cannot be selected based on the most abundant *rdhA*-gene when multiple potential dechlorinators for the same compound are present. In addition, this study demonstrated that chlorinated ethene isotope fractionation of microbial communities and enrichment cultures is not necessarily constant but can change depending on environmental and cultivation conditions.

In summary, the presented work provided conclusive evidence to assess the role of different factors which were previously proposed to cause the observed variability of microbial chlorinated ethene isotope fractionation. We confirmed that the addition-elimination mechanism is a common reaction mechanism of different PCE-transforming RdhAs. This proves the applicability of dual isotope analysis for mechanistic assessments of enzymatic transformation pathways even though measured ε values are inconclusive. For a single pure culture, ε and $\Lambda_{C/Cl}$ values of PCE were consistent despite different RdhA expression patterns prior to PCE transformation. These results indicate that OHRB predominantly catalyze chlorinated ethene dehalogenation by their specialized RdhA for the individual transformation step despite the presence of other RdhAs. Thus, changing cellular abundances of different RdhAs are not expected to affect chlorinated ethene ε values. Furthermore, this works provides important implications for the assessment of chlorinated ethene transformation at contaminated sites using CSIA. Isotope fractionation may vary in microbial communities in which multiple OHRB dehalogenate the same chlorinated ethene because dehalogenation activities of single strains can change depending on environmental conditions. To obtain representative ε values for precise quantification, practitioners should be advised to determine site specific ε values on a regular basis or under different cultivation conditions. This would allow to account for potential changes in dehalogenation activities and isotope fractionation within the present microbial community.

5.2 Outlook

The results of this work provide fundamental knowledge for future studies to assess existing research gaps. Our results indicate that masking of PCE isotope fractionation in the enzymatic multistep reaction is related to differences in RdhA enzyme structures. However, we could not attribute the differences in rate-limiting steps to characteristic structural properties of the different enzymes. The first crystal structure of a PCE-RdhA has recently become available[6] and revealed that masking of PCE isotope fractionation may be related to the amino acid composition of single enzyme parts.[3,7] Thus, amino acid sequences of the PCE-RdhAs of *Desulfitobacterium* could be compared in future studies to investigate potential effects of different sections in the sequence on enzyme structures and rate-limitation. To this end, a similar approach as described by Kunze et al.[8] can be combined with CSIA. First, PCE-RdhA structures are modeled *in silico* and parts of the enzyme that potentially cause differences in rate-limiting steps are identified. To verify their involvement in rate-limitation, these parts may be altered by site-directed mutagenesis during heterologous RdhA expression. Thereafter, CSIA is applied to investigate potential changes in isotope effects of the modified RdhA. This approach may enable to identify characteristic parts of PCE-RdhAs and the related reaction step which are rate-limiting for C-Cl-bond cleavage.

This work provided evidence that differences in RdhA expression patterns do not affect chlorinated ethene isotope fractionation of OHRB strains carrying multiple *rdhA*-genes. This indicates that the published variability of ε values for single pure cultures is not related to changing

cellular abundances of different RdhAs. It was recently demonstrated that the experimental determination of ε values is susceptible to systematic errors depending on the evaluation method for measured concentration data. It was suggested that variable ε values of single pure cultures in different experiments may arise from inconsistent consideration of chlorinated ethene mass removal during repetitive sampling.[9] Thus, a meta-study re-evaluating the raw data of published ε values while taking account of repetitive sample removal may reveal whether variable ε values for single OHRB arise from inconsistent data evaluation methods.

This study suggested that concomitant chlorinated ethene transformation by multiple OHRB strains may result in highly variable isotope fractionation of microbial communities or enrichment cultures. However, the question remains whether these more complex cultures show an analogous variability of chlorinated ethene isotope fractionation compared to the defined mixed cultures. This could be investigated by monitoring isotope fractionation of enrichment cultures from different contaminated sites using the cultivation conditions which were applied in the mixed culture experiments. In parallel, the effect of additional environmental factors such as pH value, temperature or available electron donors on isotope fractionation may be evaluated. Furthermore, we observed no correlation between strain specific dehalogenation activities and strain cell abundances. Thus, it needs to be assessed whether the reported changes of OHRB abundances in enrichment cultures during changing cultivation conditions[10–13] would significantly affect chlorinated ethene isotope fractionation (i.e. ε values). To this end, enrichment cultures with well characterized metagenomes with regard to present OHRB strains (e.g. KB-1 culture) could be cultivated under different conditions and isotope fractionation is monitored. Detailed *rdhA*-gene quantification may then reveal whether potential changes in isotope fractionation can be correlated with shifting abundances of specific *rdhA*-genes. Finally, the physiological factors determining single strain dehalogenation activities in microbial communities need to be deciphered. For this purpose, the presented mixed culture approach should be extended to more complex but still traceable mixed cultures. These should comprise also obligate OHRB such as *Dehalococcoides* strains and other important members of dehalogenating microbial communities such as acetate-producing strains. Combination of metagenomics, metatranscriptomics and metaproteomics targeting key genes and enzymes would allow to study how changing environmental conditions affect the physiological activity of single strains. CSIA should simultaneously be applied to monitor potential changes in chlorinated ethene isotope fractionation. This may enable to identify key physiological factors determining dehalogenation activities of single strains in microbial communities.

5.3 References

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Appendix

A

Supporting Information of Chapter 2

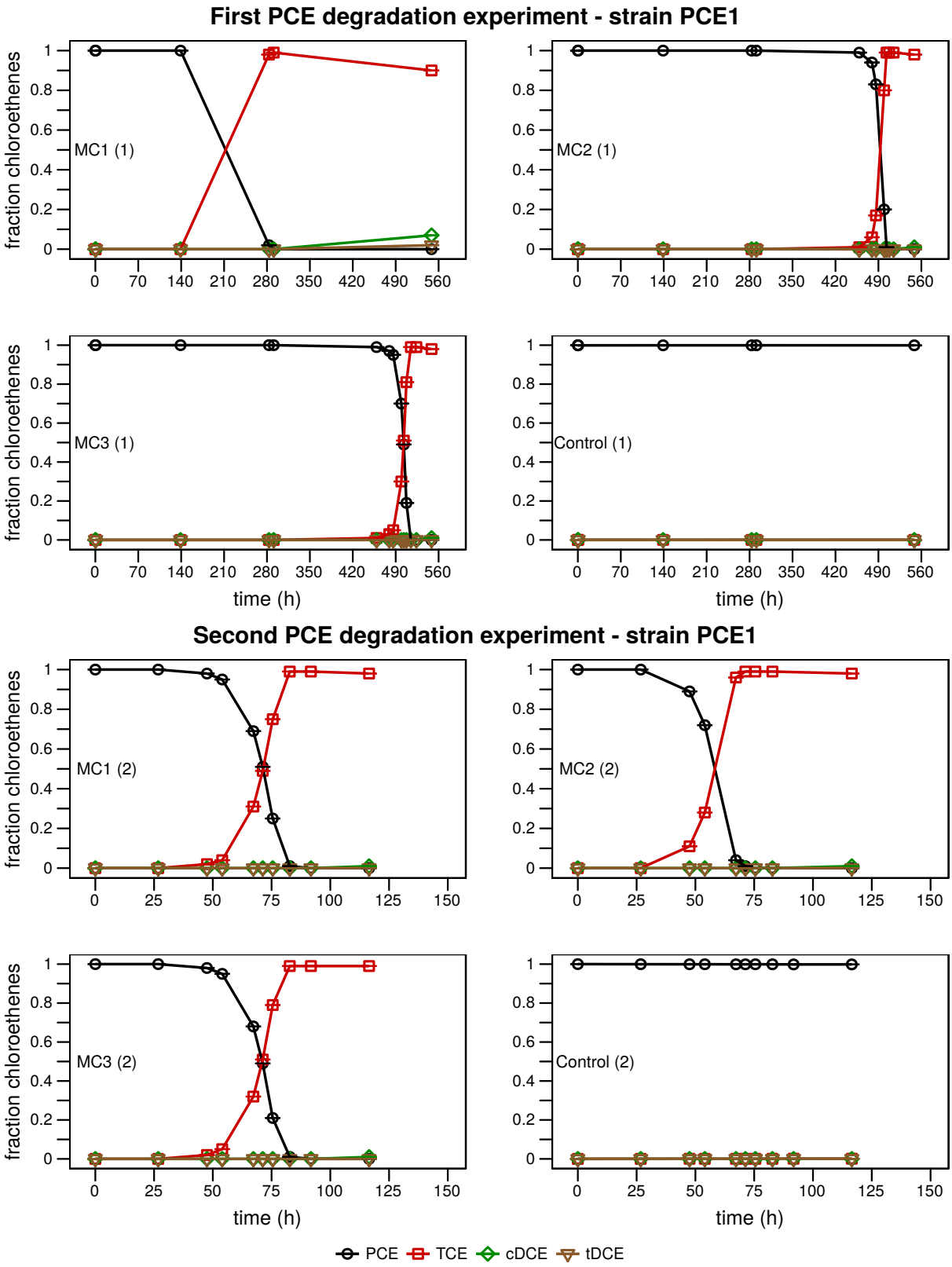

Figure A1. Fraction of chlorinated ethenes versus time for the PCE degradation experiments of *Desulfitobacterium dehalogenans* strain PCE1. Error bars represent standard deviation of calculated fractions from GC concentrations measurements (n=2).

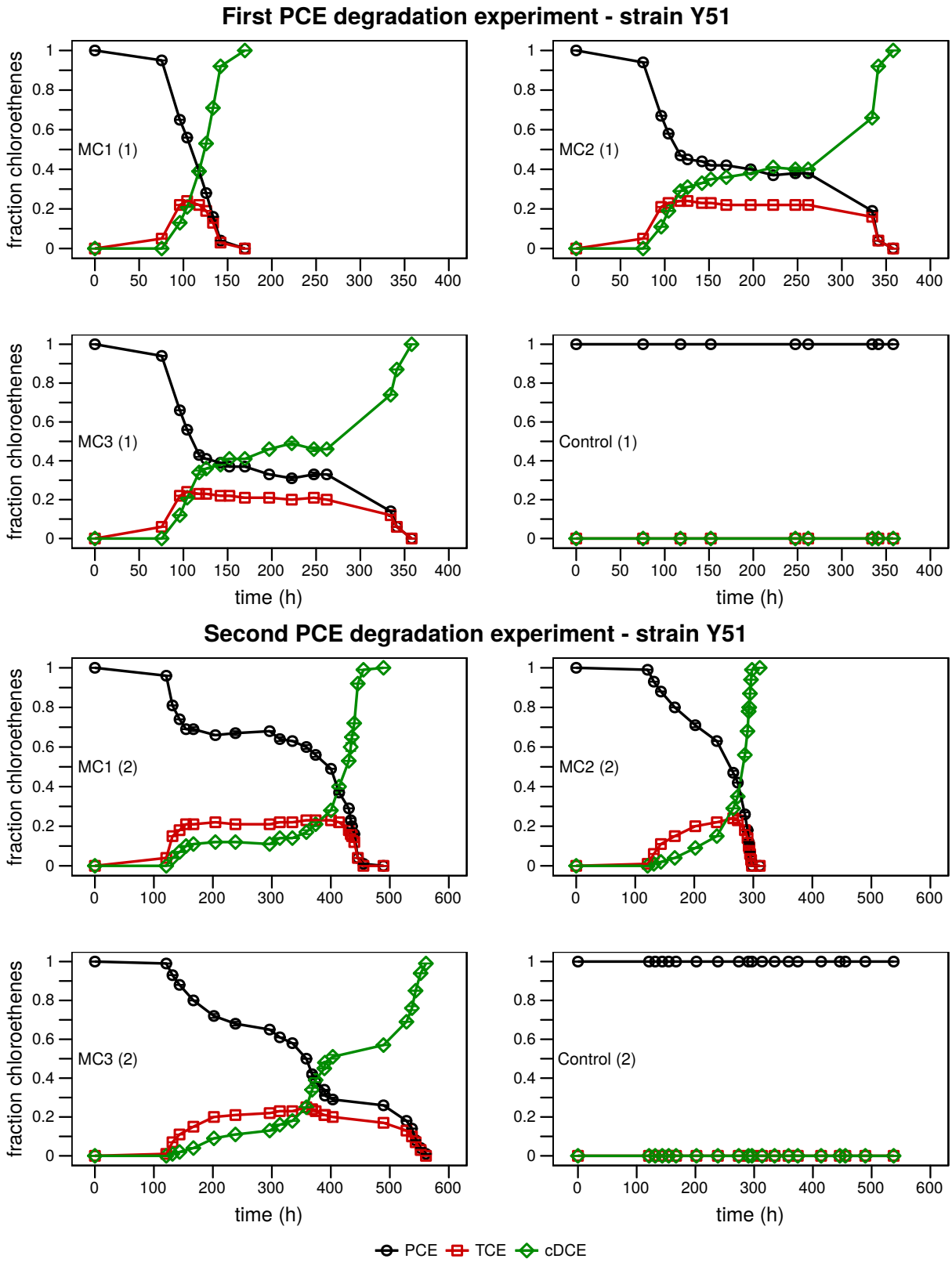

Figure A2. Fraction of chlorinated ethenes versus time for the PCE degradation experiments of *Desulfitobacterium hafniense* strain Y51. Error bars represent standard deviation of calculated fractions from GC concentrations measurements (n=2).

