Eco-physiology of anaerobic biotransformation of lower chlorinated solvents in groundwater, constructed model and laboratory systems

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Content

Summary

Lower chlorinated organic compounds such as monochlorobenzene (MCB), dichlorobenzenes (DCBs), vinyl chloride (VC), dichloroethenes (DCEs) and 1,2-dichloroethane (1,2-DCA) are among the most widespread groundwater pollutants worldwide, due to their low biodegradability and accumulation under anoxic conditions. In consequence, contaminated groundwater endangers drinking water sources and causes environmental hazards particularly when it discharges into groundwater dependent ecosystems (e.g. wetlands and rivers).

In this thesis, the anaerobic biotransformation of lower chlorinated hydrocarbons was characterised by investigating the bio-geochemical processes in laboratory studies and then implementing the gained knowledge in an integrative approach to evaluate their *in situ* biotransformation. Following the objectives and different scales (bacterial culture, model systems and contaminated aquifers), the substrate specificity and degradation pathways were investigated in pure and enriched cultures which were found to be relevant for *in situ* degradation at a contaminated field site in Bitterfeld (Germany) and in a model scale wetland.

A bacterial culture, *Dehalococcoides mccartyi* strain BTF08, originated from a enrichment culture of the contaminated field site and was shown to reductively dechlorinate diverse chlorinated hydrocarbons, including the lower chlorinated ones DCEs, VC,and 1,2-DCA, to ethene. Moreover strain BTF08 was found to play an important role in the dechlorination of *cis*-DCE in a constructed wetland fed with groundwater from the contaminated site. Carbon stable isotope fractionation was used to characterise the reaction mechanism of 1,2-DCA reductive dechlorination for strain BTF08 in comparison to *Dehalococcoides mccartyi* strain 195. Both strains formed ethene as product during 1,2-DCA dichloroelimination, whereas minor amounts of VC were produced in a branching reaction. However, only strain BTF08 was capable to metabolically convert VC to ethene. In addition to the ability to dechlorinate chloroethenes (CEs) and chloroethanes (CEs), strain BTF08 degraded higher chlorinated chlorobenzenes (CBs), e.g. penta- and hexachlorobenzene to trichlorobenzenes (TCB), but did not degrade lower chlorinated ones, e.g. DCB and MCB. However, strain BTF08 was shown to produce MCB as (end) product during degradation of HCH (hexachlorocyclohexane, Lindane), which was detected at the field site as well.

In anaerobic laboratory microcosms, set up with field site groundwater, mineralisation of MCB under nitrate and iron reducing conditions was identified for the first time. In addition, first indications for a reductive dechlorination of DCB to MCB and to benzene, with likely further mineralisation to CO_2 and CH_4, was discovered in anaerobic microcosms set up with sediment of

a model wetland, habituating the indigenous groundwater microbial community. Reductive dechlorination and mineralisation of MCB were identified as potential microbial degradation pathways resulting most likely in the observed contaminant removal in the two monitored model scale wetlands. The biotransformation of MCB in the gravel bed of the constructed wetlands was enhanced due to geochemical conditions and gradients, e.g. iron and sulphate reduction that were relevant redox processes in both wetlands. In both model gradient systems an overall MCB removal of more than 90 % was observed.

Secondly, the role of the indigenous microbial communities in the contaminant degradation was investigated, addressing different objectives, including the characterisation of the groundwater microbial community composition (Bacteria and Archaea) and its structure and dynamic in the wetland sediment. The groundwater microbial community consisted typically of methanogenic archeae, sulphate (genera *Desulfobacterium*) and iron reducing bacteria (genera *Geobacter*) as well as *Chloroflexi*, comprising bacteria capable of respiring diverse chlorinated hydrocarbons. The microbial community settled on the wetland sediment and was found to be distributed in the wetland sediment depending on present redox conditions, including the depth-related availability of oxygen.

Finally, the field scale was addressed in a contaminated aquifer in Ferrara (Italy), which has a long-term industrial tradition in manufacturing chlorinated solvents. Several contamination plumes were identified in this multi-aquifer system, which were potentially originated from uncontained former industrial and municipal dump sites or industrial spills. The aim of this study was to investigate the origin of the contamination (including mainly 1,2- DCA and VC) and to assess their biotransformation. Compound specific carbon stable isotope analysis revealed two different sources of the chlorinated solvents, most likely due to two chemical production lines. Indications for biotransformation were observed at the field site as well.

In conclusion the presented study provided the first time insights in the anaerobic biotransformation of lower chlorinated hydrocarbons by applying an integrative approach. Lower chlorinated hydrocarbons get potentially degraded *in situ* if essential hydro-geochemical and biological (microbial) conditions are present.

Zusammenfassung

Niederchlorierte organische Substanzen, wie Monochlorbenzol (MCB), Dichlorbenzole (DCB), Vinylchlorid (VC), Dichlorethen (DCE) und 1,2-Dichlorethan (1,2- DCA) gehören weltweit zu den weitverbreitetsten Umwelt- und Grundwasserschadstoffen. Ein geringes biologisches Abbaupotential führt zur Akkumulation dieser Verbindungen in der Umwelt, vorrangig in anoxischen Milieus wie zum Beispiel Grundwasser und Flusssedimenten. Infolgedessen gefährdet kontaminiertes Grundwasser unsere Trinkwasserquellen und grundwasserabhängige Ökosysteme wie Feuchtgebiete (Wetlands) und Flüsse.

