Characterisation of microbial transformation of halogenated organic contaminants using compound-specific stable isotope analysis

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Abbreviations

AKIE	Apparent stable isotope effect
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
BvcA	Vinyl chloride reductive dehalogenase
CbrA	Chlorobenzene reductive dehalogenase
CSIA	Compound specific stable isotope analysis
DCA	Dichloroethane
DCE	Dichloroethene
EA	Elemental analyser
EPA	Environmental Protection Agency
GC-C-IRMS	Gas chromatograph-combustion-isotope ratio mass spectrometer
GC-P-IRMS	Gas chromatograph-pyrolysis-isotope ratio mass spectrometer
HCH	Hexachlorocyclohexane
IRMS	Isotope ratio mass spectrometer
KIE	Kinetic isotope effect
LC	Liquid chromatography
MCB	Monochlorobenzene
MTBE	Methyl-*tert*-butylether
PCE	Perchloroethene, tetrachloroethene
PceA	Tetrachloroethene reductive dehalogenase
PCR	Polymerase chain reaction

rdh	Reductive dehalogenase gene
SIP	Stable isotope probing
TAT	Twin-arginine translocation
Tce	Trichlorethene reductive dehalogenase
TCE	Trichloroethene
TLFA`	Total lipid fatty acid
VC	Vinyl chloride
VcrA	Vinyl chloride reductive dehalogenase
V-PDB	Vienna-PeeDee Belemnite

0. Pre-face

The advance in society as result of the industrial revolution has led to an increasing anthropogenic impact on the environment. As a result of the increasing number of applications and chemical production, as well as initial lack of awareness of the potential negative impacts on the environment and human health, the environment has been subject to exploitation and contamination. After awareness arose, efforts were made to understand the fate of contaminants in the environment. Soon the importance of microorganisms as key factors influencing the removal of, e.g. groundwater contaminants, was recognised. A reliable assessment of the contribution of biodegradation to the overall natural attenuation, however, is a challenge and methods and approaches were developed allowing addressing the *in situ* activity of microorganisms. These approaches require an understanding of biotransformation of contaminants, and the relevant environmental conditions supporting these activities *in situ*.

In this work, concepts based on the application of stable isotopes were developed and investigated. These are an alternative and can serve as complementary tools to common geochemical analysis, concentration analysis, microbiology and molecular biology and are discussed in relation to the biotransformation of common halogenated groundwater contaminants. The approaches developed allow the investigation of the *in situ* activity of microorganisms, both qualitatively and quantitatively, and were used to characterize the dehalogenation reaction in organohalide-respiring bacteria. This work focuses on the enrichment of microorganisms capable of organohalide respiration, determination of their substrate spectrum, the investigation of the relevance of these organisms *in situ* and the development of stable isotope approaches to detect *in situ* biotransformation of chlorinated ethenes and monochlorobenzene. The Habilitation thesis is based on 27 publications (see chapter 7 for the complete list) and the discussion includes further recent unpublished data.

1. Contamination of groundwater by organohalides & their biodegradation

a. Sources of halogenated organics in the environment

Halogenated organics have a widespread occurrence in nature and can be either of natural or anthropogenic origin. They are produced by certain marine sponges and algae, by fungi and

particular plants. These compounds are also a by-product of wood combustion and volcanic activity (Häggblom & Bossert, 2003, van Pee & Unversucht, 2003). Additionally, they have numerous applications in industry as intermediates in chemical syntheses, as solvents, e.g. in degreasing metals, as well as for specific applications including pest control, as pharmaceuticals, flame retardants or in personal healthcare products (Häggblom & Bossert, 2003, van Pee & Unversucht, 2003). Due to improper use or disposal, these chemicals have entered the environment as point source pollutants, e.g. chlorinated ethenes used in dry-cleaning, or as diffuse pollutants, e.g. γ-hexachlorocylohexane (γ-HCH, Lindane) when used as pesticides. Depending on their chemical structure and the environmental conditions, these chemicals can persist in the environment or can be subject to transformation via biotic or abiotic reactions. Due to their high toxicity, carcinogenic properties, persistence and abundance in the environment several of the chlorinated ethenes, ethanes and benzenes have been added to priority lists of the Environmental Protection Agency in the USA (EPA; http://water.epa.gov/scitech/methods/cwa/pollutants.cfm) and Annex II of the EU Groundwater Directive (http://ec.europa.eu/environment/water/water-dangersub/pri_substances.htm).

b. Contaminated field sites

Current estimates suggest alone in Europe a total of 2.5 million potentially contaminated sites, of which 340,000 are expected to be contaminated and likely require remediation (van Liedeke *et al.*, 2014). The most frequently observed contaminants in groundwater include heavy metals, mineral oil, BTEX (benzene, toluene, ethylbenzene and xylene) and chlorinated hydrocarbons (van Liedeke *et al.*, 2014). Within my work, three field sites are included which are, amongst others, highly contaminated with halogenated organic substances, including chlorinated ethenes, ethanes and benzenes. Material from these field sites, mainly groundwater, was used for various investigations. The Bitterfeld/ Wolfen field site in Germany is an example of a large-scale contaminated site with multiple contaminants resulting from a long and varied history of chemical industry (Wycisk, 2003, Heidrich *et al.*, 2004). Similarly, the investigated area around Ferrara, Italy, is characterised by multiple contaminants and field sites where halogenated compounds were observed, which likely result from historic dumping of industrial wastes or from modern applications (Molinari *et al.*, 2007, Pasini *et al.*, 2008). Additionally, a field site in northern Germany contaminated with mainly lower chlorinated benzenes, which resulted from an accident, was investigated (Thullner & Schäfer, 1999, Stelzer *et al.*, 2009).

c. Biotransformation of organohalides

Halogenated organic compounds can be subject to transformation by microorganisms under both anoxic and oxic conditions. Higher halogenated substances, such as tetrachlorethene (PCE) or hexachlorobenzene (HCB), are usually transformed under anoxic conditions via reductive dehalogenation, but persist under oxic conditions. In contrast, lower halogenated substances are degraded more readily under oxic conditions and initially, e.g. in the case of vinyl chloride (VC) or monochlorobenzene (MCB), were thought to persist under anoxic conditions.

Thus far, transformation of chlorinated substances has been researched in depth for some classes, e.g. chlorinated ethenes and benzenes (for a review, see e.g. (Adrian & Görisch, 2002, Holliger *et al.*, 2002, Smidt & de Vos, 2004) and the sections below). Furthermore, only limited information is available for the degradation of brominated substances, while, information regarding fluorinated and iodinated substances research is mostly lacking (Häggblom & Bossert, 2003) (Smidt & de Vos, 2004). In the following sections, the focus will be on the chlorinated ethenes, dichoroethane (DCA) and lower chlorinated benzenes, especially monochlorobenzene and their biotransformation under anoxic conditions, as occurs in contaminated aquifers.

i. Chlorinated ethenes and ethanes

Chloroethanes and -ethenes are commonly used solvents and have been, accidentally or intentionally, released into the environment where they have contaminated various ecosystems, especially groundwater aquifers. For example, 1,2-DCA as well as tetra- and trichloroethene (PCE and TCE) are common groundwater contaminants that are suspected carcinogens causing severe environmental problems (www.epa.gov). In these cases, the toxic VC and, nontoxic, ethene can both result from microbial transformation under anoxic conditions (Bradley & Chapelle, 2010).

Several groups of microorganisms have been described to use these compounds as terminal electron acceptors. PCE can be dechlorinated by step-wise hydrogenolysis via TCE, dichloroethene (DCE) and VC to the non-toxic product ethene (Figure 1) under strict anoxic conditions. While most organisms from e.g. the genus *Desulfitobacterium*, *Sulfurospirillum* or *Dehalobacter* are known to be able to dehalogenate PCE and TCE to mainly *cis*-DCE, microorganisms belonging to the genus *Dehalococcoides* are the only ones reported to be

capable of the complete dechlorination of the chlorinated ethenes to ethene (for a review see (Smidt & de Vos, 2004, Taş *et al.*, 2009)).

To date only few bacterial strains have been described to reductively dechlorinate 1,2-DCA via dihaloelimination to ethene and minor amounts of VC, and these include *Dehalococcoides mccartyi* strain 195 (formerly *Dehalococcoides ethenogenes* strain 195 (Maymo-Gatell *et al*. 1999)) and *Desulfitobacterium dichloroeliminans* strain DCA1 (Figure 1) (De Wildeman *et al*. 2003).

Alternatively, the oxidation of DCE and VC under anoxic conditions was reported, however, the related reactions and the microorgansims responsible were not elucidated (Bradley & Chapelle, 1997, Bradley *et al.*, 1998, Bradley *et al.*, 1998, Bradley & Chapelle, 2010). It should be noted that under oxic conditions, the lower chlorinated ethenes are degraded, while PCE and TCE persist. Thus, vinyl chloride, which is a product of organohalide respiration, is readily transformed when it enters oxic zones such as surface waters (Coleman *et al.*, 2002, Mattes *et al.*, 2005, Jin & Mattes, 2008).

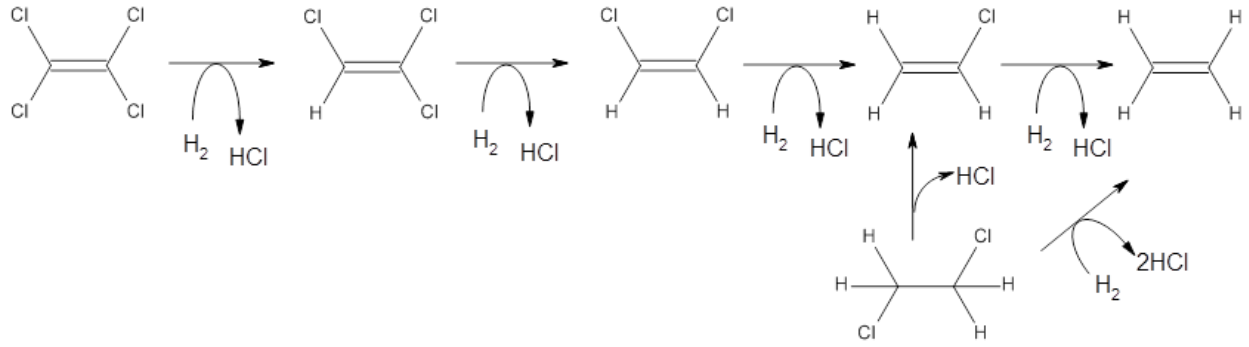

Figure 1: General pathways for reductive dechlorination of the chlorinated ethenes and 1,2-DCA.

ii. Chlorinated benzenes

Of the chlorinated benzenes, monochlorobenzene (MCB) is of the greatest concerns in contaminated aquifers, as it tends to accumulate and form long plumes as a result of its relatively high mobility and slow degradation. MCB is usually associated with contamination of higher chlorinated benzenes or hexachlorocyclohexane (HCH) (Figure 2) (Nowak *et al.*, 1996, Adrian *et al.*, 2000, Lal *et al.*, 2010).

Higher chlorinated benzenes are used during organohalide respiration by *Dehalococcoides mccartyi* strains as terminal electron acceptors in a similar way to the chlorinated ethenes, whereby tri- or dichlorobenzenes are the main products (Adrian *et al.*, 2000). More recently, the dechlorination of di- and monochlorobenzene was described for *Dehalobacter* spp. (Fung *et al.*, 2009, Nelson *et al.*, 2011). While the dechlorination of DCB supported growth, dechlorination of MCB appeared to be a co-metabolic conversion process. In addition to being a product of chlorobenzene dechlorination, MCB may also be a product of the biotransformation of hexachlorocyclohexane by anaerobic microorganisms (Boyle, *et al.*, 1999). For example, a *Dehalobacter* co-culture and several sulphate-reducing strains were shown to convert HCH into MCB and benzene as products (Boyle *et al.*, 1999, van Doesburg *et al.*, 2005). Under anoxic conditions, HCH is initially observed to be converted to the tetrachlorocyclohexene, then it is thought to proceed via a dichlorocyclohexadiene to MCB and benzene (Lal *et al.*, 2010).

The lower chlorinated benzenes can be subject to oxidation under oxic conditions, which is well documented (Agteren *et al.*, 1998), while degradation under anoxic conditions has thus far not described, although it has been hypothesised and is the subject of the work presented in this work.