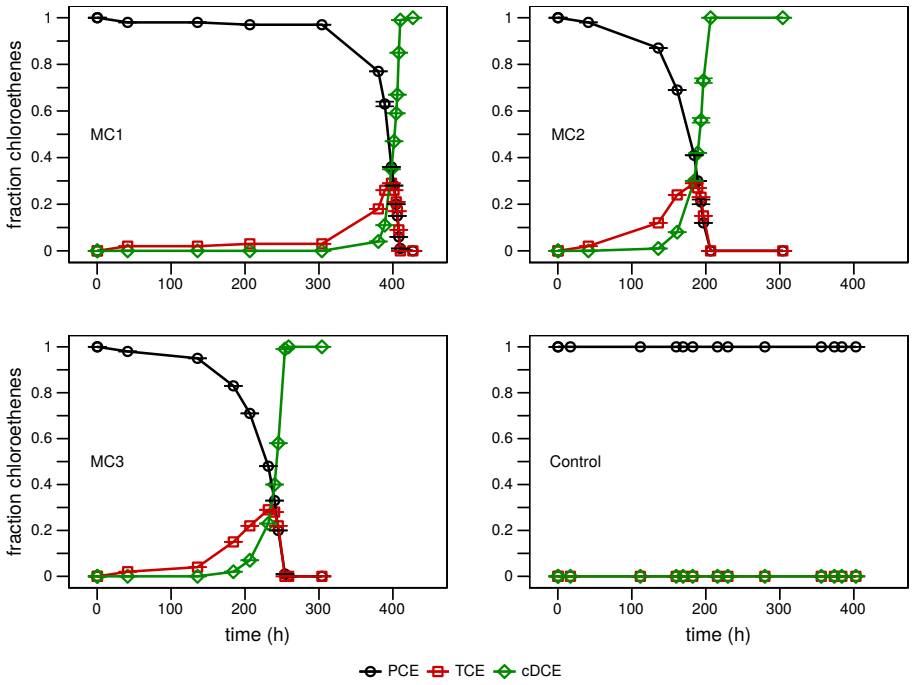

Figure A3. Fraction of chlorinated ethenes versus time for the PCE degradation experiment of *Desulfitobacterium hafniense* strain PCE-S. Error bars represent standard deviation of calculated fractions from GC concentrations measurements (n=2).

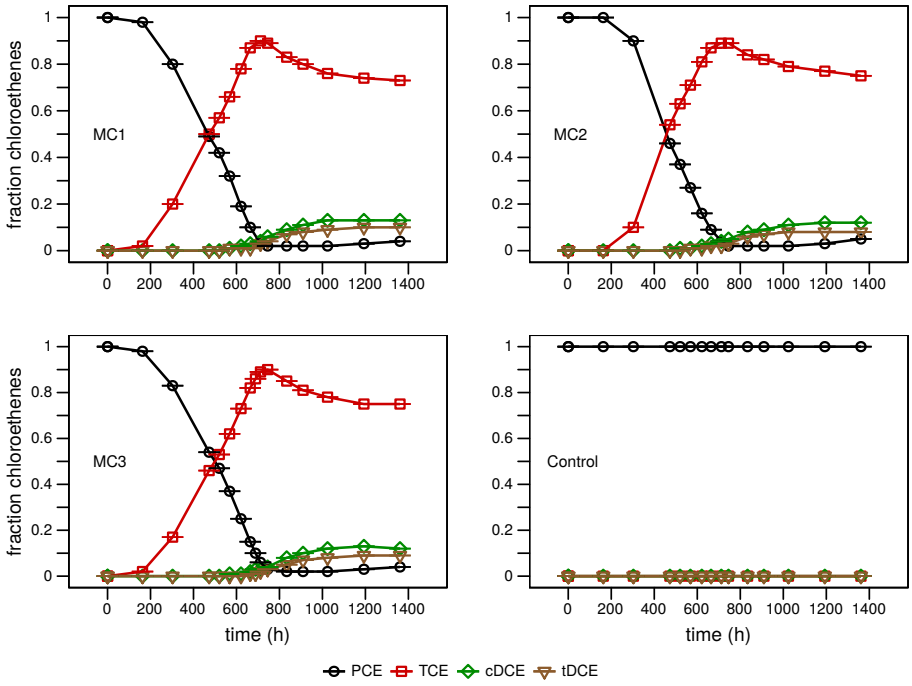

Figure A4. Fraction of chlorinated ethenes versus time for the PCE degradation experiment of *Desulfitobacterium hafniense* strain PCP-1. Error bars represent standard deviation of calculated fractions from GC concentrations measurements (n=2).

Table A1. *Z*-scores and corresponding *p*-values using two-tailed hypothesis testing for all possible pairings of dual isotope slopes.

Compared Slopes	***z*-score**	***p*-value**	**Significant different (α=0.05)**
PCE1 vs Viet1	-5.077	<0.00001	yes
PCE1 vs Y51	-6.417	<0.00001	yes
PCE1 vs PCE-S	-4.502	<0.00001	yes
PCE1 vs PCP-1	-2.203	0.027595	yes
Viet1 vs PCE1	5.077	<0.00001	yes
Viet1 vs Y51	8.485	<0.00001	yes
Viet1 vs PCE-S	7.071	<0.00001	yes
Viet1 vs PCP-1	2.121	0.033922	yes
Y51 vs PCE1	-6.417	<0.00001	yes
Y51 vs Viet1	-8.485	<0.00001	yes
Y51 vs PCE-S	-1.414	0.157362	no
Y51 vs PCP-1	-6.364	<0.00001	yes
PCE-S vs PCE1	-4.502	<0.00001	yes
PCE-S vs Viet1	-7.071	<0.00001	yes
PCE-S vs Y51	1.414	0.157362	no
PCE-S vs PCP-1	-4.950	<0.00001	yes
PCP-1 vs PCE1	2.203	0.027595	yes
PCP-1 vs Viet1	-2.121	0.033922	yes
PCP-1 vs Y51	6.364	<0.00001	yes
PCP-1 vs PCE-S	4.950	<0.00001	yes

Chart A.1. Protein sequences for the catalytic subunits of PCE reductive dehalogenases enzymes of *Desulfitobacterium hafniense* strain Y51 and strain PCE-S (both PceA) and *Desulfitobacterium dehalogenans* strain PCE1 (PrdA). The consensus twin arginine translocalisation sequence as indicator for a periplasmic location of the catalytic subunits is indicated by the green color.

>Strain Y51 PceA
MGEIN RRNFLK VSILGAAAAAAVASASAVKGMVSPLVADAADIVAPITETSEFPYKVD
AKYQRYNSLKNFFEKTFDPEANKTPIKFHYDDVSKITGKKDTGKDLPTLNAERLGIK
GRPATHTETSILFHTQHLGAMLTQRHNETGWTGLDEALNAGAWAVEFDYSGFNATG
GGPGSVIPLYPINPMTNEIANEPVMVPGLYNWDNIDVESVRQQGQQWKFESKEEASKI
VKKATRLLGADLVGIAPYDERWTYSTWGRKIYKPCKMPNGRTKYLPWDLPKMLSG
GGVEVFGHAKFEPDWEKYAGFKPKSVIVFVLEEDYEAIRTSPSVISSATVGKSYSNMA
EVAYKIAVFLRKLGYYAAPCGNDTGISVPMAVQAGLGEAGRNGLLITQKFGPRHRIA
KVYTDLELAPDKPRKFGVREFCRLCKKCADACPAQAISHEKDPKVLQPEDCEVAENP
YTEKWHLDSNRCGSFWAYNGSPCSNCVAVCSWNKVETWNHDVARVATQIPLLQDA
ARKFDEWFGYNGPVNPDERLESGYVQNMVKDFWNNPESIKQ

>Strain PCE-S PceA
MGEIN RRNFLK ASMLGAAAAAAVASASVVKGVVSPLVADAADIVAPITETSEFPYKVD
AKYQRYNSLKNFFEKTFDPEENKTPIKFHYDDVSKITGKKDTGKDLPMLNAERLGIK
GRPATHTETSILFHTQHLGAMLTQRHNETGWTGLDEALNAGAWAVEFDYSGFNAAG
GGPGSAIPLYPINPMTNEIANEPVMVPGLYNWDNIDVESVRQQGQQWKFESKEEASKI
LKKATRLLGADLVGIAPYDERWTYSTWGRKIQKPCKMPNGRTKYLPWDLPKMLSG
GGVEVFGHAKFEPDWEKYAGFKPKSVIVFVLEEDYEAIRTSPSVISSATVGKSYSNMA
EVAYKIAVFLRKLGYYAAPCGNDTGISVPMAVQAGLGEAGRNGLLITQKFGPRHRIA
KVYTDLELAPDKPRKFGVREFCRLCKKCADACPAQAISHEKDPKVLQPEDCEASENP
YTEKWHVDSERCGSFWAYNGSPCSNCVAVCSWNKVETWNHDVARVATQIPLLQDA
ARKFDEWFGYSGPVNPDERLESGYVQNMVKDFWNNPESIKQ

>Strain PCE1 PrdA
MDSEKENTLDQKEEKRSVGIS RRNFFK ASGIAAGVAALGLVTKSQPVYAGQESESAIV
NFAVQEVDQSPYNLPPFANAENLKRYELGKNAFYSKELSMDKFGGNPWHIEAEKYIV
KFIKEGVPGYSLMDNAFYDAAWASYKGTPLFSWEPLGVSNIKRAETVGKWEATPEQ
NNRYIKKVANEYGSGDTGVAVLNEQWFLSQDEKGKPYVFSTEHSKPTITEEAYYIPK
TMNRVIVMLAPMNPNMLKYAPTTLSEATVGTGYSQMAESAGKMAEFIRGLGYNAIP
MGNDASLSVPIAIDAGLGELGRHGLLVHPEYGSSVRISKVLTDLPIAPDKPISFGAAEFC
RTCMKCAEACPSESISKDKDPSDKVACASNNPGMKKWYVNTWTCLNQWVENGGGC
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DKNVPKWW

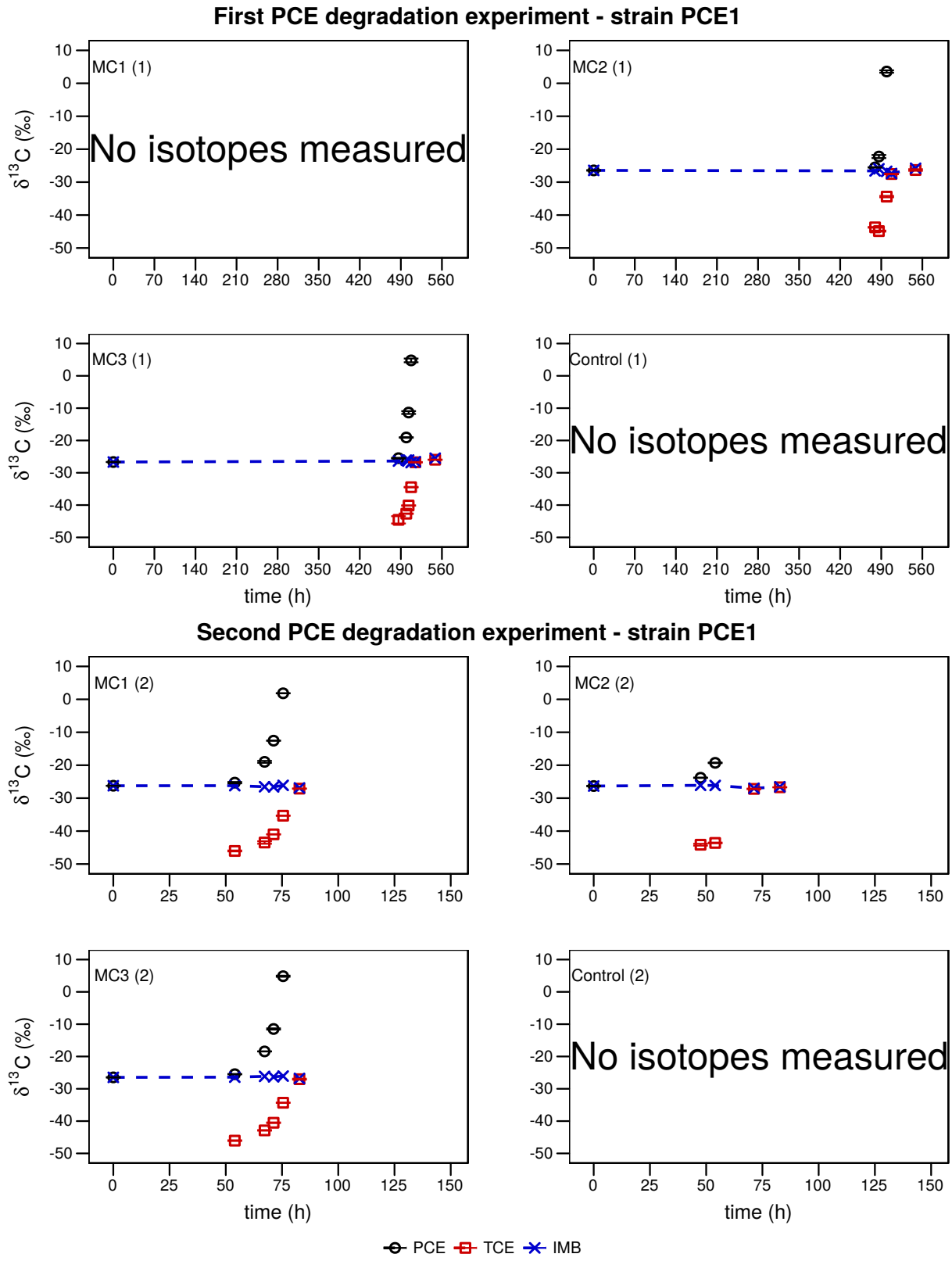


Figure A5. Measured δ^{13}C-values versus time for the PCE degradation experiments of *Desulfitobacterium dehalogenans* strain PCE1. Error bars represent standard deviation of GC-C-IRMS measurements (n=2).

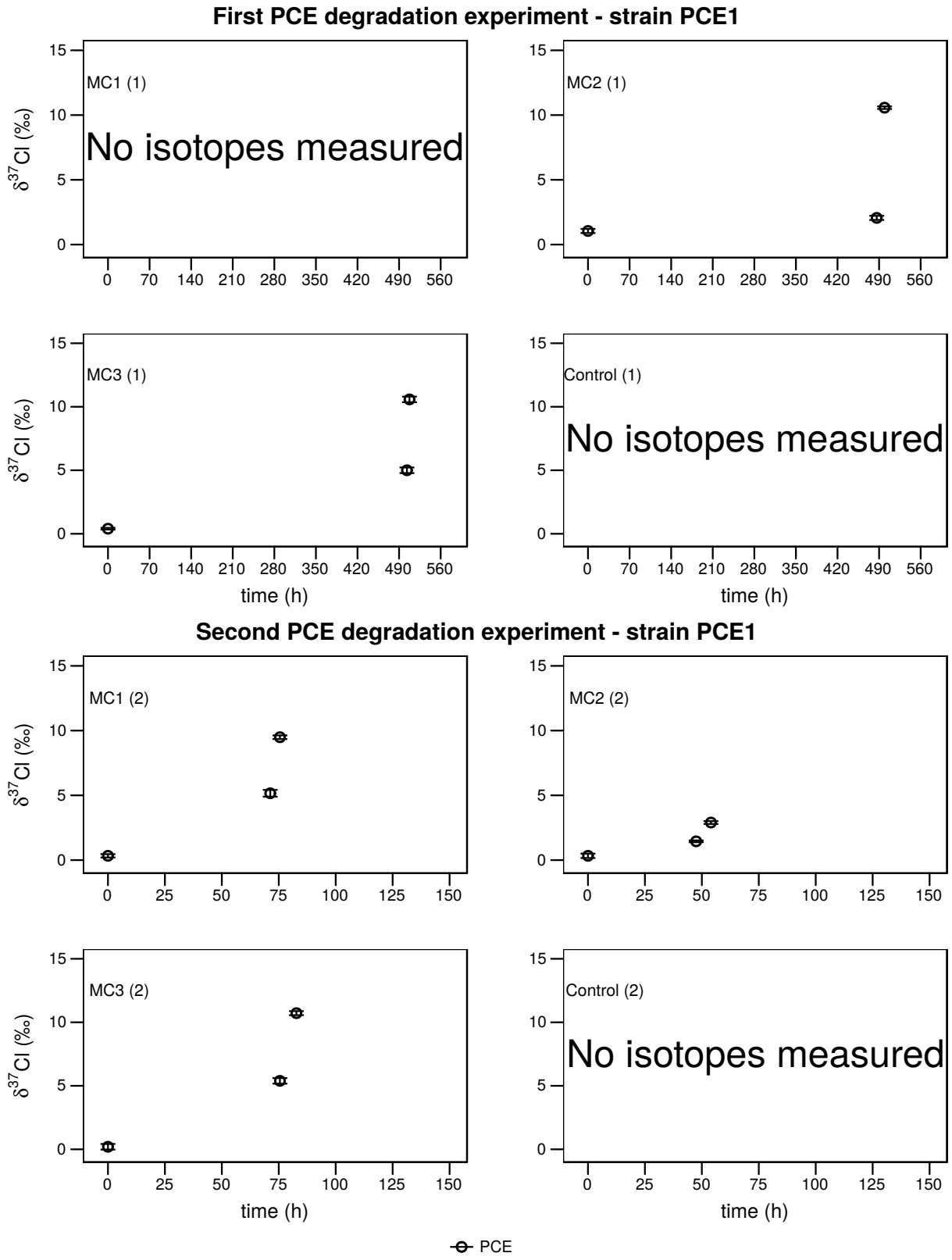


Figure A6. Measured δ^{37}Cl-values versus time for the PCE degradation experiments of *Desulfitobacterium dehalogenans* strain PCE1. Error bars represent standard deviation of GC-MS measurements (n=5).

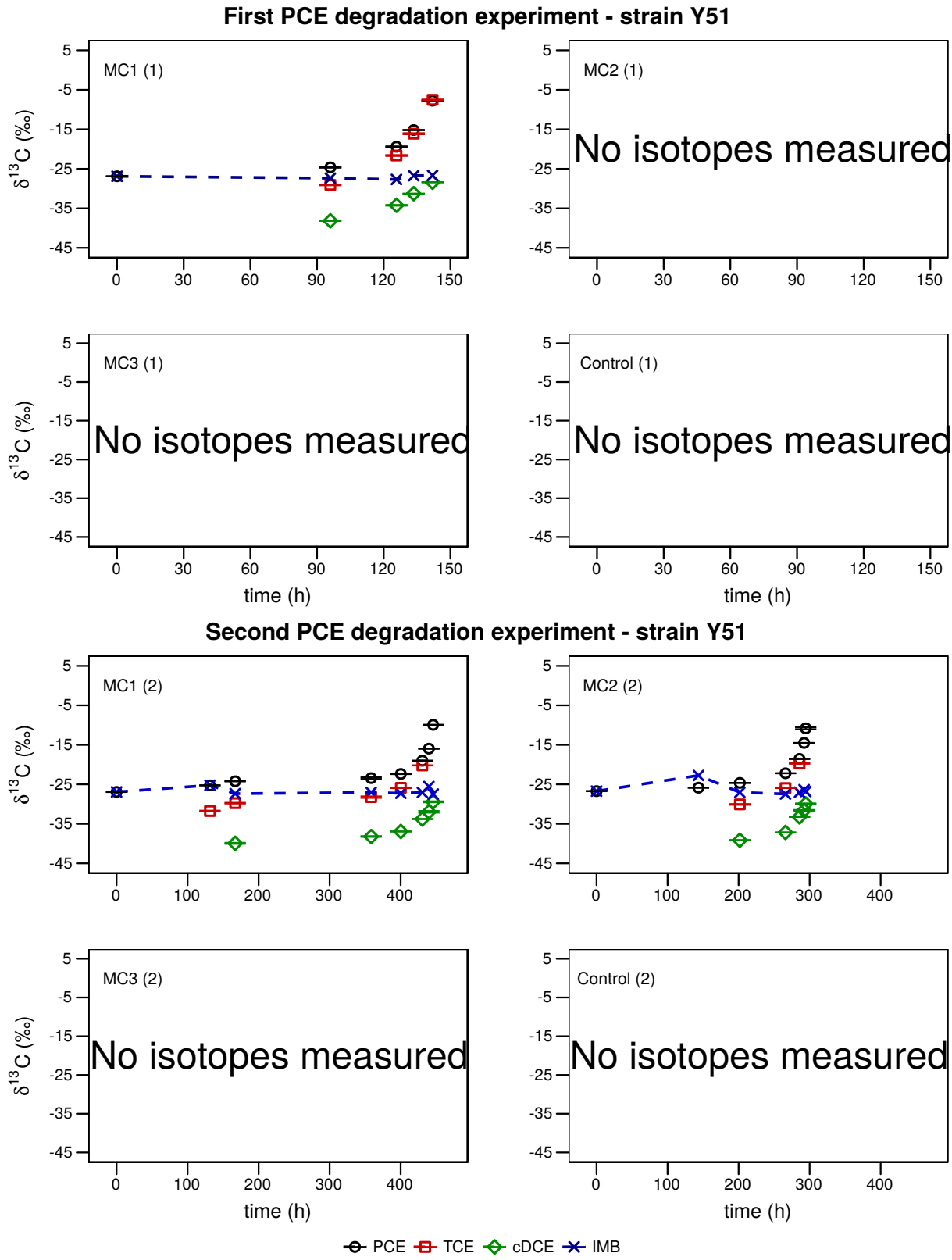


Figure A7. Measured δ^{13}C-values versus time for the PCE degradation experiments of *Desulfitobacterium hafniense* strain Y51. Error bars represent standard deviation of GC-C-IRMS measurements (n=2).

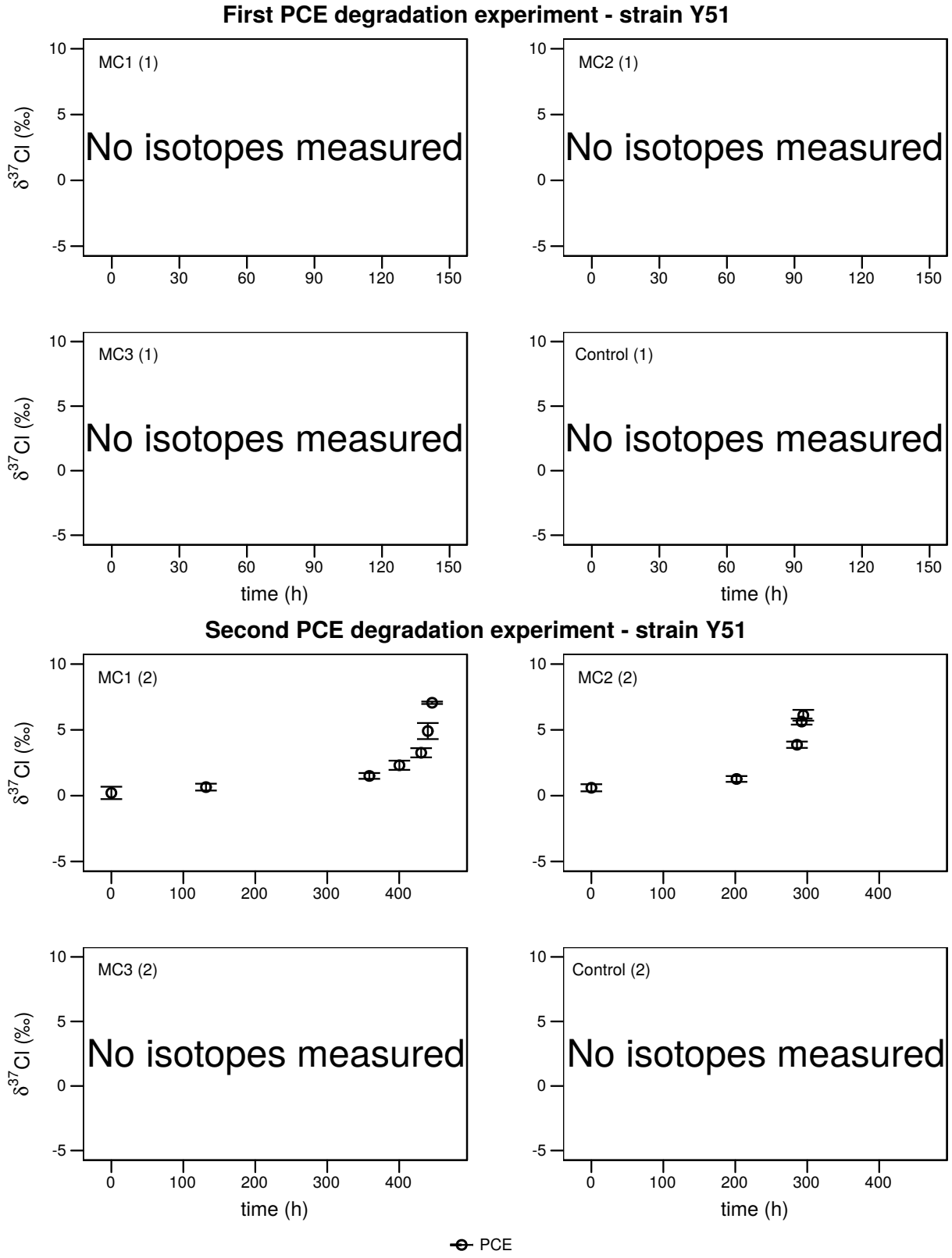


Figure A8. Measured δ^{37}Cl-values versus time for the PCE degradation experiments of *Desulfitobacterium hafniense* strain Y51. Error bars represent standard deviation of GC-MS measurements (n=5).