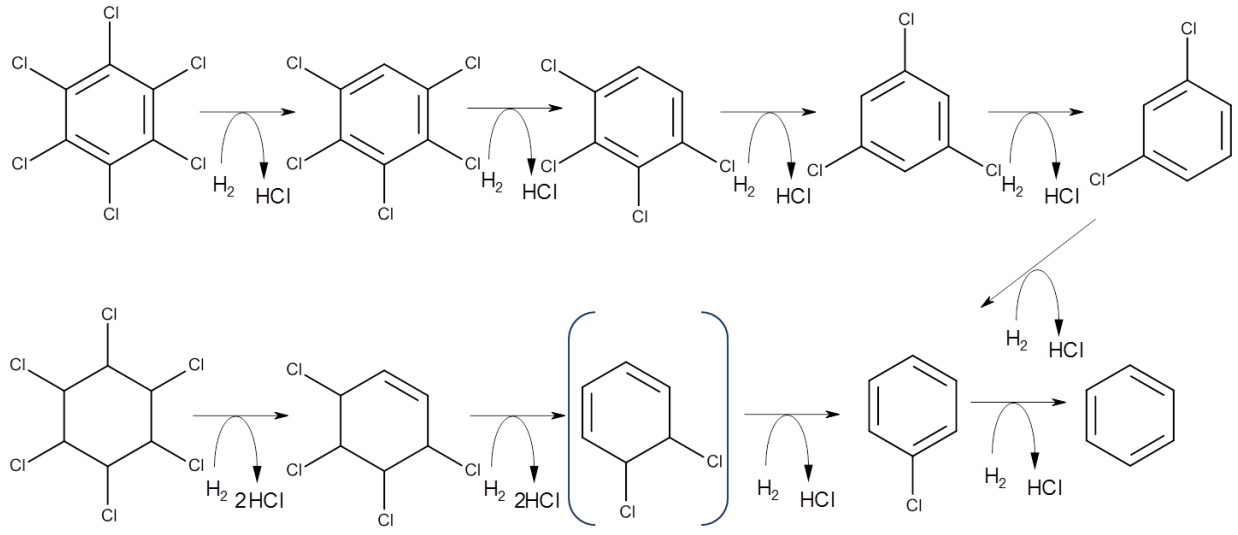

Figure 2. Reductive dehalogenation pathways for chlorinated benzenes (top) and hexachlorocyclohexane (HCH) (bottom). To date, the production of dichlorocyclodiene as intermediate from HCH has not been proven.

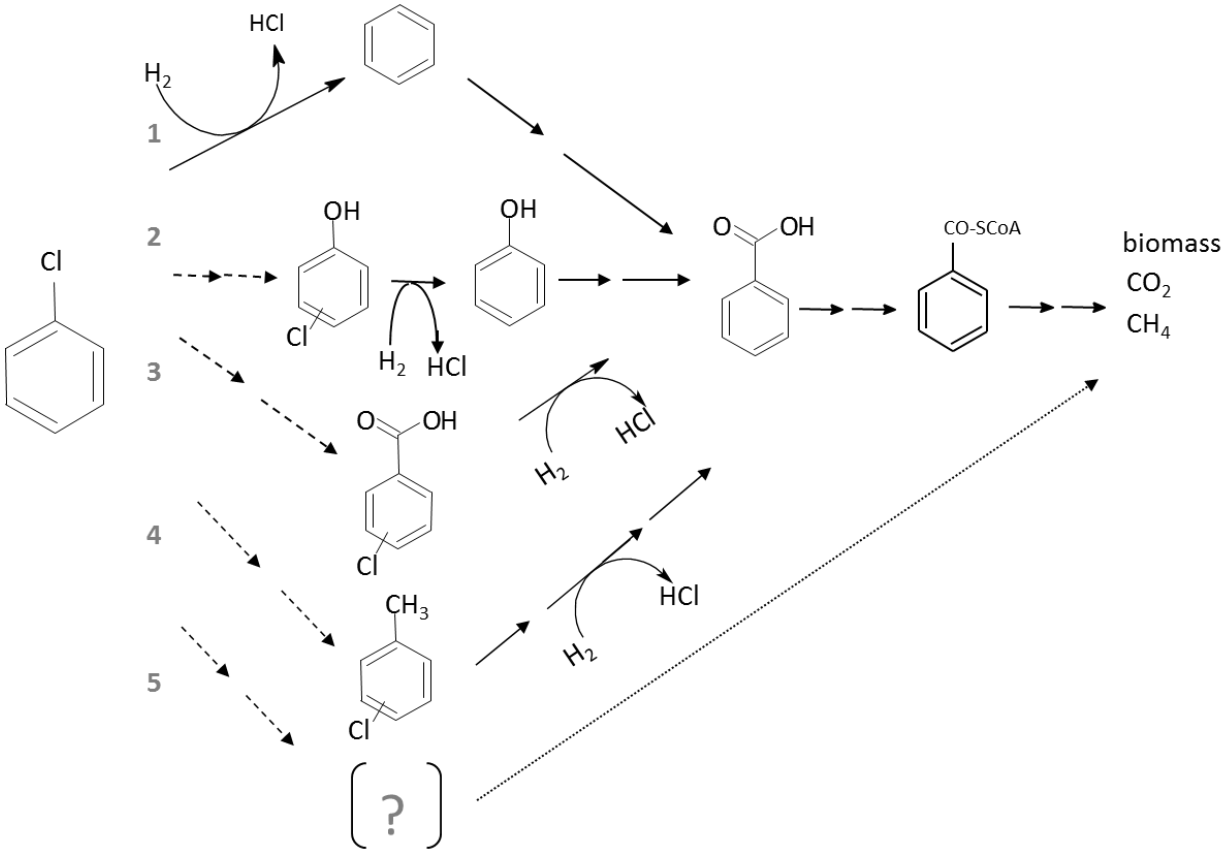

Figure 3. Observed and hypothetical degradation pathways for MCB via 1) initial dehalogenation to benzene, 2) hydroxylation to chlorophenol, 3) carboxylation to a chlorobenzoate, 4) methylation or 5) a novel intermediate. Solid arrows indicate observed reactions, dashed arrows hypothetical reactions.

For MCB, *in situ* removal under anoxic conditions may occur potentially via two principal pathways (Figure 3): dechlorination to benzene ((Nowak *et al.*, 1996, Fung *et al.*, 2009, Nelson *et al.*, 2011) with subsequent benzene degradation as described for sulfate, nitrate, iron and methanogenic conditions (Edwards *et al.*, 1992, Lovley, 2000, Phelps *et al.*, 2001, Chakraborty & Coates, 2005, Ulrich *et al.*, 2005). Indeed, by coupling these processes, degradation of chlorobenzene resulted in CO_2 and CH_4 in an artificial co-culture (Liang *et al.*, 2013). Moreover, as has been shown for benzene, MCB can be degraded via a direct attack on the benzyl ring with the formation of a substituted chlorinated aromatic intermediate such as a chlorinated phenol, benzoate or toluene. This intermediate could then be dechlorinated via reductive dechlorination, as described for chlorinated benzoate, to benzoate, e.g. by

Desulfomonile tiedje or for chlorinated phenols to phenol as described for *Desulfitobacterium* spp. or *Dehalococcoides* spp. (Mohn & Tiedje, 1990, van de Pas *et al.*, 1999, Löffler *et al.*, 2003, Adrian *et al.*, 2007). Alternatively, microorganisms such as *Thauera chlorobenzoica* can mineralise halogenated benzoates (Song *et al.*, 2001). Thus far, however, little information about the relevant pathways *in situ* is available.

iii. Enzymes and proposed reaction mechanism involved in biotransformation of organohalides

The dehalogenation reaction in organohalide-respiring microorganisms has been found to be catalysed by enzymes showing high similarity at the amino acid sequence level, the reductive dehalogenases (rdh) (for a review see (Smidt & de Vos, 2004, Taş *et al.*, 2009, Richardson, 2013)). The Rdhs that degrade PCE (PceA) were characterised in detail for several microorganisms including *Sulfurospirillum multivorans*, *Dehalobacter restrictus* and *Desulfitobacterium* sp. (Neumann *et al.*, 1995, Neumann *et al.*, 1996, Schumacher *et al.*, 1997, Miller *et al.*, 1998, Neumann *et al.*, 2002, Maillard *et al.*, 2003, John *et al.*, 2006). So far, only few Rdhs could be functionally identified in *Dehalococcoides* sp. e.g. the PCE dehalogenase PceA, the TCE dehalogenase, TceA, the vinyl chloride dehalogenases VcrA and BvcA or the chlorobenzene dehalogenase CbrA (Magnuson *et al.*, 1998, Magnuson *et al.*, 2000, Muller *et al.*, 2004, Adrian *et al.*, 2007, Tang & Edwards, 2013).

Generally, the Rdhs comprise a catalytic subunit, RdhA, and a membrane anchor subunit, RdhB (Richardson, 2013). The catalytic subunit has a twin-arginine transport (TAT)-pathway signal peptide and faces the outside of the cytoplasmic membrane because it is probably transported across the cytoplasmic membrane by the TAT machinery. Additionally, those investigated were observed to contain 2 iron sulphur clusters and a corrinoid cofactor. Corrinoids are important cofactors of Rdhs in organisms capable of organohalide respiration (Miller *et al.*, 1996, Neumann *et al.*, 1996, Miller *et al.*, 1998, Holliger *et al.*, 1999, Krasotkina *et al.*, 2001, Neumann *et al.*, 2002, Kräutler *et al.*, 2003, Maillard, 2003). The microbial dehalogenation by Rdhs has been proposed to proceed by alkylating a super-reduced corrinoid containing a Co(I) species at the reactive site with the chloroethene (Neumann *et al.*, 2002, Kräutler *et al.*, 2003). The chemical mechanism of reductive dehalogenation of chlorinated ethenes catalyzed by cobalamin has been the subject of previous studies and has been suggested to occur via a single electron transfer from a reduced cob(I)alamin leading to the formation of chloride and vinyl radicals as

intermediates (Glod *et al.*, 1997a, Glod *et al.*, 1997b, Holliger *et al.*, 1999, Shey & van der Donk, 2000, Shey *et al.*, 2002). Experiments involving chlorinated propenes suggest the involvement of a radical reaction mechanism in the case of *S. multivorans* (Schmitz *et al.*, 2007), however, the actual reaction mechanism for the chlorinated ethenes appears to differ and is still a subject of discussion (Bommer *et al.*, 2014, Payne *et al.*, 2014).

d. Methods for analysing microbial processes *in situ*

For the management of contaminated field sites, the evaluation of biodegradation and its contribution to natural attenuation is necessary. While other processes, such as sorption, dilution or evaporation, may lead to a decrease in contamination, and abiotic degradation usually is not a significant process under natural *in situ* conditions, biodegradation is the only sustainable process leading to contaminant removal from e.g. a contaminated aquifer. Currently, several methods are routinely available to analyse microbial processes in the environment (Table 1). Based on the recommendations of the United States Environmental Protection Agency (US EPA) (www.epa.gov, (Lu *et al.*, 2006, Wilson, 2010)), the decrease in contaminant concentration, an increase of metabolites and prevailing redox conditions are analysed. These, however, are not direct evidence for biodegradation as the concentration decrease may be due to other factors such as sorption or dilution, metabolites might be present as co-contaminants and redox processes, e.g. nitrate or sulphate reduction, might be linked to the biotransformation of other organic substances. Traditionally, cultivation of microorganisms has been used to investigate the biodegradation of specific substances of interest. While this allows the analysis of microbial processes in detail, including an understanding of the biochemistry and physiology, not all microorganisms of interest are available in axenic culture and the behaviour of a microorganism under laboratory conditions may not correspond to its activity *in situ*. Over the last few decades, however, cultivation-independent methods have been developed, which allow investigation of the microorganisms *in situ.* Molecular biological, e.g. PCR-based, methods allowing detection of the presence of a specific microorganism or genes of interest signifying a variety of environmental processes have been published. These approaches give information about the presence of a microbial group with known function although these methods cannot give direct information about the activity. Analysis of the complete environmental transcriptome or proteome is still currently challenging and can be hampered by non-specific expression of genes under stress conditions and isolation of target molecules from complex matrices, nonetheless

there are promising methods for the analysis of *in situ* processes (Chuang *et al.*, 2010, von Bergen *et al.*, 2013, Fletcher, 2011). It is, however, possible to deduce the specific activity and function of microorganisms *in situ* using a combination of the above approaches, e.g. by determining contaminant concentration and geochemical conditions. In the last decades, however, additional approaches and concepts have been developed that are based on the application of compound-specific stable isotope analysis (CSIA) including fractionation and tracer approaches, which allows a more direct investigation of *in situ* biodegradation (Meckenstock *et al.*, 2004, Whiteley *et al.*, 2006, Bombach *et al.*, 2010, Hofstetter & Berg, 2011, von Bergen *et al.*, 2013).

In the next chapters, these different approaches are briefly discussed in the context of the work of the author with a focus on the chlorinated ethenes, 1,2-DCA and MCB as groundwater contaminants.

Table 1: Currently available and commonly used methods for the evaluation of *in situ* biodegradation of contaminants (after recommendation of the EPA, www.epa.gov; (Bombach *et al.*, 2010).

Method	**direct/indirect proof**	**advantage**	**challenges**	**section 1.c.**
Contamination pattern	direct	easy to analyse (GC-FID/MS; HPLC etc)	co-contaminations, decrease of concentration due to sorption, dilution etc.	*i.*
Redox processes	indirect	easy to analyse (Iron, sulphate, nitrate etc)	process may be linked to other substrates	*i.*
Metabolite detection	direct	proof for pathway	Metabolite may be present as co-contaminant or as background substance from other processes	*i.*
Laboratory microcosms	direct evidence for the potential	direct evidence for the potential capacity of the *in situ* community	long time-frame; change of environment (laboratory vs. *in situ*)	*ii.*
Molecular biology	direct	rapid identification of organisms present	not an indicator of activity; only known organisms and genes can be detected	*iii.*
Compound-specific stable isotope analysis	direct	qualitative and potentially quantitative assessment of biodegradation	equipment not universally accessible and applicable complementary methods needed for quantitative assessment; reference laboratory studies not always available	*iv.; Chapter 2*
Application of *in situ* stable isotope tracers (BACTRAP)	direct	direct evidence for use as growth substrate (analysis biomass), pathways (metabolites, protein-SIP), identification of microbial community involved (DNA/RNA SIP)	only usable if the contaminant is used as the carbon source; still under development	*iv.; Chapter 2*

i. Geochemical and contaminant analysis

In the case of the chlorinated ethenes, PCE and TCE are usually the main contaminants with the DCEs and VC being possible co-contaminants or products of dehalogenation. The presence of DCE and VC alone, therefore, does not indicate the biotransformation of the chlorinated ethenes. However, the presence of ethene can be used as such an indicator. Another challenge may result from the presence of co-contaminants such as 1,2-DCA, which might result in the same degradation products, VC and ethene, so that it cannot be determined which contaminant is subject to biotransformation. The analysis of the different redox processes will aid in identifying whether the proper conditions for reductive dehalogenation are met, in particular if methane as result from methanogenesis, sulphide from sulphate reduction or ferrous iron from iron reduction, signifies the presence of strict anoxic conditions needed for activity of organohalide-respiring strains (Lu *et al.*, 2006). However, these redox processes may simultaneously compete with dehalogenation for the electron donors present (Fennell *et al.*, 1997).

At the field-site in Bitterfeld, anoxic conditions were apparently present as indicated by the presence of sulphide, ferrous iron and methane. In addition, besides PCE and TCE, the DCEs, VC and ethene were present as potential products (Nijenhuis *et al.*, 2007, Imfeld *et al.*, 2008, Imfeld *et al.*, 2011). The DCEs and VC were potential co-contaminants, however, ethene could be excluded as a co-contaminant, thus providing evidence for the reductive dechlorination of the chlorinated ethenes at some stage. It is not possible to determine, however, if dehalogenation was taking place as an active process on the date of sampling, which would require sampling at several different time points. Similarly, ethene was observed as product in the greater Ferrara area. However, VC was also thought to be a primary contaminant and cannot therefore be used as an indicator of dehalogenation (Pasini *et al.*, 2008, Nijenhuis *et al.*, 2013).

The identification of biodegradation of MCB is more complex using geochemical and contaminant indicators. MCB is usually a product of the dechlorination of higher chlorinated benzenes or hexachlorobenzene, accumulating in the environment. MCB might simultaneously be subject to biodegradation and production as well as be subject to potentially different types of biotransformation pathways. Therefore, providing evidence for the *in situ* biodegradation of MCB is a challenging task.

In the anoxic, mainly MCB, plume in Bitterfeld, anoxic conditions were indicated by the presence of sulphide and ferrous iron. Concurrently, concentrations of MCB decreased over the flow path, with minor amounts of benzene, suggesting a potential dechlorination pathway (Kaschl *et al.*, 2005). Under more defined conditions in a model wetland system, the decrease in MCB was concurrent with the mobilisation of iron (Schmidt *et al.*, 2014). These results suggest the biotransformation of MCB under iron- or sulphate-reducing conditions; however, due to the absence of products no direct proof for this proposal could be obtained. Additionally, the benzene detected could be a result of co-contamination (Heidrich *et al.*, 2004)

ii. Microcosm & cultivation of microorganisms capable of organohalide respiration

Traditional microbiological approaches such as the preparation of microcosms and/or plating provide the ultimate proof for biodegradation of a specific substrate and allow not only for the identification of microorganisms capable of performing a process, but also for the investigation of the physiology and biochemistry of a reaction. However, cultivation can be very tedious, with long incubation times and in many cases the microorganisms responsible for a process elude cultivation or can only be maintained in culture as part of a microbial consortium. Additionally, the cultivated microorganisms might not be the ones responsible for biodegradation *in situ* since it is difficult to replicate the field conditions in laboratory settings. In the case of the chlorinated ethenes, initially, it was thought dehalogenation could not proceed past *cis*-DCE until the discovery of the strictly anaerobic, microorganism responsible, which was *D. mccartyi* strain 195 (formerly *D. ethenogenes* strain 195) by Jim Gossett and Steve Zinder and co-workers (DiStefano *et al.*, 1991, DiStefano *et al.*, 1992, Maymo-Gatell *et al.*, 1995, Maymo-Gatell *et al.*, 1997). The microorganisms belonging to the *Dehalococcoides* genus turned out to be a group of organisms that are challenging to cultivate, requiring patience and persistence of the researchers to achieve successful growth. The first strain described to be capable of the dehalogenation of PCE to mainly VC, which was strain 195 (Maymo-Gatell *et al.*, 1997), was quickly followed by the identification of other strains such as strain CBDB1 (Adrian *et al.*, 2000), which is capable of dechlorination of chlorinated benzenes and strain BAV1 (He *et al.*, 2003), which is capable of growth with VC. Currently, there are detailed descriptions of *D. mccartyi* strains in the literature and the genomes of several of these have meanwhile been sequenced (Löffler *et al.*, 2013, Richardson, 2013).

D. mccartyi strain BTF08 was enriched from the Bitterfeld region, and was shown to be capable of the full metabolic dechlorination of the chlorinated ethenes to ethene (Cichocka *et al.*, 2010). Investigation of its substrate spectrum showed that it could dechlorinate hexa-, penta and tetra-chlorobenzenes to trichlorobenzene and γ-hexachlorocyclohexane (γ-HCH; Lindane) to monochlorobenzene. Moreover, it can dehalogenate 1,2-DCA to ethene (Kaufhold *et al.*, 2013). Its genome sequence was annotated and compared to strain DCMB5, which was isolated from Spittelwasser sediments (Bunge *et al.*, 2008, Pöritz *et al.*, 2013). The genome of strain BTF08 encodes 20 Rdhs, and is the first example of a genome containing all three genes (*pceA*, *tceA* and *vcrA*) encoding enzymes necessary to couple the complete reductive dechlorination of PCE to growth. The *tceA* and *vcrA* genes encode Rdhs specific for trichloroethene and vinyl chloride, respectively, and these are located within mobile genetic elements suggesting their recent acquisition via horizontal gene transfer. Interestingly, *Dehalococcoides* spp. were also identified as important key players *in situ* for the removal of halogenated contaminants such as the chlorinated ethenes, as indicated in the next section.

Very recently, it could be demonstrated that *Dehalobacter* spp. are capable of dechlorinating the lower chlorinated benzenes, DCB and MCB; however, although the reduction of DCB to MCB was metabolic, the further dechlorination of MCB appeared to be co-metabolic (Fung *et al.*, 2009, Nelson *et al.*, 2011). Our initial investigations involving MCB were hampered due to abiotic loss of the compound and the lack of detectable products (Nijenhuis *et al.*, 2007). Application of stable isotope (^{13}C) tracers, however, demonstrated that a very slow biotransformation of the molecule occurred (see Chapter 2) (Nijenhuis *et al.*, 2007, Stelzer *et al.*, 2009). Recent microcosm investigations suggest the presence of parallel pathways for both dechlorination and oxidation *in situ* and benzene could be identified as a direct product from MCB (Markantonis, 2014). A next step would be to isolate the microorganisms with these capabilities and to determine the activity and relevance of the isolated strains *in situ* e.g. using molecular biological approaches.

iv. Molecular biology

Over the last fifteen years molecular biological methods have been applied for the detection of microorganisms capable of specific dechlorination reactions. PCR methods were developed for the detection of taxa of organohalide-respiring microorganisms as well as for the detection of putative Rdh genes (Löffler *et al.*, 2000, Hendrickson *et al.*, 2002, Amos *et al.*, 2007).

Furthermore, fluorescence *in situ* hybridisation (FISH) and microarray approaches have been developed (Aulenta *et al.*, 2004, Dijk *et al.*, 2008, Matturro *et al.*, 2013). While the PCR-based approaches are now routinely applied and indeed have been developed further, FISH detection of *Dehalococcoides* spp. is hampered by the low copy number of 16S rRNA in each cell as well as by the low cell numbers *in situ*. Their detection requires specialised equipment and microarray analysis is currently mainly applied using laboratory cultures (Hug *et al.*, 2011, Lee *et al.*, 2012, Matturro *et al.*, 2013, Men *et al.*, 2013, West *et al.*, 2013).
Meanwhile PCR detection is used routinely for the qualitative and quantitative detection of microorganisms or genes. These approaches are mainly DNA-based as extraction of RNA from environmental samples quantitatively is highly challenging and stress conditions were found to affect the level of RNA transcribed (Fletcher *et al.*, 2011).

At the field site in Bitterfeld, a taxon-specific PCR approach indicated the presence of *Dehalococcoides*, *Desulfuromonas*, and *Dehalobacter*, which was confirmed by sequencing (Nijenhuis *et al.*, 2007, Imfeld *et al.*, 2008); however, this method does not allow to identify if the microorganisms are metabolically active *in situ*. Moreover, as *Dehalococcoides* spp. were found to be highly similar based on 16S rRNA gene sequence, but highly variable in their inventory of putative Rdh genes, the presence of *Dehalococcoides* does not allow any inferences to made regarding their respective activities (Taş *et al.*, 2009). Therefore, functional gene-specific approaches should be implemented to improve the analysis. Indeed, an analysis of the 16S rRNA gene sequence of *Dehalococcoides* spp. in groundwater samples isolated from a model wetland system and microcosms all derived from different locations at the same field site in Bitterfeld, confirm the uniform presence of a single *Dehalococcoides* sp. based on 16 rRNA gene sequence. Nevertheless, the analysis of vinyl chloride reductase genes, *vcrA* and *bvcA*, suggested a diversity of not only these genes in the samples but also the inventory of the specific *Dehalococcoides* sub-species (Mészáros *et al.*, 2013). At this field site, *tceA*, which encodes TCE dehalogenase, was not found although *D. mccartyi* strain BTF08, which is capable of TCE dehalogenation, could be isolated from these samples and corresponding dechlorination activity was observed in microcosms (Cichocka *et al.*, 2010, Kaufhold *et al.*, 2013, Mészáros *et al.*, 2013). The subsequent sequencing of the strain (Pöritz *et al.*, 2013) confirmed the presence of the *tceA* gene indicating that the PCR approach was not effective in this case as a means of

detecting the presence of the bacteria. This is possibly due to the presence of multiple non-specific binding sites for one of the primers used (Mészáros *et al.*, 2013). Therefore, great care must be taken when interpreting environmental data and the failure to detect a gene does not necessarily indicate the absence of that gene. Several approaches should be applied to confirm the data. Furthermore, application of the molecular biological tools described here is only possible if a specific microorganism or gene has been clearly identified to be uniquely responsible for a specific process. Thus, for example, methods for the analysis of anaerobic MCB degradation are not yet available. By combining the approaches discussed above, it is possible to obtain qualitative indicators for *in situ* biodegradation; however, a quantitative analysis still requires the development of additional tools, e.g. by using stable isotope fractionation concepts.

iv. *Isotope tracer and fractionation approaches*

In the last decades stable isotope approaches have been developed for the assessment of *in situ* biodegradation. Two main types of approaches have been developed. The first applies stable isotope tracers and the second employs the concept of stable isotope fractionation.

Due to the naturally low abundance of the heavier stable isotope, e.g. carbon has a ^{12}C:^{13}C ratio of 98.9:1.1, stable isotopes can be applied in a tracer approach. Thus, using a ^{13}C-labelled substance product formation can be followed, intermediates can be identified and additionally, due to the incorporation of ^{13}C into biomass, the microorganisms and enzymes responsible for the biotransformation can be identified using total lipid fatty acids (TLFA), DNA-, RNA- or protein-stable isotope probing (SIP) approaches.

Stable isotope fractionation approaches rely on the higher activation energy and resulting lower reaction rates of compounds with the heavier stable isotope (^{13}C, ^{2}D) compared to the lighter stable isotope (^{12}C, ^{1}H). This results in an enrichment of the heavier stable isotope in a residual fraction of a substance subject to biodegradation. At the same time, the product is depleted from the original heavier stable isotope fraction, which proves to be an excellent indicator for transformation processes. In the following sections, these approaches are described in more detail in the context of the research of the author and the current literature.