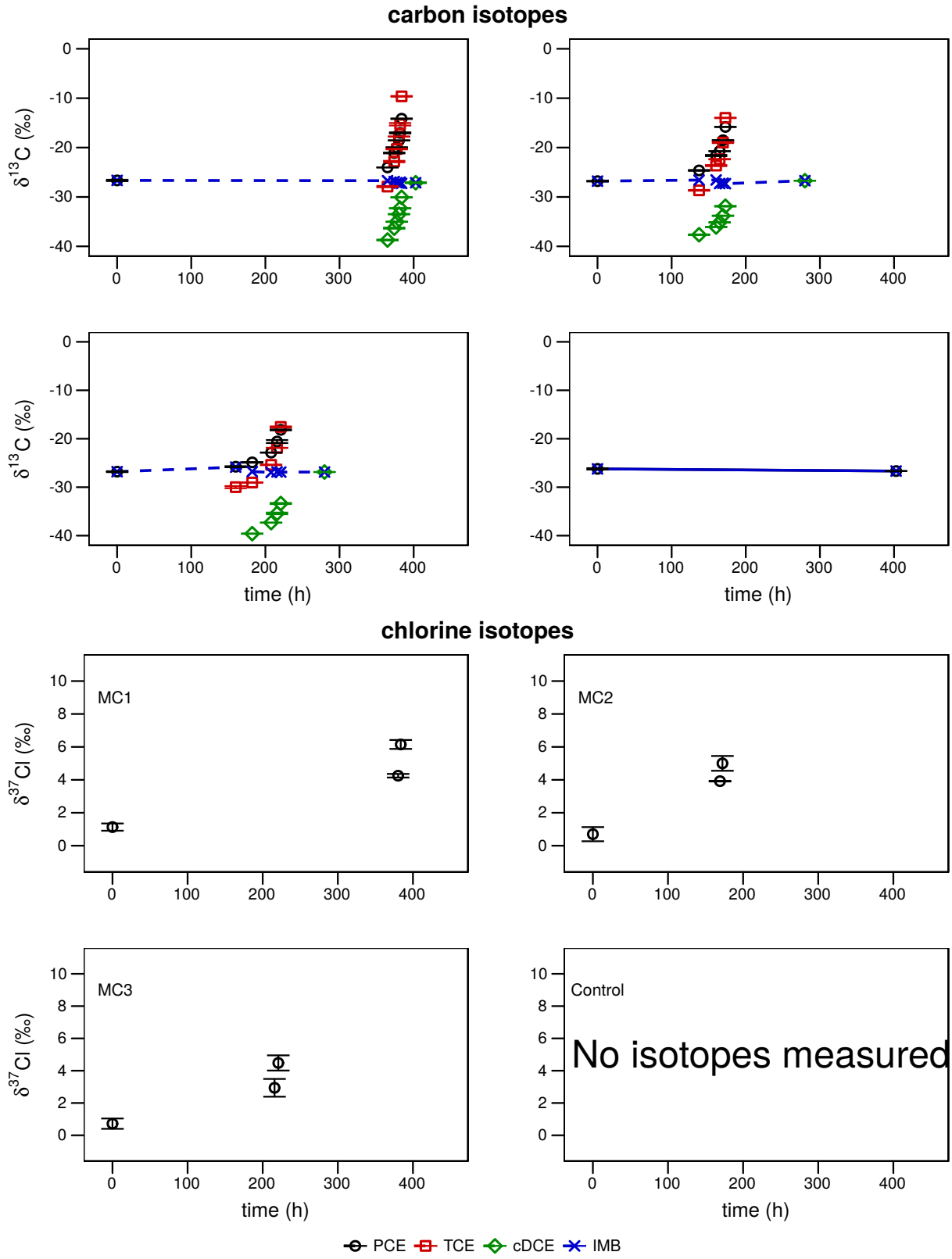


Figure A9. Measured δ^{13}C-values (upper part) and δ^{37}Cl-values (lower part) versus time for the PCE degradation experiment of *Desulfitobacterium hafniense* strain PCE-S. Error bars represent standard deviation of GC-C-IRMS measurements (n=2) for carbon and of GC-MS measurements for chlorine (n=5).

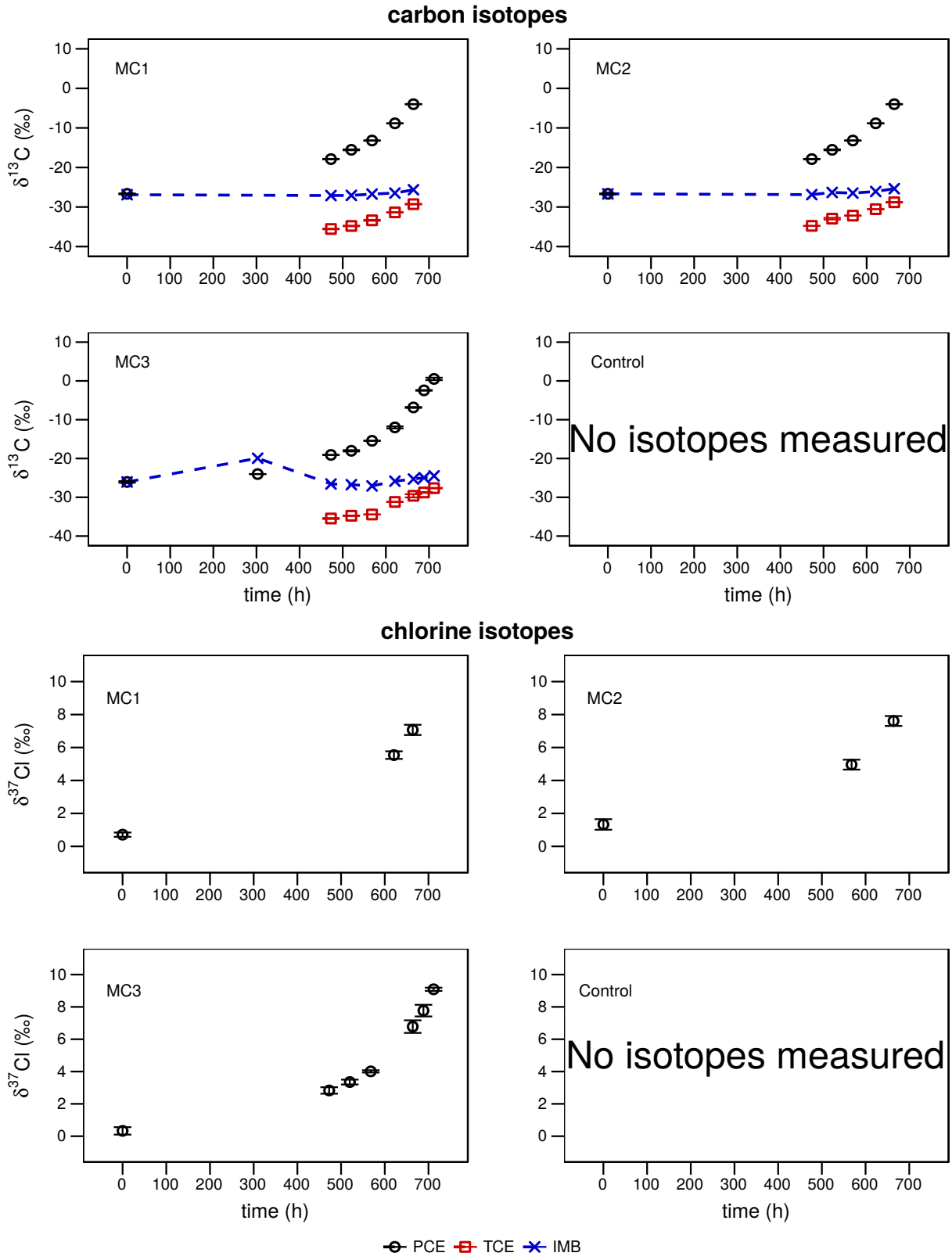


Figure A10. Measured δ^{13}C-values (upper part) and δ^{37}Cl-values (lower part) versus time for the PCE degradation experiment of *Desulfitobacterium hafniense* strain PCP-1. Error bars represent standard deviation of GC-C-IRMS measurements (n=2) for carbon and of GC-MS measurements for chlorine (n=5).

B

Supporting Information of Chapter 3

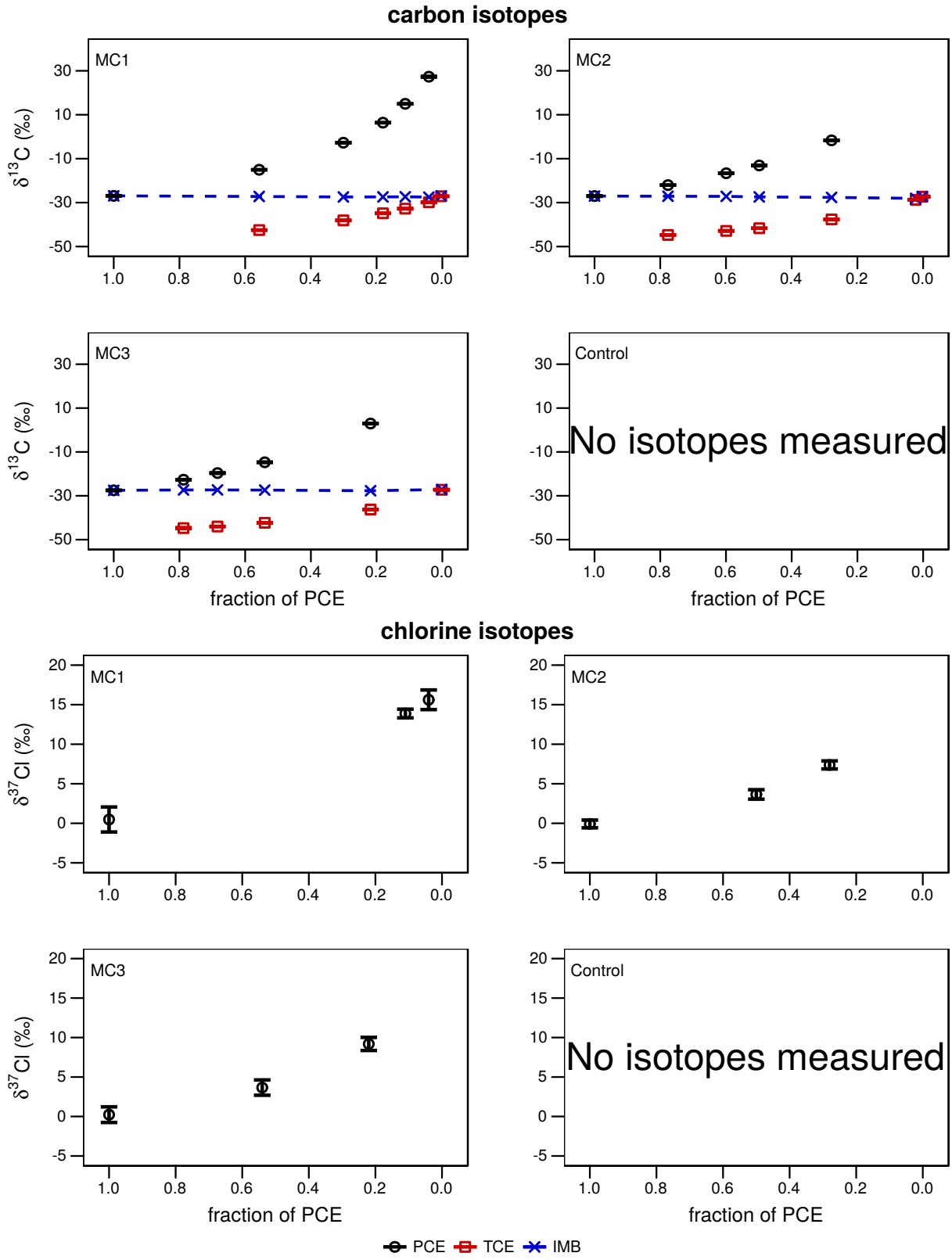


Figure B1. Measured δ^{13}C-values (upper part) and δ^{37}Cl-values (lower part) versus time for TS_{PCE}. Error bars represent standard deviation of GC-C-IRMS measurements (n=2) for carbon and of GC-MS measurements for chlorine (n=5).