2. Stable isotope tracers

a. Theory and current applications

In recent years, the range of applications involving stable isotope tracers for the analysis of microbial degradation processes has been greatly extended and currently includes the analysis of: i) products and metabolites; ii) phospholipids fatty acids; iii) nucleic acids (RNA or DNA); and iv) proteins (Kästner *et al.*, 2006, Stelzer *et al.*, 2006, Neufeld *et al.*, 2007, Whiteley *et al.*, 2007, Bombach *et al.*, 2010, Jehmlich *et al.*, 2010). These stable isotope probing (SIP) methods have the advantage of providing direct proof for the existence of particular anabolic or catabolic pathways with a specific substrate, or the identity of the microorganisms and enzymes involved in a process. However, as a prerequisite, the substrate must be used as a carbon source. Approaches where a co-substrate for growth e.g. acetate or ammonium have also been used but in such cases the overall activity of the microbial community is identified, but not the specific community responsible for the biodegradation (Kittelmann & Friedrich, 2008a, Kittelmann & Friedrich, 2008b) (Siegert *et al.*, 2013). For example, the involvement of *Dehalococcoides*-related microorganisms in the dehalogenation of PCE in a tidal flat and river sediments was suggested by incorporation of the carbon derived from ^{13}C-labeled acetate into the biomass (Kittelmann & Friedrich, 2008a, Kittelmann & Friedrich, 2008b). Substrates investigated using SIP approaches over the last years included common groundwater contaminants such as BTEX and MTBE (Lueders *et al.*, 2004, Bombach *et al.*, 2010, Herrmann *et al.*, 2010, Winderl *et al.*, 2010, Bastida *et al.*, 2011, Bozinovski *et al.*, 2012), (Morasch *et al.*, 2007). In the case of the chlorinated ethenes, which are used as electron acceptors and not as carbon sources, stable isotope tracers are generally not applicable.

Stable isotope tracer approaches have, however, proven to be a valuable tool for the investigation of biodegradation of MCB (Nijenhuis *et al.*, 2007, Stelzer *et al.*, 2009, Martinez-Lavanchy *et al.*, 2011, Schmidt *et al.*, 2014). While the decrease in concentration of MCB (see section 1.c. ii) suggested its removal from the contaminated aquifer in Bitterfeld, no direct evidence was available supporting on-going microbial degradation. Because the carbon derived from MCB, either in the form of MCB directly or as result of its dechlorination via benzene, might potentially be incorporated into microbial biomass, several approaches employing stable isotope

tracers have been applied both in the laboratory and using *in situ* approaches to test this hypothesis.

b. Laboratory studies

In laboratory studies, stable-isotope experiments using [$^{13}C_6$]-labelled MCB were applied to investigate its mineralisation after it was observed that, when using standard methods, the concentration of MCB decreased due to abiotic processes such as through sorption. This hampered obtaining a direct proof of its biodegradation. Groundwater from the field-site in Bitterfeld containing mainly MCB was used and $^{13}CO_2$ production was detected. Furthermore, investigation of the total lipid fatty acids in these microcosms confirmed the incorporation of MCB-derived carbon into biomass (Nijenhuis *et al.*, 2007). Similarly, mineralisation of MCB was shown in groundwater from a field-site in northern Germany (Stelzer *et al.*, 2009). However, in both cases, the groundwater was directly used from the field site and the specific redox process linked to MCB mineralisation was not identified. Therefore, the conditions which supported MCB mineralisation were subsequently investigated by amendment of specific substrates (Schmidt *et al.*, 2014). Addition of iron oxides or nitrate significantly stimulated MCB mineralisation, as indicated by the increased $^{13}CO_2$ production, while sulphate had no effect. No indications for the potential reductive dehalogenation of the chlorinated benzenes MCB or DCB could be obtained in this investigation, suggesting that dechlorination of MCB was not an important process or the resulting product, benzene, was degraded relatively quickly. Indeed, the microbial community in the groundwater was capable of the mineralisation of benzene, a potential end product of chlorobenzene dechlorination, in the presence of ferric iron, nitrate and sulphate (Schmidt *et al.*, 2014).

Additionally, cultivation-independent analyses combined with SIP were applied to study the structural diversity of the bacterial community, which developed in groundwater-inoculated microcosms actively metabolising monochlorobenzene (MCB) under anoxic conditions (Martinez-Lavanchy *et al.*, 2011). Nine distinguishable contributors to the single-strand conformation polymorphism (SSCP)-profiles were characterised by DNA sequencing. These data revealed the presence of different members from the phyla *Proteobacteria*, *Fibrobacteres* and from the candidate division OD1, which potentially contributed to the MCB degradation (Harris *et al.*, 2004, Ransom-Jones *et al.*, 2012). Therefore, DNA-SIP was applied to distinguish

the actual MCB-metabolising bacteria from the other community members. Unfortunately, this approach did not allow identification of a specific degrading community member due to the low ^{13}C incorporation and the long incubation times in addition to likely post-contamination complications. As well as demonstrating the limitations of SIP approaches to detect bacteria that slowly metabolize carbon sources under anaerobic conditions, the study nevertheless revealed for the first time the structural diversity of an anaerobic MCB-metabolising bacterial community (Martinez-Lavanchy *et al.*, 2011).

c. *In situ* (BACTRAPs)

In addition to laboratory approaches, development of *in situ* microcosm approaches involving the application of a ^{13}C-labelled contaminant in recent years has facilitated the detection of microbial activity directly in a contaminated aquifer (Kästner *et al.*, 2006). These *in situ* microcosms, also called 'BACTRAP®s' ('bacterial traps'), were developed and applied to a variety of substrates including BTEX, MTBE or PAH (Bombach *et al.*, 2010). BACTRAPs can also be used to provide evidence for the *in situ* biodegradation of halogenated substrates, e.g. in instances where microorganisms use carbon derived from MCB to generate biomass. These systems consist of a sorbent material, e.g. activated charcoal, onto which the substrate of interest is adsorbed. The BACTRAPs are then subsequently incubated directly in the environment of interest, e.g in a groundwater monitoring well or directly in the sediment (Kästner *et al.*, 2006, Bombach *et al.*, 2010). The biomass developed on the sorbent material *in situ* can be extracted after incubation and the total lipid fatty acid (TLFA), metabolite, DNA, RNA or protein content and composition can be analysed.

Thus far, the TLFA fraction has mainly been analysed to demonstrate incorporation of contaminant-derived carbon into biomass (Bombach *et al.*, 2010). Application of BACTRAPs and the subsequent analysis of TLFA delivered evidence for complete degradation of MCB at the contaminated aquifers in Bitterfeld and at a field site in northern Germany (Nijenhuis *et al.*, 2007, Stelzer *et al.*, 2009). In both cases, the total lipid fatty acid fraction extracted from the sorbent material of the BACTRAP was significantly enriched in ^{13}C indicating that the microorganisms used the contaminants as a carbon source for growth.

Incubation of these systems in different zones of a plume or by amendment of the BACTRAP material with a potential electron acceptor might provide further insights into the biodegradation

of a specific contaminant. For example, the incubation of these systems with MCB in a sulphate- vs. nitrate-reducing zone within the same plume allowed assessment of the similarities and differences in the microbial community and the overall activity of that community (Martinez-Lavanchy *et al* in prep.). Incubation of BACTRAPs within a model wetland system further allowed analysis of the effect of iron oxides on the microbial activity and preliminary results suggest indeed that the stimulation of MCB degradation by iron oxides *in situ* occurs, as indicated by the increase in the overall biomass as well as the ^{13}C-content in the biomass (Wolfram *et al* in prep.). These results confirm the link between MCB degradation with the iron cycle observed in laboratory microcosms (Schmidt *et al.*, 2014). Future SIP analyses might potentially allow identification of specific microorganisms and possible key enzymes. However, this could be hampered by the low amount of biomass formed on the *in situ* microcosms, as well as due to the problematic extraction of proteins and nucleic acids in the presence of a sorbent such as activated charcoal. In all these cases, however, [$^{13}C_6$]-benzene could be identified as a potential metabolite, although at this stage the levels were not significantly higher compared to the amount present as contamination within the commercially available [$^{13}C_6$]-MCB. This precludes any conclusions being drawn at this stage about the pathway involved in MCB degradation.

Although these approaches allow the direct identification of microbial activity, the processes cannot be quantified because the described *in situ* microcosms function as an open system. Provided certain requirements are taken into account, however, approaches based on compound-specific stable isotope analysis and stable isotope fractionation might allow not only quantification but also an identification of the underlying reaction mechanisms. This is described in the next chapter.

3. Stable isotope fractionation concepts

a. Background

During the last decade, stable isotope fractionation concepts have been developed that allow qualitative and quantitative assessment of the *in situ* biodegradation, source identification and analysis of the reaction mechanism of organic contaminants. Currently, concepts are available

for the common groundwater contaminants such as BTEX, MTBE and chlorinated ethenes (Meckenstock *et al.*, 2004, Elsner *et al.*, 2005, Bombach *et al.*, 2010).

These concepts and applications were made possible due to the development of compound-specific stable isotope analytical methods, which include currently GC-C/P-IRMS (gas chromatography – combustion/pyrolysis – isotope ratio mass spectrometry) (Aelion *et al.*, 2010). After separation of the substances in a sample via gas or liquid chromatography, the substance is converted to a simple gas: e.g CO_2 for C isotope analysis or H_2 for hydrogen stable isotope analysis. These are then separated by mass difference (Aelion *et al.*, 2010). Principally, GC-amenable groundwater contaminants such as BTEX, chlorinated ethenes or MTBE can be analysed using this approach; however, there are some limitations. For example, the presence of a halogen in a substance leads to the formation of an acid, i.e. HCl, during pyrolysis, which prevents the routine analysis of hydrogen isotope composition of organohalides, although progress is being made in this field (Chartrand *et al.*, 2007, Kuder *et al.*, 2013). More recently, LC-C-IRMS methods based on a pre-separation using liquid chromatography have been developed and these allow the analysis of polar substances. Although polar substances can be made amenable for GC-analysis by derivatisation, introduction of carbon or hydrogen into a molecule is undesirable, as it must be ascertained that the derivatisation does not result in an isotope effect. Moreover, later adjustments are needed to correct for the dilution of the isotope signal. The LC approaches should allow the analysis of substance classes currently listed as 'emerging contaminants' such as pharmaceuticals and polar pesticides.

Overall, the analysis requires well separated peaks of substances and a very low background. As all carbon entering the combustion reactor is converted without discrimination, poor separation and high background lead to the determination of an isotope signature derived from mixing of compounds. Therefore, the analysis of a specific compound from a complex matrix, e.g in the case of groundwater from a contaminated field-site, can be challenging.

Currently, methods are well developed for carbon isotope analysis of halogenated substances. However, methods for determination of Cl and H are still challenging and are not yet available for routine analysis. Therefore, the following sections will mainly focus on carbon stable isotope analysis with some specific examples including Cl isotope analysis.

b. Theory & definitions

The carbon stable isotope composition is reported as $\delta^{13}C$ ‰ vs. an international standard, i.e. Vienna-PeeDee Belemnite (V-PDB) (Coplen *et al.*, 2006) as follows:

Equation 1: $$\delta^{13}C = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} * 1000$$

where R_{sample} corresponds to the $^{13}C/^{12}C$ ratio determined via IRMS and R_{standard} is the international standard. The standardisation allows comparison of isotope signatures between different laboratories and experiments and each GC analysis is standardised by using an internal reference CO_2 peak with known isotope composition. Additionally, due to the small differences that are analysed and other potential analytical problems, laboratories have routinely started to use compound-specific standards to improve quality control.

Compound-specific stable isotope analysis (CSIA) can be used to obtain information on sources, transport routes, degradation pathways and sinks of organic chemicals in the environment. Multi-element isotope fingerprinting of chemically complex substances can also be used to analyse transformation pathways. This makes use of isotope fractionation processes altering the reactive position, as well as in the analysis of the isotope composition of reactive and non-reactive entities of an organic molecule to track sources. The microbial or chemical degradation of contaminants leads to kinetic isotope fractionation processes, which may alter the isotope composition of organic chemicals. Kinetic isotope fractionation is a process that separates stable isotopes from each other by their mass during unidirectional reactions resulting in an enrichment of usually the heavy stable isotope in the residual, non-reacted, fraction of a chemical being degraded (Northrop, 1981). Kinetic isotope fractionation characterises the transition-state of a chemical bond cleavage and provides opportunities to analyse the reaction mechanism (Northrop, 1981, Paneth, 2003, Dybala-Defratyka *et al.*, 2008). The Rayleigh model (see section 3.e) can be used to relate changes in isotope composition to changes in concentration under closed system conditions making use of the isotope enrichment factor (ε). The latter allows quantification of degradation processes *in situ* (Sherwood Lollar *et al.*, 2001, Richnow *et al.*, 2003, Griebler *et al.*, 2004, Morrill *et al.*, 2005). These concepts offer opportunities to analyse quantitatively the fate of organic compounds in the environment.

BOX I: Definitions:
Isotopes are forms of the same chemical element having different atomic masses as variation in the number of neutrons.
Stable Isotopes are isotopes which are not radioactive
CSIA compound-specific stable isotope analysis
Isotope forensics encompasses the application of isotope methods to determine the origin of bioorganic and anthropogenic organic compounds in the environment as well as to trace reactive transport processes governing persistence. For example, isotope analysis permits the authentication of chemicals and identification of producers important for the 'polluter-pays' principle of the EU.
Stable isotope fractionation is the process in which one of the stable isotopes is enriched relative to the other during a (bio) chemical or physical process. Due to the different reaction kinetics, light isotopes usually react relatively faster compared to the heavy ones resulting in an enrichment of heavy stable isotopes in the residual, non-degraded, fraction of a compound.
Multi-element isotope fingerprinting is the parallel analysis of the stable isotope ratios for several elements within the same molecule: for example the analysis of $^{13}C/^{12}C$ and $^{1}H/^{2}D$ ratios in toluene. Multi-isotope analysis allows the identification of reaction mechanisms and may permit the parallel assessment of (bio)transformation and source identification. During production, synthesis pathways and starting materials will leave a typical isotope signature in chemicals, allowing delineation of sources.

c. Stable isotope composition as an indicator for sources & production lines

Although the chemical structure of any organic contaminant provides information on the chemical identity, which allows deduction of its synthetic pathway (Ertl *et al.*, 1998, McRae *et al.*, 1999, Shouakar-Stash *et al.*, 2003), the origin of a chemical is often not clear since worldwide production and complex distribution pathways result in many potential production sites. In contrast, the stable isotope composition of defined chemical substances reveals specific information on the substrates used for synthesis as well as the pathways employed for production. These typical isotope patterns offer the opportunity to fingerprint the origin of chemicals, and trace the production source (McRae *et al.*, 1999) especially when using multiple isotopic elements bound within the structure of chemicals (Shouakar-Stash *et al.*, 2003). In case chemicals are produced by different production pathways or when different ground substrates were used for the synthesis, typical isotope signatures may result representing different producers and/or production periods. The typical isotope signatures then may allow identifying responsible polluters.

For example, at a production area in the Po region of Italy (Ferrara), chloromethanes were formerly produced from methane with chloroethenes as by-product. These were subsequently discarded at municipal dumps resulting in severe groundwater contamination (Pasini *et al.*, 2008,

Gargini *et al.*, 2011). Moreover, industry used chlorinated ethenes in their production processes and these chlorinated ethenes were derived from modern production lines using petrochemical substrates for synthesis. As both the methane and the petrochemical compounds used have typical isotope signatures with $\delta^{13}C$ = <-60 and -20 ‰, respectively, the resulting isotope signatures of the chlorinated ethenes were expected to be distinct depending on production process (van Warmerdam *et al.*, 1995, Whiticar, 1999, Doherty, 2000, Doherty, 2000). In the area surrounding Ferrara several contamination hot-spots were found, which could have resulted from either historical dumping of wastes or as spillage of solvents from modern industrial activity. However, for some of these contaminations it was not clear which was the source. The determination of isotope signatures of the chlorinated contaminants including the chlorinated ethenes, ethanes and methanes allowed the identification of the different types of sources and pinpointed the original source of contamination. The typical isotope signatures were approximately between -20 and -30 ‰ for the modern contamination vs. highly depleted with $\delta^{13}C$ = < -60‰ for the historic production of the chlorinated ethenes as a by-product of chloromethane synthesis (Shouakar-Stash *et al.*, 2009, Nijenhuis *et al.*, 2013).

d. Stable isotope composition as indicator for in situ biodegradation

Over the last decade, concepts based on carbon stable isotope analysis of chlorinated compounds have been developed to assess whether biotransformation has contributed to the removal of these contaminants *in situ*. To develop this approach, laboratory and field evaluations were performed (Hunkeler *et al.*, 1999, Sherwood Lollar *et al.*, 1999, Bloom *et al.*, 2000, Sherwood Lollar *et al.*, 2001, Meckenstock *et al.*, 2004, Mundle *et al.*, 2012) (Vieth *et al.*, 2003). These initial field investigations confirmed that enrichment in carbon stable isotope composition could be used as an indicator for biodegradation for PCE (Hunkeler *et al.*, 1999, Vieth *et al.*, 2003). Indeed, at the field sites in Bitterfeld, Northern Germany and Ferrara distinct patterns of the carbon isotope composition not only of the chlorinated ethenes but also of lower chlorinated benzenes could be identified, suggesting *in situ* biodegradation (Kaschl *et al.*, 2005, Nijenhuis *et al.*, 2007, Imfeld *et al.*, 2008, Stelzer *et al.*, 2009, Imfeld *et al.*, 2011). For the chlorinated ethenes, an enrichment could be observed for PCE and a corresponding ^{13}C-depleted ethene was found concurrently. Similarly, an enrichment was observed for the chlorinated benzenes suggesting *in situ* biodegradation. However, due to the potential simultaneous production and degradation of TCE,

DCE and VC, the stable isotope composition of these metabolites was not always easy to interpret.

Because of the different extents of isotope fractionation, TCE can become more enriched in ^{13}C compared to PCE during a sequential dehalogenation process (Nijenhuis *et al.*, 2005). Therefore, to examine the overall removal of halogenated contaminants, the calculation of an average isotope composition of all the substances in a dehalogenation sequence, e.g. the chlorinated ethenes or benzenes, must be applied:

Equation 2:
$$\delta^{13}C_{avg}(‰) = \frac{\Sigma(C_i \times \delta^{13}C_i)}{C_{sum}}$$

where the $\delta^{13}C_{avg}$ is the calculated average isotope composition of all compounds in a sequence, $\delta^{13}C_i$ and C_i are the isotope composition and concentrations, respectively, of each individual compound (i) and C_{sum} is the sum concentration of all compounds in a sequence. In the case of a closed system, and the inclusion of all parent and daughter products in a dehalogenation sequence, the $\delta^{13}C_{avg}$ is expected to remain constant at different time-points or locations within a contaminant plume. When the average isotope signature becomes enriched, the overall degradation or production of an unknown product is implied. For example, at a field site in Northern Germany analysis of the average isotope composition of chlorinated benzenes suggested that dehalogenation to benzene, transformation to other products or overall mineralisation had occurred (Stelzer *et al.*, 2009). Similarly, the average isotope composition of chlorinated ethenes in the Ferrara region suggested a complete degradation, beyond VC and ethene (Nijenhuis *et al.*, 2013).

e. Quantification of biodegradation

CSIA may also be used to quantify the *in situ* biodegradation. However, some limits and prerequisites need to be taken into account. The quantification of biodegradation requires knowledge of e.g. the groundwater flow path, the predominant degradation pathway *in situ*, as well as the respective corresponding extent of isotope fractionation, usually expressed as the enrichment factor ε or the isotope fractionation factor α where ε (‰) = (α-1)*1000. In the next sections, only the enrichment factor ε is used.

The carbon isotope enrichment factor (ε_c) expresses the extent of isotope fractionation, which can be determined from the linearized logarithmic form of the Rayleigh equation (Meckenstock *et al.*, 2004):

Equation 3: $$\ln \frac{(\delta_t{}^{13}C+1)}{(\delta_0{}^{13}C+1)} = \varepsilon_c \ln \frac{C_t}{C_0}$$

Plotting $\ln(C_t/C_0)$ versus $\ln[(\delta_t{}^{13}C+1)/(\delta_0{}^{13}C+1)]$ yields ε_c from the slope of the linear regression ($m = \varepsilon_c$). Since carbon isotope enrichment factors are typically small, ε_c-values are reported as parts per thousand (‰).

Conventionally, the extent of biodegradation (B) is estimated by using the observed concentrations at different times t (or location x):

Equation 4: $$B[\%] = \left(1 - \frac{C_t}{C_o}\right) \times 100$$

However, this approach includes processes like adsorption or dilution. To exclude these physical processes, the fraction of biodegraded contaminant *in situ* can be estimated by using the modified Rayleigh equation (Meckenstock *et al.*, 2004):

Equation 5: $$B(\%) = \left[1 - \left(\frac{R_t}{R_0}\right)^{\left(\frac{1}{\varepsilon}\right)}\right] \times 100$$

Here, R_t and R_0 represent $(\delta_t{}^{13}C+1)$ and $(\delta_0{}^{13}C+1)$, respectively. As mentioned above, the enrichment factor is usually determined in laboratory studies. Thus far, values are available for several groundwater contaminants including the chlorinated ethenes, BTEX, MTBE as well as some pesticides (Meckenstock *et al.*, 2004, Elsner *et al.*, 2005, Aelion *et al.*, 2010), and these can be used to perform a quantitative assessment. However, the selection of an enrichment factor requires detailed information about the prevailing redox conditions and processes *in situ* or the active microorganisms. For example, in the case of toluene the concurrent redox processes should be determined to select an appropriate factor (Morasch *et al.*, 2002, Morasch *et al.*, 2004)

while for chlorinated ethenes knowledge of the microbial community involved in degrading the compound is essential due to the observed variability in carbon isotope fractionation (Lee *et al.*, 2007, Cichocka *et al.*, 2008) (see section 3.g for a detailed discussion).

The isotope enrichment factors for PCE and TCE were observed to be highly diverse when a range of organohalide-respiring microorganisms was tested; the range was from not significant to -16.7±4.5 ‰ for PCE and -3.3 to -20‰ for TCE. This variability appeared to be dependent on the microbial genus, and partially on the strain (see also below, sections 3.g) (Cichocka *et al.*, 2007, Lee *et al.*, 2007, Cichocka *et al.*, 2008). In contrast, carbon isotope fractionation of the dichloroethenes and vinyl chloride for five different *Dehalococcoides mccartyi* strains was less variable, with ε= -6.3±1.2 ‰, -18.4±2.8 ‰, -21.1± 4.5 ‰ and -23.2±1.1 ‰ on average for 1,1-DCE, *cis*-DCE, *trans*-DCE and VC, respectively (Fletcher *et al.*, 2011). Interestingly though, enrichment factors determined for chlorinated ethenes with *D. mccartyi* strain BTF08 were -28.8±1.5 ‰ (VC), -30.5±1.5 ‰ (*cis*-DCE) and -12.4±1.1 ‰ (1,1-DCE) correlating with enrichment observed in a model constructed wetland, suggesting that similar enzymes and strains were involved in the dechlorination (Imfeld *et al.*, 2008, Imfeld *et al.*, 2010, Schmidt *et al.*, 2014).

Therefore, we developed a novel conceptual approach (Figure 4) to allow quantification of biodegradation of chlorinated ethenes *in situ* combining CSIA, microcosm studies and molecular marker detection (Nijenhuis *et al.*, 2007, Cichocka *et al.*, 2008).

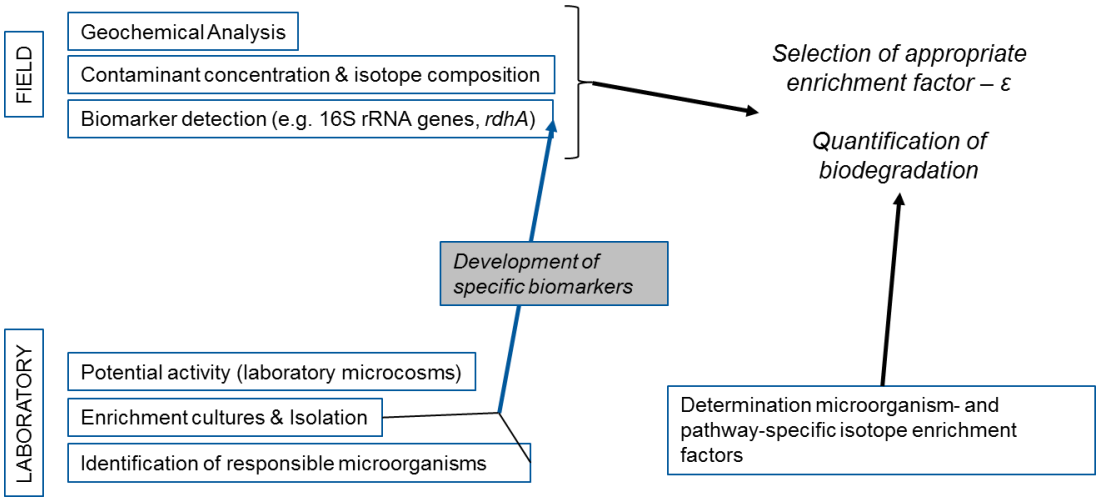

Figure 4: Conceptual approach for the quantification of biodegradation of chlorinated solvents using CSIA.

At the field site in Bitterfeld, the product range and isotope composition of chlorinated ethenes suggested dehalogenation *in situ*, however, since DCE could have resulted from PCE/TCE dehalogenation, or as co-contamination, the evaluation was not straightforward. Therefore, microcosms were set-up to test the potential for chloroethene conversion. Indeed, PCE was dechlorinated to ethene proving that *in situ* degradation had occurred (Nijenhuis *et al.*, 2007). Additionally, analysis of 16S rRNA gene biomarkers was used specifically to detect microbial taxa and *Dehalococcoides*-, *Desulfuromonas*-, *Desulfitobacterium*- and *Dehalobacter*–related microorganisms could be detected showing that organohalide-respiring microorganisms potentially capable of chloroethene degradation were present. The approach could be further refined in the future by analysing for the presence of functional genes or proteins. Taking this combined information into consideration, an appropriate enrichment factor representative for the field site can be selected and the extent of biodegradation can be estimated as illustrated in Figure 5.