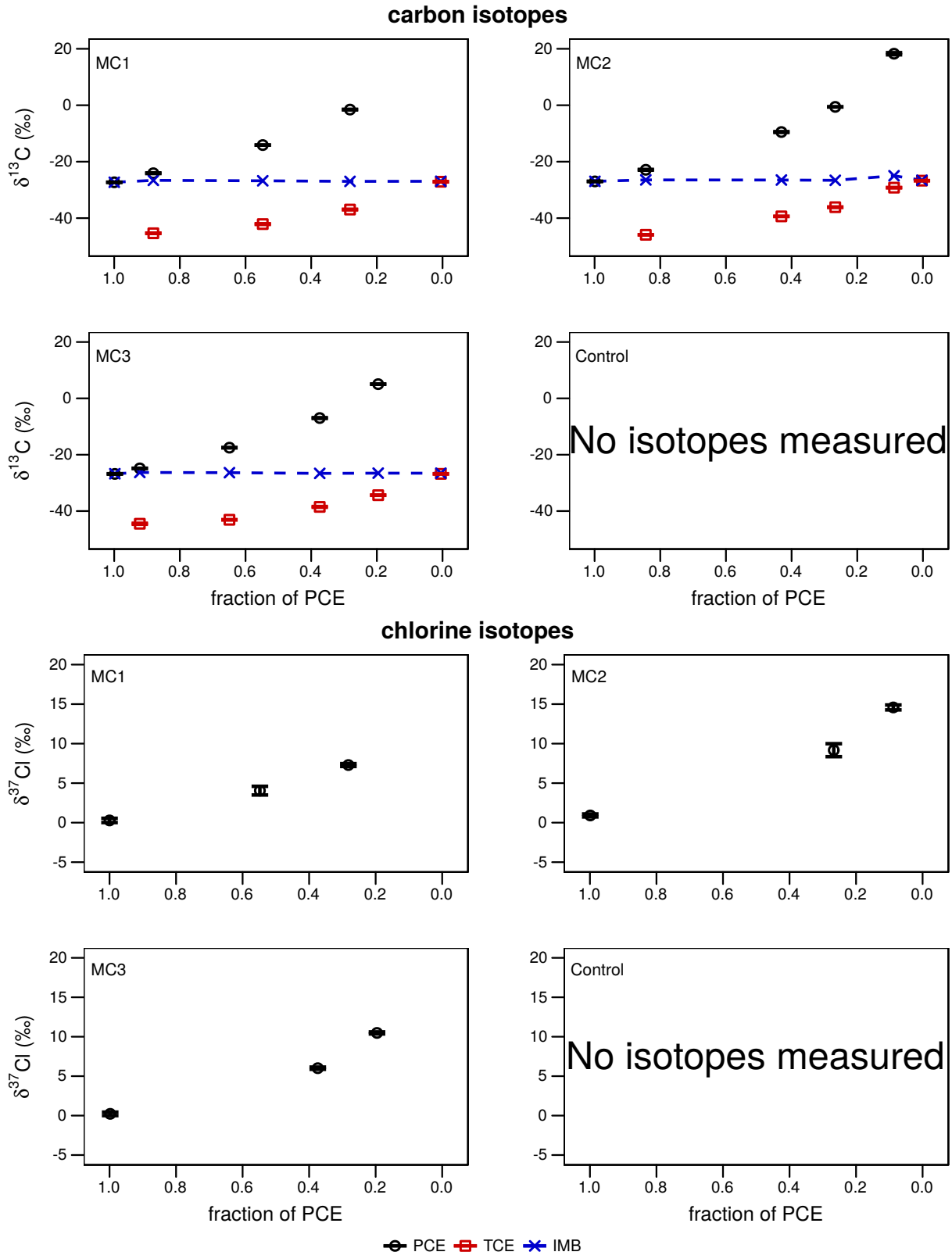


Figure B2. Measured δ^{13}C-values (upper part) and δ^{37}Cl-values (lower part) versus time for TS_{OHPA}. Error bars represent standard deviation of GC-C-IRMS measurements (n=2) for carbon and of GC-MS measurements for chlorine (n=5).

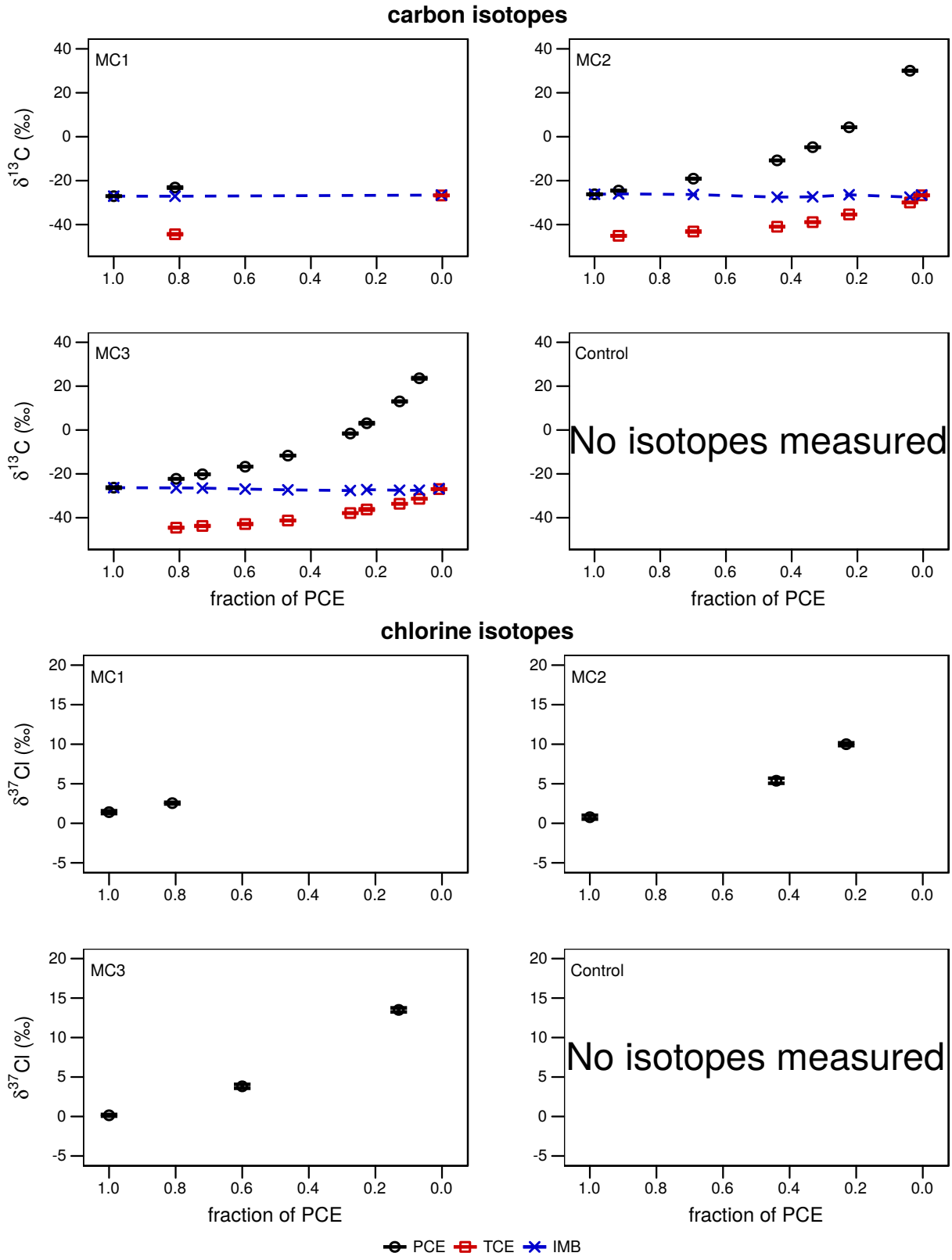
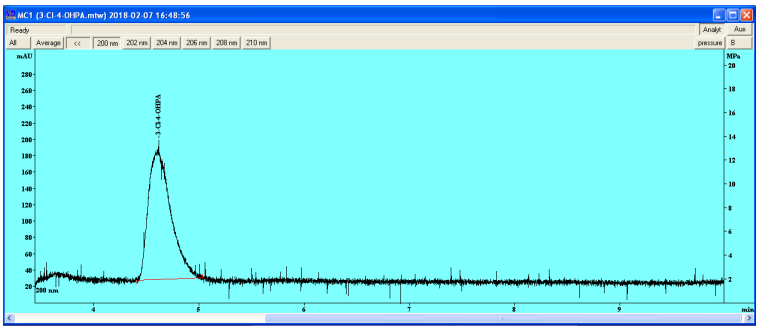
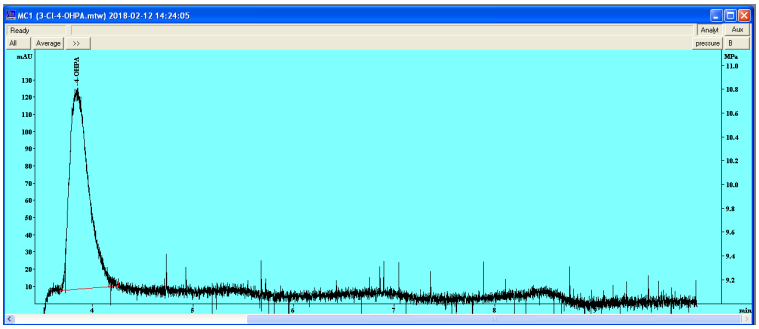


Figure B3. Measured δ^{13}C-values (upper part) and δ^{37}Cl-values (lower part) versus time for TS_{TCP}. Error bars represent standard deviation of GC-C-IRMS measurements (n=2) for carbon and of GC-MS measurements for chlorine (n=5).

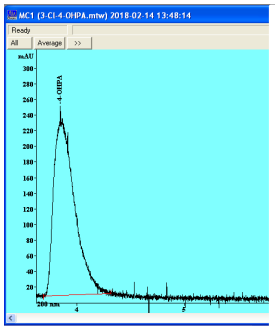
1) Inoculation (07.02.18):
1 Vol.-% pre-culture,
1 mM 3-Cl-4-OHPA,
1 mM pyruvate

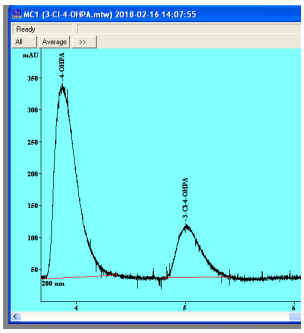


2) 1st complete degradation (12.02.18) & Respike:
1 mM 3-Cl-4-OHPA,
1 mM pyruvate



3) 2nd complete degradation (14.02.18, left) & Respike (16.02.18, right):
1 mM 3-Cl-4-OHPA,
1 mM pyruvate





4) 3rd complete degradation (19.02.18, left) & Respike (20.02.18, right):
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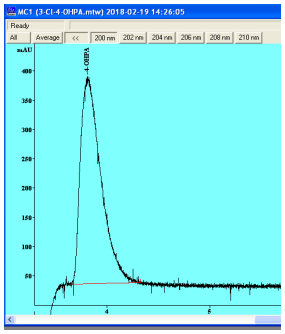
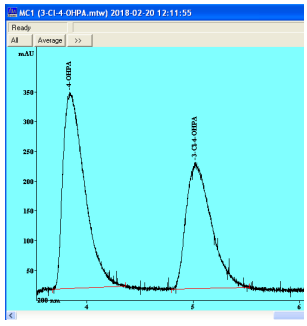
1 mM pyruvate





5) 4th complete degradation (22.02.18) & provision of PCE spike solution

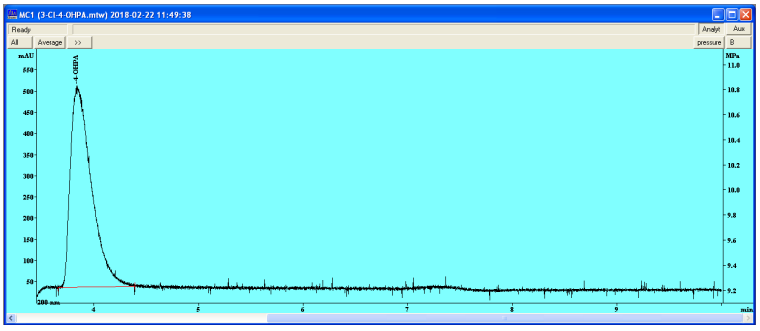
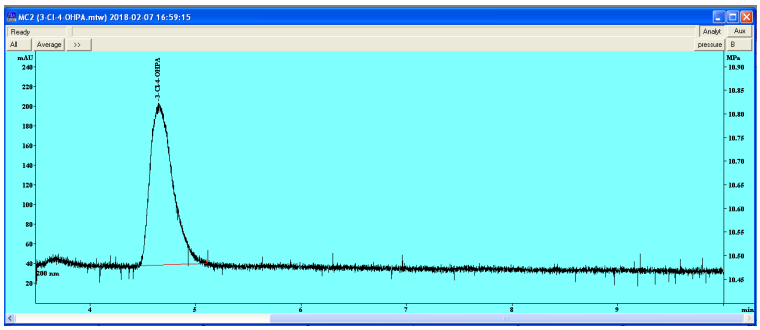
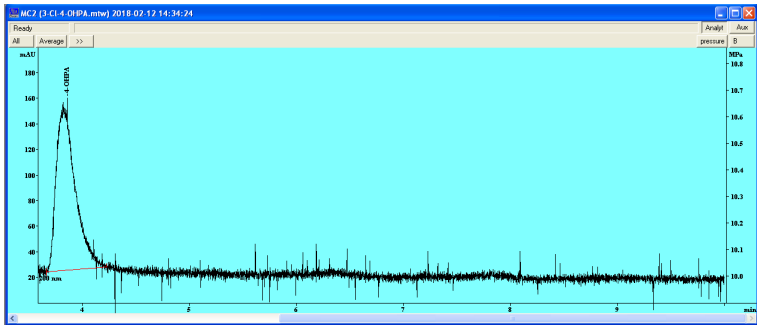


Figure B4. Qualitative HPLC-UV/VIS measurements of MC1 of TS_{OHPA} to verify complete 3-Cl-4-OHPA dechlorination.