It should be kept in mind, however, that the quantification using the Rayleigh approach is only valid in closed systems and requires knowledge of groundwater flow. While estimates will still have a relatively large uncertainty, these values are a significant improvement compared to estimates of biodegradation solely based on concentration data.

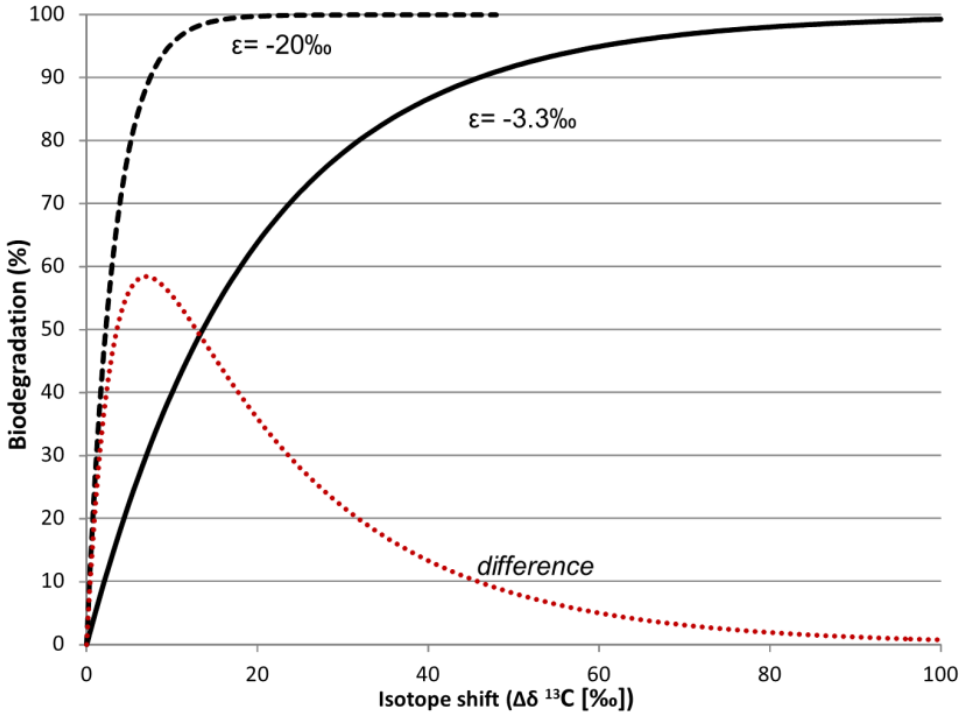

Figure 5: Quantification of biodegradation using compound-specific stable isotope analysis applying equation 6 and the two extreme isotope enrichment factors for TCE: ε = -3.3‰ and ε = -20‰. Additionally, the difference between the estimates is shown and represents the degree of uncertainty of the method.

f. Characterisation of biochemical reactions

Apparent kinetic isotope effects - In environmental and biological science, kinetic isotope fractionation has been used to characterize the transition-state of chemical reactions to obtain information on the chemical mechanism or to identify rate-limiting steps in complex biochemical transformation reactions (Northrop, 1981). The extent of the kinetic isotope effect (KIE) depends on rate limitation during the isotope-sensitive cleavage of the chemical bond after which the reaction becomes irreversible (commitment to catalysis). The KIE has been used to obtain information on the chemical mechanism of bond cleavage and expresses the relative reaction rates of the lighter (^{l}k) vs. the heavier isotope (^{h}k) of an element E:

Equation 6: $$KIE_E = \frac{l_k}{h_k}$$

The apparent kinetic isotope effect (AKIE) can be calculated from the determined bulk enrichment factor for the reaction mechanisms potentially involved and then compared to the theoretical KIE values, i.e. Streitwieser limits (Northrop, 1981, Huskey, 1991, Elsner *et al.*, 2005).

Equation 7: $$AKIE = \frac{1}{1 + \left(z \times \left(\frac{n}{x} \right) \times \frac{\varepsilon_{bulk}}{1{,}000} \right)}$$

The bulk ε does not consider the non-reacting positions and the intra-molecular competition in the molecule, whereas calculating the AKIE values provides estimates of position-specific kinetic isotope effects where *n* is the number of atoms of an element, *x* is the number of atoms at reacting positions and *z* is the number of indistinguishable reactive sites (reactive positions between which intra-molecular isotopic competition exists). These corrections allow the comparison between compounds with different numbers of carbon, which are thought to be subject to the same reaction, and can be used to evaluate reaction mechanisms taking place by comparing the values with reactions having a known mechanism and to theoretical KIE values.

This approach was used to evaluate the reaction during 1,2-dichloropropane and 1,2-DCA dichloroelimination by *Dehalococcoides* spp. (Fletcher *et al.*, 2009). The isotope fractionation of 1,2-dichloropropane during dichloroelimination to propene by two distinct *Dehalococcoides*-containing cultures was explored in laboratory experiments. The ε_{bulk} factors calculated in the two cultures were statistically identical, -10.8 ± 0.9 and -11.3 ± 0.8‰, even though the cultures were derived from geographically distinct locations, suggesting similar reaction mechanisms. AKIE values for 1,2-dichloropropane dichloroelimination, and assuming stepwise and concerted reaction mechanisms, were approximately 1.033 and 1.017, respectively. These values were within the range of previously reported values for dichloroelimination reactions and were equivalent to values reported for biotic 1,2-dichloroethane and abiotic 1,1,2,2,-tetrachloroethane

and pentachloroethane dichloroelimination reactions, which support the overall similarity in reaction of abiotic and biotic dichloroelimination (Elsner *et al.*, 2007, Hirschorn *et al.*, 2007, Vanstone *et al.*, 2008, Hofstetter & Berg, 2011).

In a later study, the reaction during 1,2-DCA dichlorelimination was analysed in more detail and compared to hydrogenolysis of DCE and VC dechlorination by two *Dehalococcoides* strains, *D. mccartyi* strain 195 and *D. mccartyi* strain BTF08 (Schmidt *et al.*, 2014). These reactions are likely catalysed by similar enzymes, or even by the same enzyme. For example, enzyme assays with Rdhs from *Dehalococcoides mccartyi* spp. indicated that the dehalogenation of TCE and 1,2-DCA was catalysed by TceA in strain 195 and DCEs, VC and 1,2-DCA was catalysed by BvcA in strain BAV1 (Magnuson *et al.*, 1998, Magnuson *et al.*, 2000, Tang & Edwards, 2013, Tang *et al.*, 2013). Therefore, a similarity in the reaction was assumed. The carbon isotope enrichment factors (ε_{bulk}) determined for 1,2-DCA were -30.9 ± 3.5 ‰ and -29.0 ± 3.0 ‰ for *D. mccartyi* strain BTF08 and *D. mccartyi* strain 195, respectively. Here, calculated $AKIE_{12\text{-}DCA}$ values were 1.066 and 1.062 for the stepwise and 1.032 and 1.030 for the concerted reaction for *D. mccartyi* strain BTF08 and 195, respectively. For the stepwise dechlorination of *cis*-DCE, AKIE was 1.065 for strain BTF08 and 1.044 for strain 195. Calculated AKIE values did not allow a clear distinction to be made for the reaction for 1,2-DCA, however, the data fit with the stepwise reaction as occurs for the chlorinated ethenes. The generally higher AKIE values for 1,2-DCA compared to 1,2-dichloropropane, however, suggests that the actual reaction of 1,2-dichloropropane at the enzyme may not be the rate-determining step. Steps preceding the actual reaction, such as substrate uptake and transport to the enzyme, might be limiting the overall rate of the reaction (see also section 3g).

Product related enrichment factors

To evaluate the reaction further and to assess whether the reaction of 1,2-DCA to ethene was a direct reaction, without an intermediate, or if an intermediate was generated, e.g. VC, the product-related enrichment factors were calculated. These product-related enrichment factors were then compared with the substrate-related factors in order to investigate whether a single- or a multiple-step reaction was taking place. Furthermore, this analysis could reveal whether ethene was a direct product of 1,2-DCA dichloroelimination. It was assumed that when substrate- and product-related isotope enrichment factors were similar, a single step reaction was involved.

However, in a multiple-step reaction, where all steps have a significant isotope effect, the substrate-related enrichment factor was expected to differ significantly from the product-related one.

Equation 8 was used to determine the enrichment factors ($\varepsilon C_{substrate \rightarrow product}$) from product isotope signatures, where the product isotope value is $\delta_{product}$ and the initial isotope value of the substrate is $\delta_{0,substrate}$; f is $\ln(C_t/C_0)$ based on the concentrations at time t and time 0 and $D(\delta_{product})$ represents $\delta_{product} - \delta_{product,\ average}$, which defines the deviation of the specific product δ value from the average product δ (Elsner *et al*., 2008).

Equation 8:
$$\frac{1000+\delta_{product}}{1000+\delta_{0,substrate}} = \left(1+\frac{D(\delta_{product})}{1000}\right)\frac{1-f^{((\varepsilon C)+1)}}{1-f}$$

The product-related enrichment factor was calculated from the parameters $D(\delta_{product})$ and εC according to equation 3 (Elsner *et al*., 2008):

Equation 9:
$$\varepsilon C_{substrate \rightarrow product} = D(\delta_{product}) + \varepsilon C$$

Equation 9 is solely based on isotope measurements of a given product. The product-related enrichment factor $\varepsilon C_{\ substrate \rightarrow product}$ can be determined without knowledge of the absolute reaction and product formation rates. The fitting parameter $D\delta_{product}$ was calculated from the slope obtained with Eq. 8. Product, ethene-related enrichment factors ($\varepsilon C_{1,2\text{-DCA-ethene}}$) calculated for 1,2-DCA (-34.1 and -32.3 ‰ for strain BTF08 and strain 195, respectively) were similar to substrate-based enrichment factors ($\varepsilon C_{1,2\text{-DCA}}$). This supports the hypothesis that ethene is the direct product of 1,2-DCA dichloroelimination but that VC was a side product as result of branching in the reaction.

Dual-element stable isotope analysis

Like pure chemical reactions, biochemical reactions may be characterised using the calculation of AKIE or product-related enrichment factors. However, it should be taken into account that

rate-limiting steps prior to the isotope-sensitive bond cleavage (see Section 3.g for details), such as substrate uptake, transport and binding to an enzyme may reduce the observed kinetic isotope fractionation effects (O'Leary, 1981, Nijenhuis *et al.*, 2005, Cichocka *et al.*, 2007, Lollar *et al.*, 2010). Here, the isotope analysis of a single element in a molecule might not suffice to gain insight into the reaction mechanism. Additional information can be obtained from the analysis of a 2nd or even 3rd element e.g. H/D or Cl, which is assumed to allow for cancelling out of rate-limiting effects where the whole molecule, therefore all elements, should be similarly affected as illustrated in Figure 6. A similarity of the dual-element slope would suggest similar reaction mechanisms while different slopes would imply different reaction mechanisms. In the first case, a similarity in the slopes implies that steps preceding the bond-cleavage are rate-limiting in the case where a variability of the single element fractionation was observed (see further discussion in the next sections). The dual-element isotope fractionation for carbon and chlorine is usually presented as $\varepsilon_C/\varepsilon_{Cl}$, which represents the relative isotope effects on carbon vs. that on chlorine during a reaction.

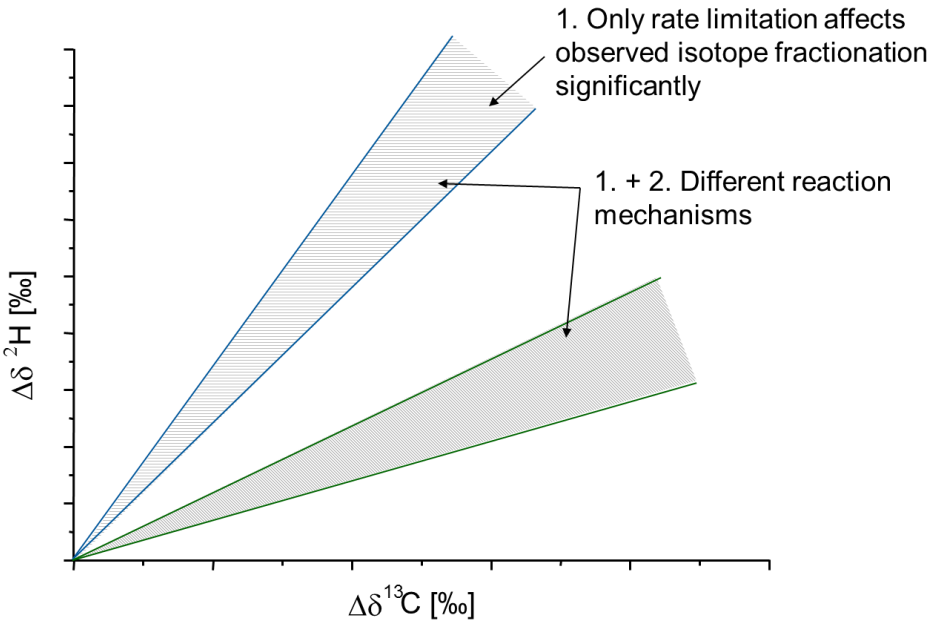

Figure 6: Conceptual figure explaining the use of dual-element isotope analysis, i.e. C and H. Differences in slope represent different pathways and reaction mechanism. If the data lie on a similar slope, the reaction mechanism is likely to be similar for the tested systems while different reaction mechanisms likely result in different slopes.

Previously, the dual-element isotope analysis of toluene or benzene allowed the assessment of the different pathways as distinct patterns were observed when comparing aerobic vs. anaerobic or facultative vs. strict anaerobic biodegradation (Fischer *et al.*, 2008, Fischer *et al.*, 2008, Vogt *et al.*, 2008). The recently developed analytical methods made the application of dual-element isotope approach also possible for PCE and TCE (Cretnik *et al.*, 2013, Cretnik *et al.*, 2014). These concepts allow a more detailed investigation of the reaction involved in the reductive dehalogenation, as discussed in the next sections.

g. Factors affecting the observed isotope effects during microbial dehalogenation

As mentioned above, carbon stable isotope fractionation of the chlorinated ethenes was highly variable with enrichment factors ranging from insignificant to -16.7‰ for PCE and from -3.3 to -20‰ for TCE (Nijenhuis *et al.*, 2005, Cichocka *et al.*, 2007, Lee *et al.*, 2007, Cichocka *et al.*, 2008). Additionally, variations in the carbon isotope fractionation during the dechlorination of PCE, TCE, DCE and VC were observed despite the reaction taking place in related *D. mccartyi* strains with similar complements of enzymes (Taş *et al.*, 2009, Marco-Urrea *et al.*, 2011, Schmidt *et al.*, 2014).

As all Rdhs investigated thus far are highly similar with iron-sulfur clusters and corrinoid cofactors (Hug *et al.*, 2013), the chemical reaction mechanism was assumed to be similar for the tested microorganisms with resulting similar isotope effects. Since these differences were not expected, further investigations into the reasons for this discrepancy were carried out.

Previously, such a variability of observed effects was also reported for CO_2 fixation pathways in plants (O'Leary, 1981). Here, uptake of carbon dioxide into the cell was found to be the rate-limiting step during photosynthesis in C4 plants, but not in C3 plants. Similar effects were observed during microbial aerobic toluene degradation under iron-limiting conditions, and were thought to be due to differences in substrate-enzyme binding (Mancini *et al.*, 2006), and during aerobic dichloromethane degradation and were correlated to differences in enzyme structure (Nikolausz *et al.*, 2006). While isotope fractionation is caused at the first irreversible step where a bond is broken, steps preceding this actual reaction might cause transport limitation (Figure 7).

In the case of reductive dehalogenation potential rate-limiting steps might be at the level of substrate uptake through the outer membrane and/or cell wall, or at the level of transport and binding to the Rdh before the actual dehalogenation reaction takes place in the active site of the enzyme as illustrated in Figure 8. The isotope effects of the bond cleavage might therefore be masked in the case where transport of the substrate to the active site of the enzyme is limited by preceding steps. In other words, the reaction of the enzyme is faster than diffusion of the substrate to the active site. Therefore, rate-limitation results in a non-homogeneous system and the observed (bulk) isotope effect is lower compared to the actual effect of the bond cleavage at the enzyme. In the case where the bond-cleavage is the only rate-determining step, the observed (bulk) isotope effect would approach the actual isotope effect of the chemical reaction as a result of an overall equilibrium in the system.

Additionally, in the case of substrates with low availability, such as halogenated dioxins, the dissolution of the substrate may also play an important role and affect the observed isotope effect as the non-bioavailable substrate fraction 'dilutes' the isotope composition of the reacting, bioavailable, fraction. In the next sections, each step is considered separately as a cause for the observed variability in isotope effects starting from the actual reaction at the enzyme towards each rate-limiting step.

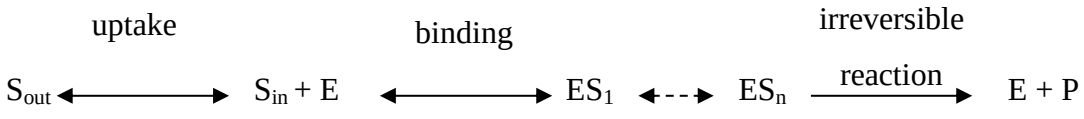

Figure 7: Scheme showing that the resistance of membranes can modify the substrate flow into the cell and may lead to non-isotope equilibrium conditions between substrate in the cell (S_{in}) and the medium (S_{out}). Furthermore, the formation of the enzyme-substrate complex can be a rate-limiting step in the reaction (ES_1, ES_n) before the biochemical reaction takes place.

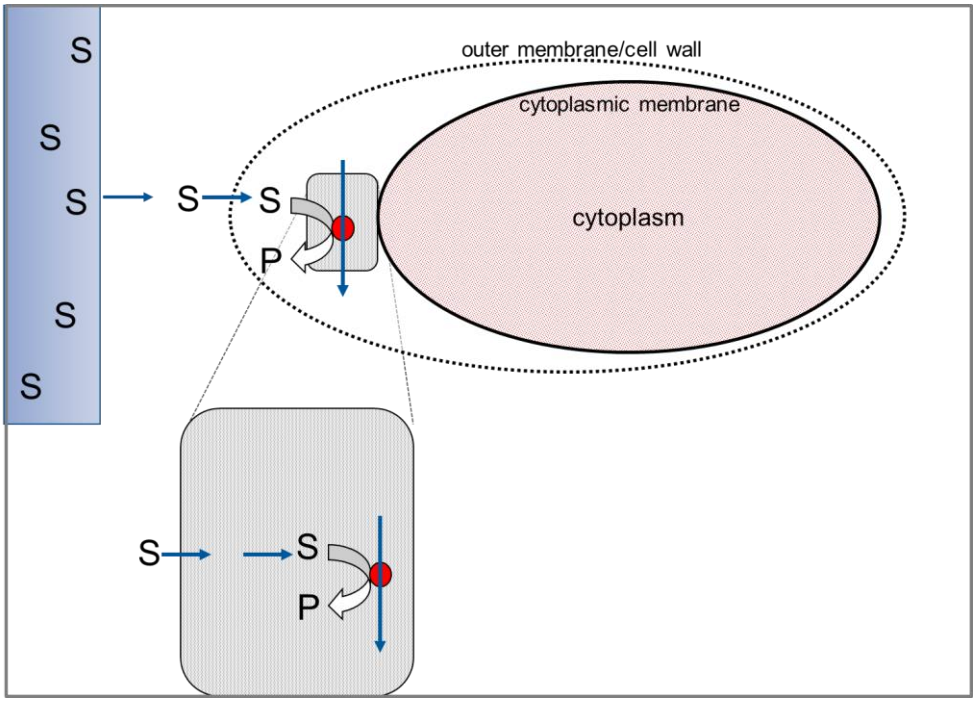

Figure 8: Schematic of potentially rate-limiting steps preceding a biochemical reaction. The reaction is assumed to be periplasmic, similar to that of Rdhs. Initially, a substrate (S) will be present as 2nd phase, where dissolution into the aqueous phase is limiting, and it needs to be transported across the outer membrane and to the enzyme (inset). P = product.

i. Effects of the corrinoid cofactor

The actual (chemical) reaction, C-Cl bond cleavage, during microbial dehalogenation is thought to take place at the corrinoid cofactor. The mechanism of the enzyme is not known but is proposed to proceed via nucleophilic substitution, nucleophilic addition or single electron transfer (Cretnik *et al.*, 2013, Kuder *et al.*, 2013). A simultaneous reaction via different mechanisms is also hypothesised because during the reaction of TCE at corrinoids a mixture of products is formed (Shey & van der Donk, 2000, Nonnenberg *et al.*, 2002), although this only occurs to a limited extent in enzymes (i.e. a mixture of *cis*- and *trans*-DCE is usually formed during microbial dehalogenation). In recent years, the type of corrinoid cofactor integrated in the Rdh within different strains was investigated and diversity in its structure was observed. For example, *norpseudovitamin* B_{12} was determined to occur in *Sulfurospirillum multivorans* (Kräutler *et al.*, 2003). The corrinoid structure is thought to play an important role in the substrate specificity as well as the actual reaction and Keller *et al*. (Keller *et al.*, 2013) observed a decrease in function when the *norpseudo*-B_{12} cofactor, which has an adenine as the lower

ligand base, was replaced with *nor*-B_{12}, which has a dimethylbenzimidazole as the lower ligand base. Furthermore, activity of *Dehalococcoides mccartyi* strains BAV1 and FL2 was apparently dependent on the production of a specific cobamide in *Geobacter* co-cultures (Yan *et al.*, 2012). Therefore, diversity in the type of corrinoids present in different dehalogenases is considered to result in the variability of isotope fractionation for the chlorinated ethenes.

Recently, (Cretnik *et al.*, 2013) performed a dual C/Cl-isotope investigation for TCE dehalogenation and observed similar fractionation for cobalamin and in selected microorganisms, namely *Geobacter lovelyi* and *Desulfitobacterium hafniense*. Further, we observed similar slopes for the dual-element isotope analysis for corrinoids and *Sulfurospirillum multivorans,* and therefore concluded that a similar reaction takes place for the dechlorination of TCE at selected representative and structurally diverse corrinoids and cobamide (Renpenning *et al.*, 2014). Both studies confirmed a similarity in the observed dual-element slopes for corrinoids and that of representative microorganisms. Therefore, a similarity in reaction was concluded in both microbial and abiotic reference systems for TCE.

In contrast, significant differences were observed for PCE with the different corrinoids tested. The dual-element slope ($\varepsilon_C/\varepsilon_{Cl}$) for PCE depended on the corrinoid type and particularly on its lower ligand: $\varepsilon_C/\varepsilon_{Cl}$ values of 4.6 and 5.0 for vitamin B_{12} and *nor*vitamin B_{12} were significantly different compared to values of 6.9 and 7.0 for *norpseudo*-vitamin B_{12} and dicyanocobinamide, respectively. Therefore, corrinoids with a bulkier lower ligand (dimethylbenzimidazole) could be distinguished from those with a smaller lower ligand (*norpseudo*-vitamin B_{12}*;* adenine) and cobinamide, lacking a lower ligand. It is assumed that the dissociation of the lower ligand affects the reaction and therefore the resulting observed isotope effect. In the case of *S. multivorans* dissociation of the lower ligand is thought not to play a role as the adenine appears fixed within the enzyme structure in a base-off position (Bommer *et al.*, 2014).
Overall, these results suggest distinct differences in reaction of PCE at different corrinoids but not of TCE. Interestingly, again significantly different slopes were observed for *S. multivorans* containing either *nor*- or *norpseudo*-vitamin B_{12}, as discussed in the next section.

ii. Effects of the enzyme scaffold

Before a reaction occurs, a substrate needs to associate with and bind to the active site of an enzyme. In recent studies, two patterns of dual-element fractionation were observed for PCE dechlorination in enrichment cultures and microcosms, which was proposed to be related to the enzyme's structure (Wiegert *et al.*, 2013, Badin *et al.*, 2014). Even though we observed differences for the pure corrinoids for PCE, interestingly, dual-element isotope slopes (2.2 to 2.8) for *S. multivorans* were similar, regardless of whether *norpseudo*- or *nor*vitamin B_{12} was present as cofactor. This slope was significantly different again from the corrinoids. On the one hand, this suggests a limited role of the corrinoid cofactor in the dehalogenation reaction of PCE but a dominant role of the enzyme scaffold on the resulting isotope effect. On the other hand, we suggest that dehalogenation takes place as a minimally two-step reaction with overlapping isotope signatures for the different steps (Renpenning *et al.*, 2014). Assuming the reaction mechanism at the corrinoid in pure form or as cofactor is the same, dechlorination of PCE might be relatively fast in the actual bond-cleavage while an initial, e.g. binding step, is relatively slow and isotope-sensitive. For TCE, in contrast, the reaction at pure corrinoids was found to be ten-fold slower, which may result in equilibrium during the first step of the binding reaction and the full isotope effect of the actual bond-cleavage is observed. Recently, the elucidation of the enzyme structure suggested a very tight hydrophobic channel in the Rdh of *S. multivorans*, which would allow entry of only a single PCE at a time (Bommer *et al.*, 2014). Assuming a higher interaction of PCE compared to TCE (see also next section), such a restrictive channel could explain the observed isotope effects assuming the association with the channel causes a distinct dual-element isotope effect. Schematically, enzymatic dehalogenation may be proposed to proceed as depicted in Figure 9 via a multi-step reaction.