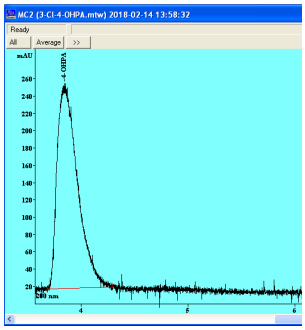
1) Inoculation (07.02.18):
1 Vol.-% pre-culture,
1 mM 3-Cl-4-OHPA,
1 mM pyruvate

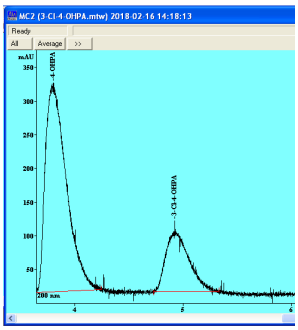


2) 1st complete degradation
(12.02.18) & Respike:
1 mM 3-Cl-4-OHPA,
1 mM pyruvate

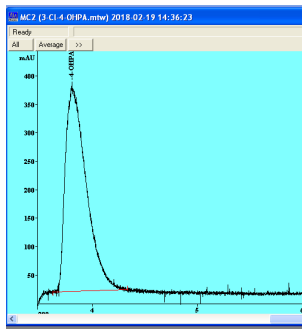


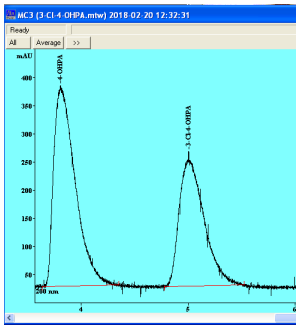
3) 2nd complete degradation
(14.02.18, left) &
Respike (16.02.18, right):
1 mM 3-Cl-4-OHPA,
1 mM pyruvate





4) 3rd complete degradation
(19.02.18, left) &
Respike (20.02.18, right):
1 mM 3-Cl-4-OHPA,
1 mM pyruvate





5) 4th complete degradation
(22.02.18) & provision of PCE
spike solution

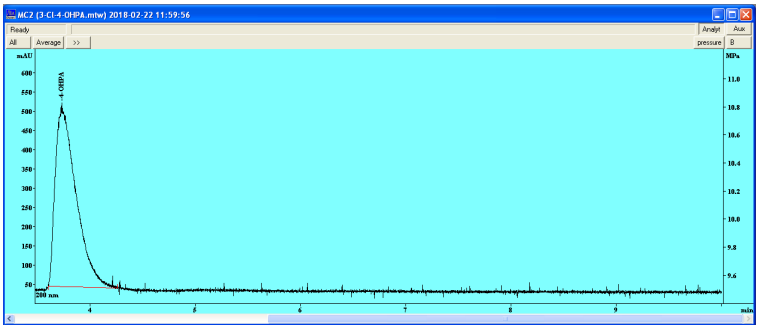


Figure B5. Qualitative HPLC-UV/VIS measurements of MC2 of TS_{OHPA} to verify complete 3-Cl-4-OHPA dechlorination.

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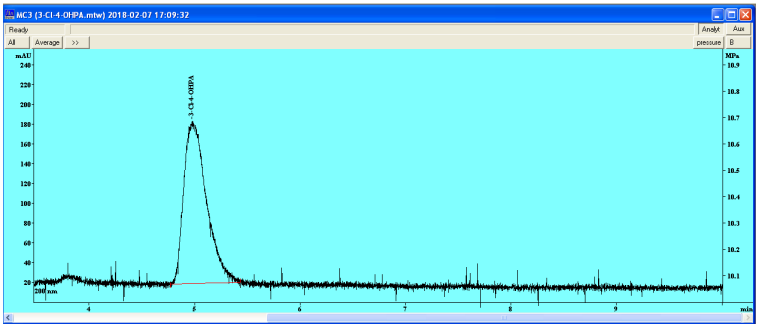
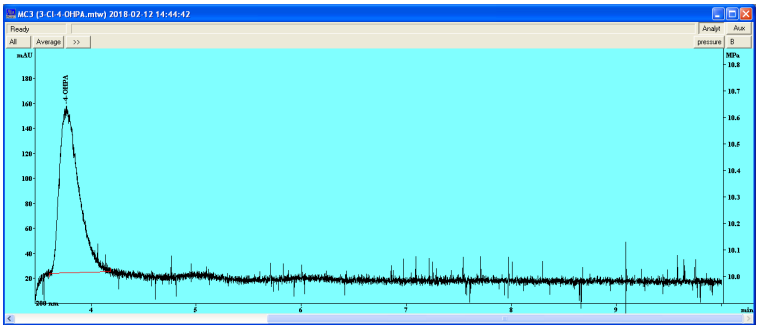
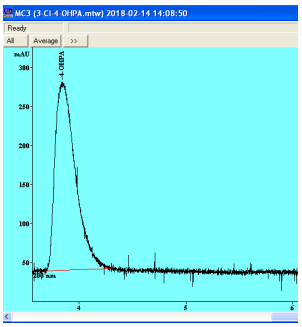
1 mM 3-Cl-4-OHPA,
1 mM pyruvate

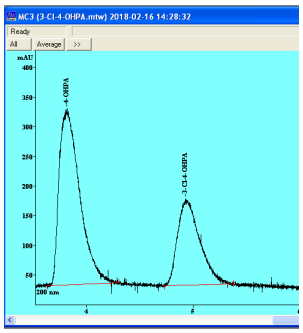


2) 1st complete degradation
(12.02.18) & Respike:
1 mM 3-Cl-4-OHPA,
1 mM pyruvate

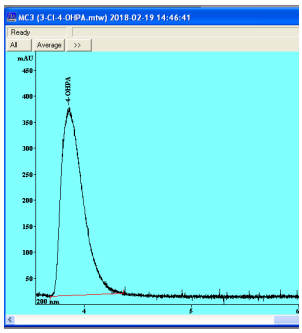


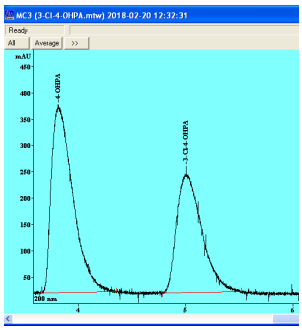
3) 2nd complete degradation
(14.02.18, left) &
Respike (16.02.18, right):
1 mM 3-Cl-4-OHPA,
1 mM pyruvate





4) 3rd complete degradation
(19.02.18, left) &
Respike (20.02.18, right):
1 mM 3-Cl-4-OHPA,
1 mM pyruvate





5) 4th complete degradation
(22.02.18) & provision of PCE
spike solution

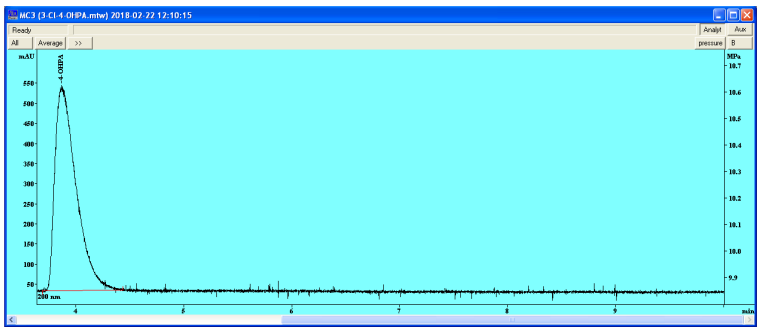


Figure B6. Qualitative HPLC-UV/VIS measurements of MC3 of TS_{OHPA} to verify complete 3-Cl-4-OHPA dechlorination.

C

Supporting Information of Chapter 4

Calculation of error terms (er) for error propagation of averaged strain fractions of total cell numbers

$$er_{PCE1-MC1} = \left(\frac{cells_{Y51-MC1} + cells_{Y51-MC2}}{(cells_{Y51-MC1} + cells_{Y51-MC2} + cells_{PCE1-MC1} + cells_{PCE1-MC2})^2} * \Delta_{PCE1-MC1} \right)^2$$

$$er_{PCE1-MC2} = \left(\frac{cells_{Y51-MC1} + cells_{Y51-MC2}}{(cells_{Y51-MC1} + cells_{Y51-MC2} + cells_{PCE1-MC1} + cells_{PCE1-MC2})^2} * \Delta_{PCE1-MC2} \right)^2$$

$$er_{Y51-MC1} = \left(\frac{cells_{PCE1-MC1} + cells_{PCE1-MC2}}{(cells_{Y51-MC1} + cells_{Y51-MC2} + cells_{PCE1-MC1} + cells_{PCE1-MC2})^2} * \Delta_{Y51-MC1} \right)^2$$

$$er_{Y51-MC2} = \left(\frac{cells_{PCE1-MC1} + cells_{PCE1-MC2}}{(cells_{Y51-MC1} + cells_{Y51-MC2} + cells_{PCE1-MC1} + cells_{PCE1-MC2})^2} * \Delta_{Y51-MC2} \right)^2$$

Error Propagation for Calculation of 95%-Confidence Interval of Strain Specific Contributions

$$\sqrt{(er_{\varepsilon-mix})^2 + (er_{\varepsilon-PCE1})^2 + (er_{\varepsilon-Y51})^2} * 100$$

$$er_{\varepsilon-mix} = \left(\frac{1}{\varepsilon_{Y51} - \varepsilon_{PCE1}} \right) * \Delta\varepsilon_{mix}$$

$$er_{\varepsilon-PCE1} = \left(\frac{\varepsilon_{mix} - \varepsilon_{Y51}}{(\varepsilon_{Y51} - \varepsilon_{PCE1})^2} \right) * \Delta\varepsilon_{PCE1}$$

$$er_{\varepsilon-Y51} = \left(\frac{\varepsilon_{PCE1} - \varepsilon_{mix}}{(\varepsilon_{Y51} - \varepsilon_{PCE1})^2} \right) * \Delta\varepsilon_{Y51}$$

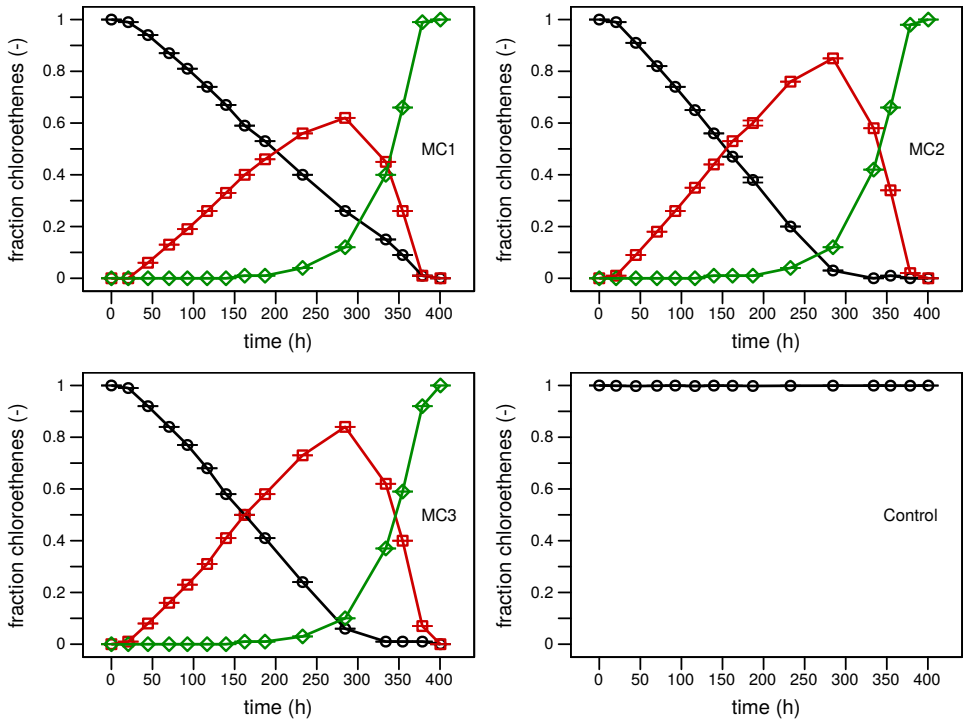


Figure C1. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of MC_{NA}. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