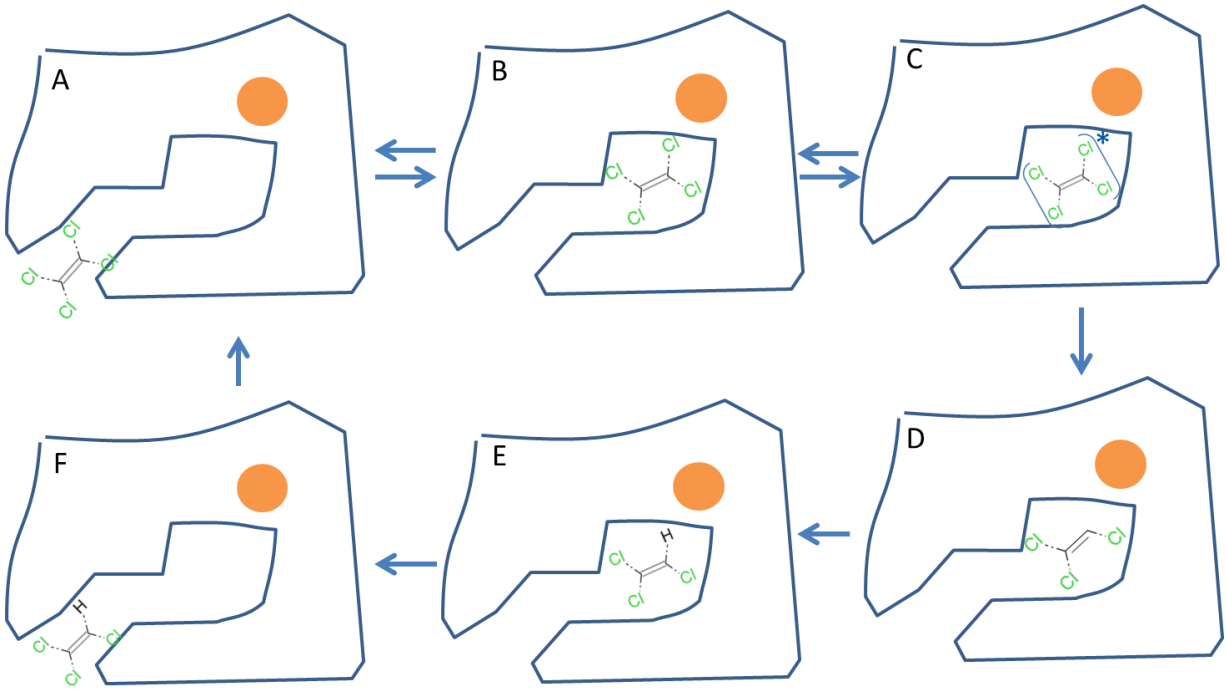

Figure 9: The suggested sequence of the multi-step dehalogenation reaction for PCE with: A) initial binding to the enzyme; B) transport & binding in the active site; C) initial reaction step with a transition state; D) dechlorination; E) protonation; and F) release of the product. The orange dot indicates the reactive centre with corrinoid cofactor.

Multi-step reactions were observed to affect the isotope effects during the abiotic hydrolysis of the herbicide isoproturon where the pattern was explained by initial (reversible) zwitterion formation and subsequent irreversible reaction (Penning *et al.*, 2008). Potentially, during microbial reductive dehalogenation, the initial step may be the (reversible) binding to the enzyme or the corrinoid, as effects of the different corrinoid structures were also observed, and then irreversible dehalogenation (Figure 7 section g.).

v. Effects of the cell envelope structure

Finally, the initial, potentially rate-limiting, step might be the uptake and transport of the substrate across the cell envelope. Due to the location of Rdhs on the outer face of the cytoplasmic membrane, potentially the cell wall and/or outer membrane might be expected to affect the transport of e.g. PCE or TCE. Our initial investigations showed that the destruction of

the cell envelope in *Sulfurospirillum multivorans* and *Desulfitobacterium* strain PCE-S significantly increased the observed isotope fractionation of PCE suggesting that the transport of PCE limits the rate of the reaction (Nijenhuis *et al.*, 2005, Cichocka *et al.*, 2007). Moreover, the reaction at the purified enzyme had an even stronger fractionation suggesting that the presence of membrane fragments hampered transport. Similar effects were observed during the dehalogenation of 1,1,1-trichloroethane by a *Dehalobacter* culture, when comparing cell-free extracts with the intact cells and the pure chemical reaction (Lollar *et al.*, 2010).

Interestingly, when comparing microorganisms with a gram-positive cell envelope (e.g. *D. hafniense,* and *D. restrictus*) vs. a gram-negative envelope (*S. multivorans, G. lovleyi*, *D. michiganensis*), a systematic difference was observed with higher rate-limiting effects for PCE in gram-negative strains correlating with the higher content of hydrophobic components (e.g. phospholipids) especially in the outer membrane (Rapp, 2013, Renpenning *et al.*, 2015). These effects were not observed for TCE, which corresponds with the lower hydrophobicity of this substrate compared to PCE. Furthermore, specific sorption tests were performed to evaluate the interactions of PCE vs. TCE in the membrane. PCE adsorbed to cell material by approximately one order of magnitude more strongly compared to TCE, confirming that the observed isotope masking effects mainly are due to the properties of the substrate. Additionally, modification of the location of PceA into mainly the cytoplasm of *S. multivorans*, resulted in an additional depression of the observed isotope effects for PCE, but not TCE, correlating with the presence of an additional barrier, the cytoplasmic membrane (Renpenning *et al.*, 2015).

Based on the results presented, a conceptual model of dehalogenation can be constructed and includes the rate-limiting steps at membranes and resulting isotope masking (Figure 10). Overall, the reaction of PCE is thought to be relatively rapid compared to TCE, as well as to the overall transport rate resulting in the masking of isotope effects. In contrast, the TCE reaction is comparatively slow with relatively fast transport so that isotope effects are not masked.

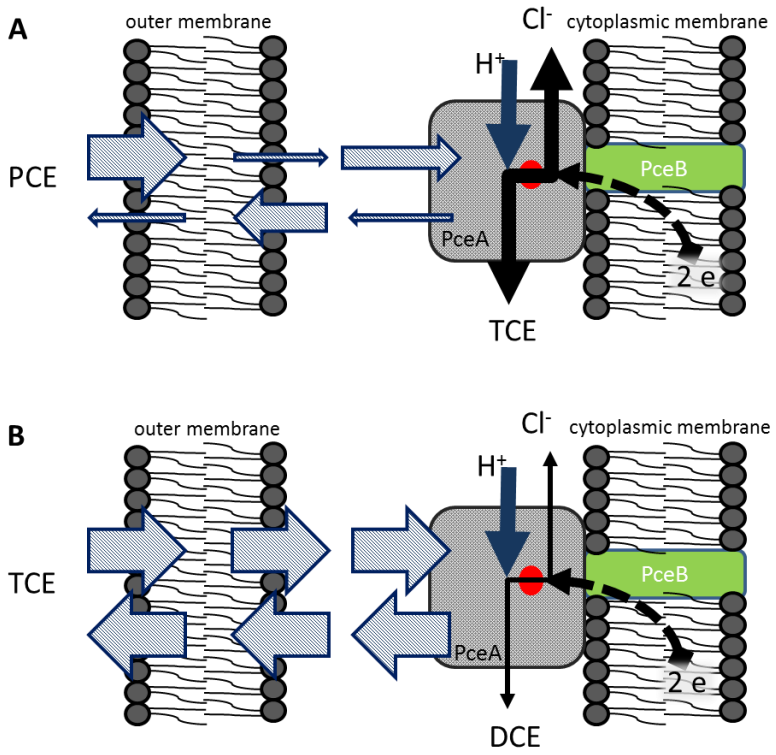

Figure 10: Conceptual scheme of the rate-limiting step of A) PCE and B) TCE dehalogenation in *Sulfurospirillum multivorans*. The size of the arrows indicates the rate of each step. While PCE adsorbs preferentially to the lipid membranes and enzyme, TCE does not, resulting in an equilibrium situation for TCE but not for PCE.

vi. Effect of bioavailability

Bioavailability of a substrate can significantly reduce the observed isotope effects, although this is usually not relevant for the chlorinated ethenes, which have a relatively high solubility. For substances such as chlorinated dioxins, these effects may be relevant when the substance is not completely dissolved. However, provided that the substances are completely dissolved, relevant isotope enrichment factors may be obtained during the dechlorination of dioxins by *Dehalococcoides* spp. (Ewald *et al.*, 2007, Liu *et al.*, 2010). That the bioavailability significantly affects the isotope fractionation was shown in experiments with *S. multivorans* in the presence or absence of a second, organic, phase, which functioned as a TCE reservoir (Aeppli *et al.*, 2009). Here, it could be shown that the presence of tetradecane depressed signficantly the observed isotope effects for the dechlorination of TCE. Therefore, in addition to the microbial factors

discussed above, the chemical's properties need to be carefully considered and taken into account when evaluating isotope effects.

4. Conclusions & Outlook

The assessment of *in situ* biodegradation and the characterisation of the associated reactions is currently still a great challenge. Advances in equipment, methods and approaches have improved our understanding of microbial reactions. However, when used individually, these methods are severely limiting, therefore, a conceptual approach including several methods and approaches is advised. In particular, the development of compound-specific stable isotope analytical approaches, which include tracer and fractionation concepts, allow the evaluation of *in situ* microbial element cycles, as well as the characterisation of pathways and mechanisms involved. These concepts are still under development and are challenging to use for field investigations and, in many cases, reference laboratory studies are required for a reliable and conclusive analysis.

In this work, the microbial transformation of mainly the chlorinated ethenes and MCB is discussed, however, emerging contaminants, such as pharmaceuticals, brominated flame retardants and pesticides have come into focus and their environmental fate is currently still poorly understood. To understand the biodegradation of these compounds, it is not only necessary to cultivate and investigate the microorganisms capable of the transformation, but analytical methods allowing the *in situ* evaluation also must be developed simultaneously. For example, the development of reliable methods for the carbon stable isotope composition of polar substances via LC-IRMS, as well as multi-element approaches for Cl, Br, N, S and H in these emerging contaminants is required. Therefore, methods allowing the routine compound-specific analysis of Cl in organic compounds need to be developed as initiated recently by (Hitzfeld *et al.*, 2011) and currently further developed (Renpenning *et al.*, 2015). Additionally, the development of Br isotope analysis (Bernstein *et al.*, 2013) will allow the detailed investigation of microbial dehalogenation reactions *in situ*. Of special interest is the comparison of brominated and chlorinated analogues by related enzymes, which may allow us to derive rules and transfer of the knowledge from one class of substances to another. At the same time, additional analysis using e.g. enantiomer-selective degradation might provide further information on the pathways involved, such as is currently available for α-HCH (Bashir *et al.*, 2013).

In addition, isotope concepts may allow an understanding of microbial reaction mechanisms as well as the overall process of contaminant degradation. In particular, it is of great interest to obtain a deeper understanding of the reactions taking place during organohalide respiration. Multi-element isotope analysis should allow investigation of the respective reactions directly at (purified) enzymes. Besides the analysis introduced in this study, Kuder *et al.* recently presented a method allowing the analysis of H in halogenated ethenes (Kuder *et al.*, 2013). However, routine methods are thus far not commercially available. Furthermore, it would be of interest to understand how two distinct reactions, such as the hydrogenolysis of chloroethenes and dichloroelimination of 1,2-DCA, take place at the same or at very similar enzymes. On the one hand, to achieve this, the substrate specificities of the different putative dehalogenases in the different *Dehalococcoides mccartyi* strains should be determined and the *in vivo* activity should be addressed e.g. via proteomic approaches. On the other hand, the triple-element-isotope analysis discussed above would allow a direct comparison of the reactions. The recently resolved crystal structures of Rdhs will allow the development of a molecular model for the dehalogenation reaction, which then can be compared with experimental data as was done for aerobic degradation of hexachlorocyclohexanes (Manna & Dybala-Defratyka, 2013, Bashir, 2013). Together, these approaches will enable elucidation of the reaction mechanism and important steps in the dehalogenation.

Furthermore, together with computational biologists, it should be possible to develop a conceptual model for the transport of PCE across membranes and to investigate the bottlenecks and rate-limiting steps in more detail. These complementary theoretical studies should allow elucidation of the observed isotope fractionation and underlying reaction mechanism, as well as the overall reaction steps.

Overall, compound-specific isotope approaches provide promising opportunities both to evaluate *in situ* biotransformations as well as to investigate reactions in depth; however, it will be imperative to adopt a conceptual approach that involves multiple methods.

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7. List of peer-reviewed scientific publications of the author on which the habiliation-thesis is based

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8. Curriculum Vitae

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9. Affidavit

This habilitation thesis was written independently and without outside help. Other than the indicated sources and aids were not used. Whole or in substance adopted sections were indicated as citations.

Leipzig, 10.03. 2015

Ivonne Nijenhuis