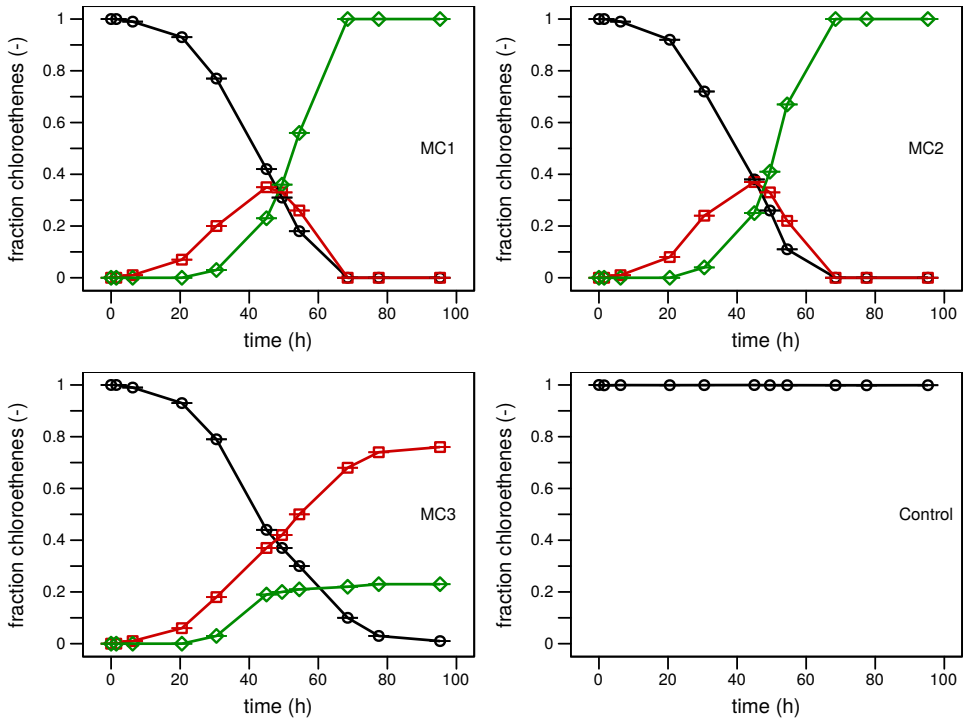


Figure C2. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of MC_{PA}. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

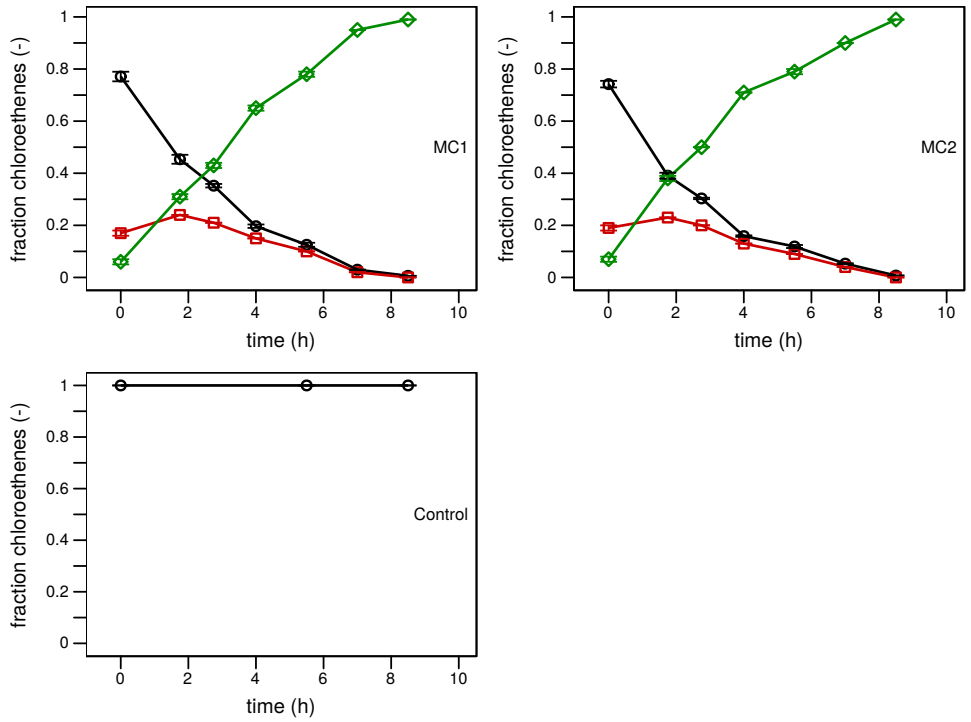


Figure C3. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of MC_{Y51}. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

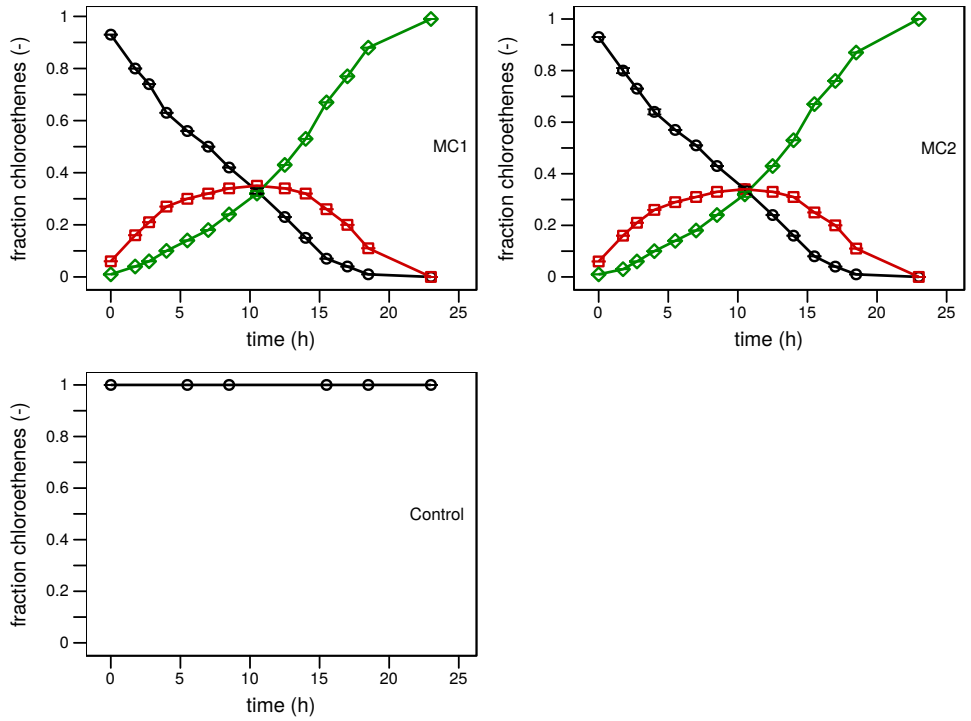


Figure C4. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of MC_{PCE1}. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

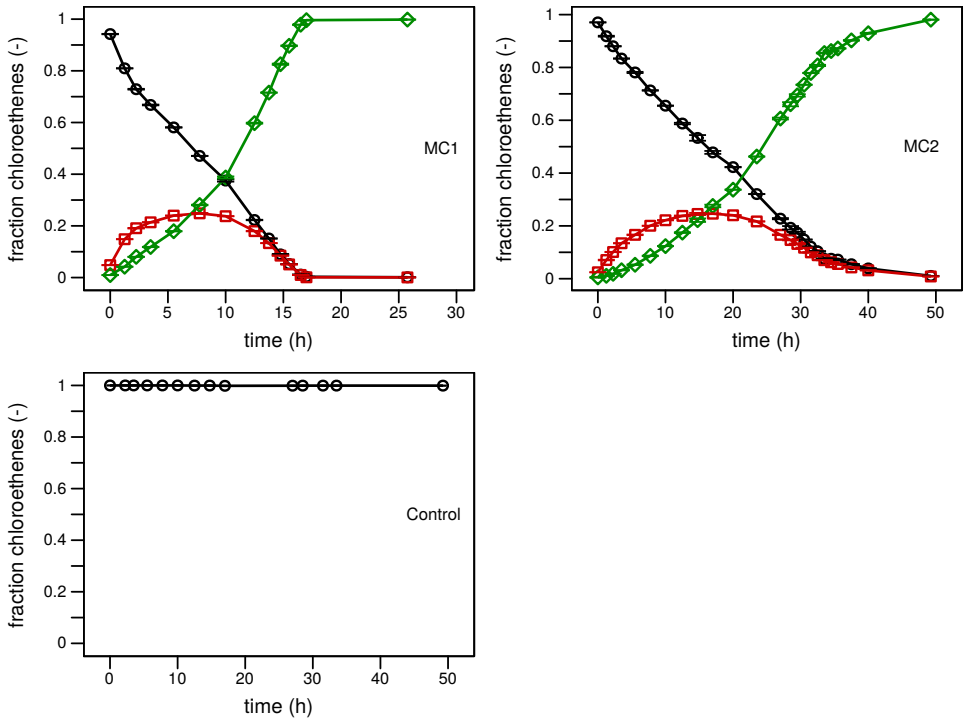


Figure C5. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-I. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

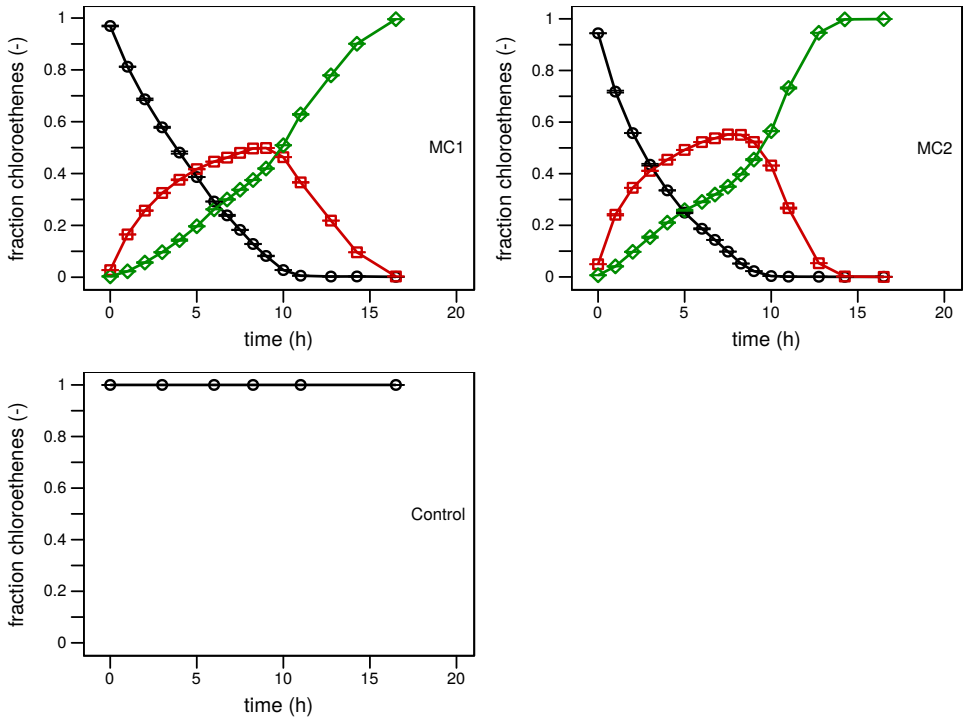


Figure C6. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-II. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

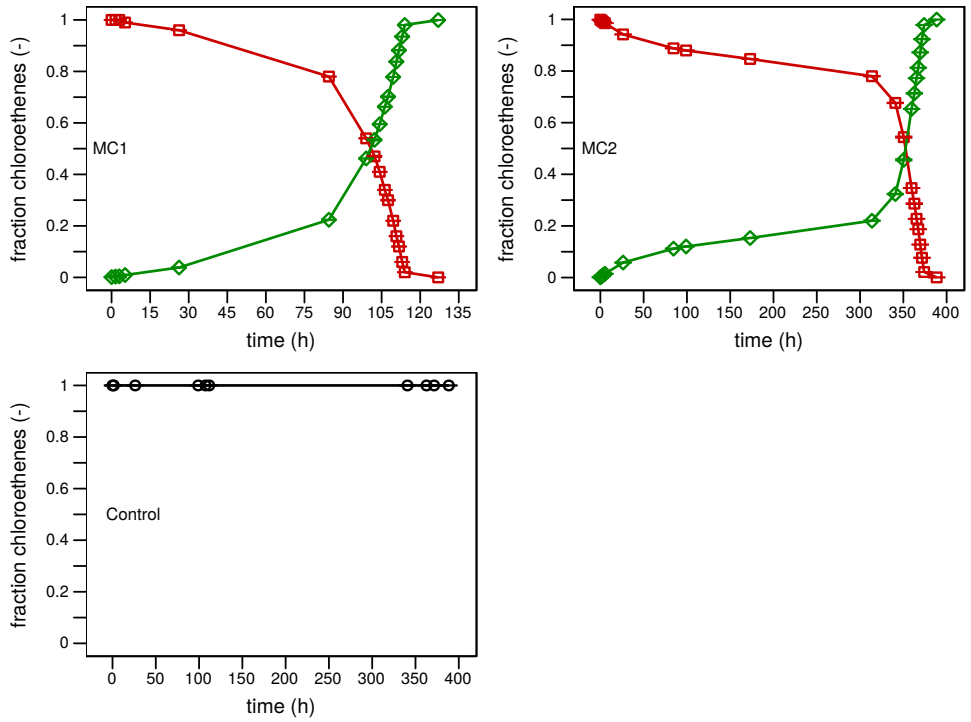


Figure C7. Fraction of chlorinated ethenes (TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-III. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

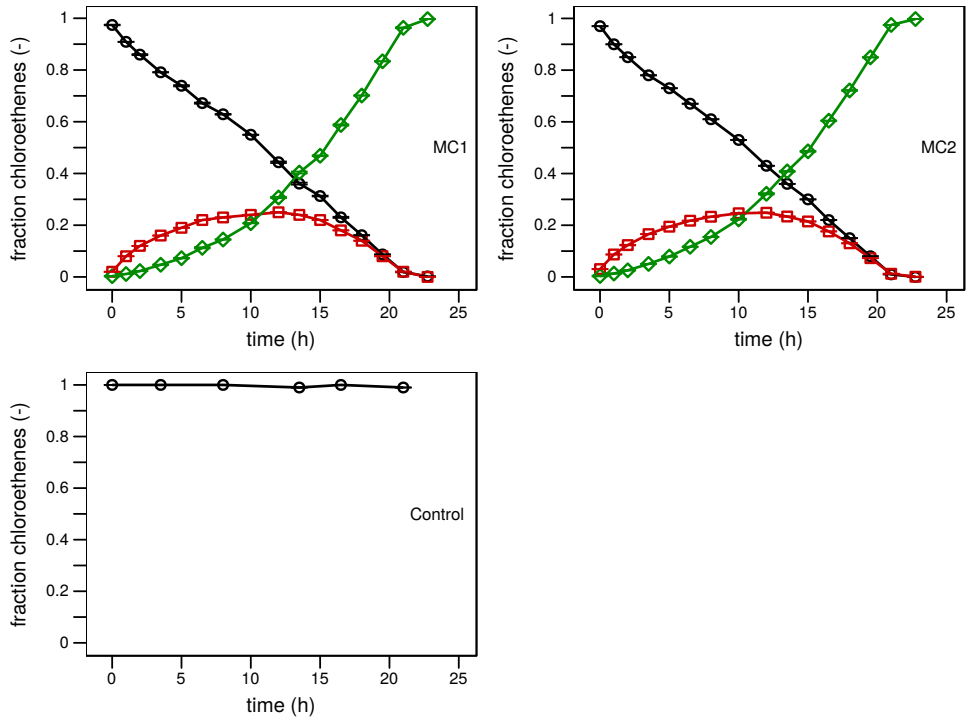


Figure C8. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-IV. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

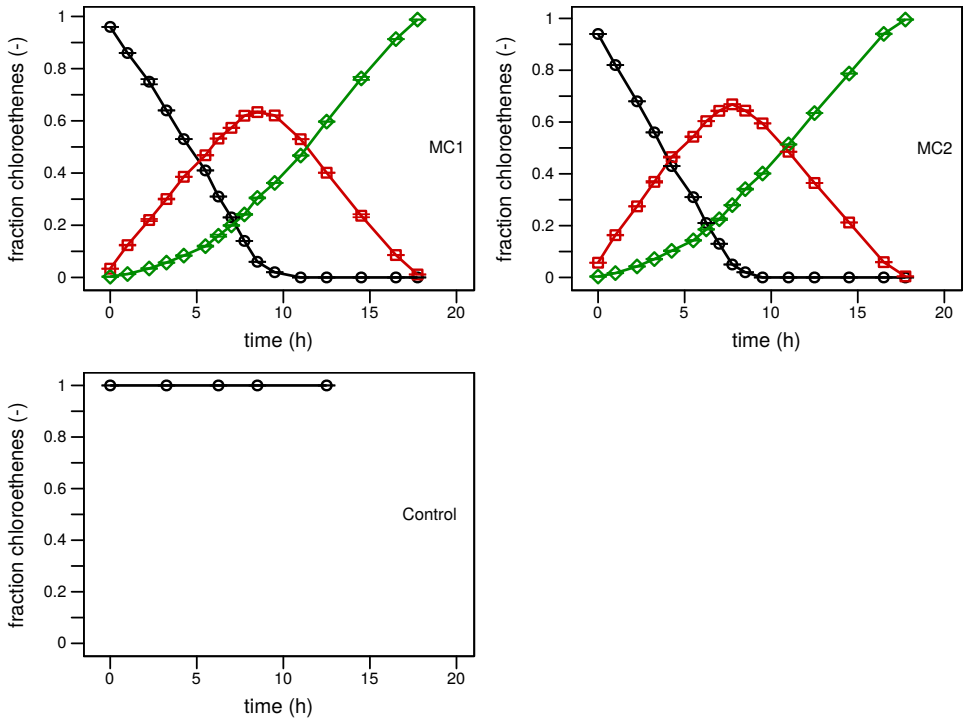


Figure C9. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-V. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

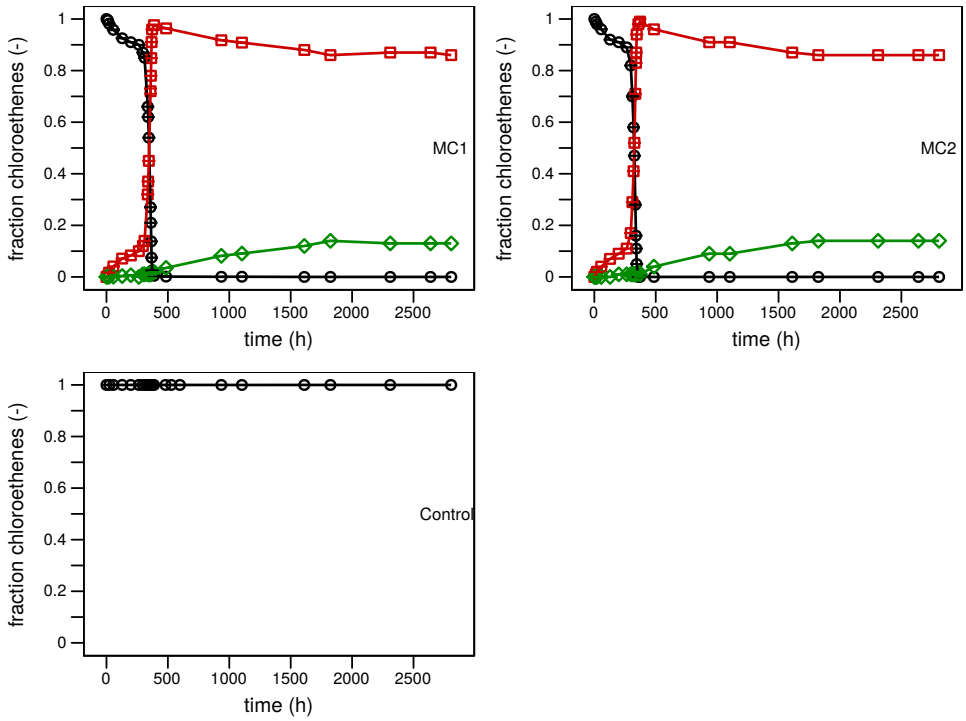


Figure C10. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-VI-HCC. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

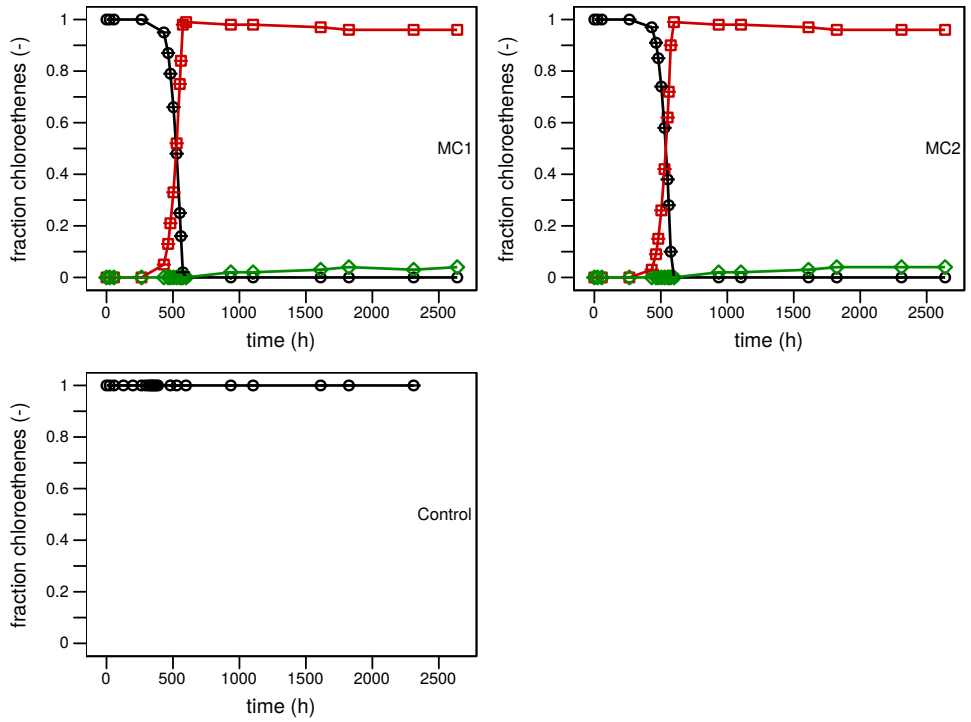


Figure C11. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-VI-LCC. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

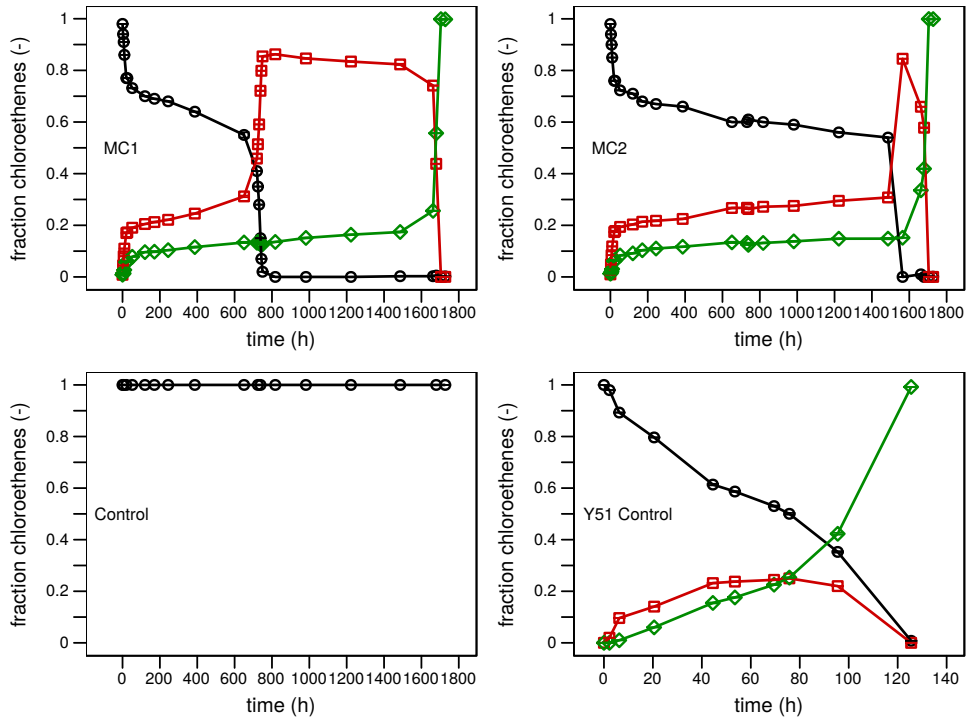


Figure C12. Fraction of chlorinated ethenes (PCE: ○, TCE: □, *cis*-DCE: ◇) versus time for the degradation experiment of RSP-VII. Error bars represent standard deviation of calculated fractions from GC-MS concentrations measurements (n=2).

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