In dieser Arbeit wurde die anaerobe Biotransformation von niederchlorierten Kohlenwasserstoffen charakterisiert. Um deren *in-situ* Abbau zu bewerten, wurden biogeochemische Abbauprozesse in Laborexperimenten untersucht und die gewonnenen Erkenntnisse anschließend in einem integrativen Ansatz angewandt. Den Zielen und verschiedenen experimentellen Maßstäben/Ansätzen (Bakterienkultur, Modellsystem und kontaminierter Grundwasserleiter) folgend, wurden zunächst die Substratspezifität und die relevanten Abbauwege an einem kontaminierten Feldstandort in Bitterfeld sowie in einem Modell-Wetland untersucht.

Für die obligat anaerobe Bakterienkultur *Dehalococcoides mccartyi* Stamm BTF08, isoliert aus einer Anreicherungskultur vom Feldstandort Bitterfeld, wurde die Aktivität der reduktiven Dechlorierung für diverse chlorierte Kohlenwasserstoffe wie DCEs, VC, und 1,2- DCA nachgewiesen. Darüber hinaus scheint Stamm BTF08 eine wichtige Rolle bei der Dechlorierung von DCE in einem Model-Wetland zu spielen, welches mit belastetem Grundwasser beschickt wurde. Des Weiteren wurde das Prinzip der stabilen Isotopenfraktionierung genutzt, um den Reaktionsmechanismus der reduktiven Dechlorierung von 1,2-DCA für Stamm BTF08 im Vergleich zum *D. mccartyi* Stamm 195 aufzuklären. Beide Stämme bildeten Ethen als Produkt einer Dichlor-Eliminierung, während nur geringe Mengen an VC in einer Nebenreaktion gebildet wurden. Ausschließlich Stamm BTF08 kann VC zu Ethen metabolisch reduktiv dechlorieren. Neben Chlorethenen und Chlorethanen wies Stamm BTF08 eine Aktivität der reduktiven Dechlorierung auch für höher chlorierte Chlorbenzole auf, wie z. B. Penta- und Hexachlorbenzol zu Trichlorbenzol (TCB). Ein Abbau von niederchlorierten Benzolen wie DCB und MCB wurde nicht nachgewiesen. Im Gegensatz dazu bildet Stamm BTF08 MCB als Endprodukt im anaeroben Abbau von HCH (γ-Hexachlorozyklohexan, Lindan), welches auch am Feldstandort Bitterfeld detektiert wurde.

Eine vollständige Mineralisierung von MCB unter nitrat- und eisenreduzierenden Bedingungen wurde erstmals in anaeroben Labor-Grundwassermikrokosmen, welche mit kontaminiertem Feldstandort-Grundwasser angesetzt wurden, nachgewiesen. Zusätzlich wurden in anaeroben Labor-Sedimentmikrokosmen erste Hinweise für eine reduktive Dechlorierung von DCB zu MCB und Benzol, gefolgt von einer möglichen vollständigen Mineralisierung zu CO_2 und CH_4, erbracht. Diese Mikrokosmen wurden mit Sediment aus den Modell-Wetlands angesetzt, welches ein Habitat für die indigene mikrobielle Gemeinschaft des kontaminierten Grundwassers darstellt. Reduktive Dechlorierung und Mineralisierung von MCB wurden als potenzielle mikrobielle Abbauwege festgestellt, die wahrscheinlich zum Abbau der Grundwasserkontaminanten in den zwei Modell-Wetlands führten. Die Biotransformation von MCB im Kiesbett der Modell-Wetlands wurde durch die vorherrschenden geochemischen Bedingungen und Gradienten unterstützt, wobei Eisen- und Sulfatreduktion die relevanten Redoxprozesse in beiden Modell-Systemen darstellten. In beiden Modell-Wetlands wurden insgesamt mehr als 90 % der initialen MCB-Konzentration beseitigt.

Des Weiteren wurde die Rolle der indigenen mikrobiellen Grundwassergemeinschaften im Schadstoffabbau untersucht. Es wurden dabei die Ziele der Charakterisierung der mikrobiellen Grundwassergemeinschaft (Bakterien und Archaeen) sowie der Untersuchung der Zusammensetzung, Struktur und Dynamik im Wetland-Kiesbett verfolgt. Die indigene Mikrobengemeinschaft des Grundwasser umfasste methanogene Archeen, Sulfat- (Gattung: *Desulfobacterium*) und Eisenreduzierende Bakterien (Gattung: *Geobacter,* welche auch halogenierte Substanzen dechlorieren) sowie *Chloroflexi. Chloroflexi* ist ein Phylum, in dem Bakterien vertreten sind, die zur reduktiven Dechlorierung von diversen Chlorkohlenwasserstoffen fähig sind. Die Besiedlung und Verteilung der mikrobiellen Gemeinschaft im Kiesbett des Modell-Wetlands war abhängig von den vorherrschenden Redoxbedingungen, einschließlich der Verfügbarkeit von Sauerstoff mit zunehmender Tiefe.

Abschließend wurde in einem Feldversuch ein Grundwasserleiter in Ferrara (Italien) hinsichtlich der Kontamination mit niederchlorierten Ethenen und Ethanen untersucht. Dieser Standort hat eine langjährige Tradition in der industriellen Herstellung von chlorierten Lösungsmitteln. Mehrere Kontaminationsfahnen wurden in diesem Multi-Aquifer-System identifiziert, welche möglicherweise ehemaligen industriellen und kommunalen Deponien oder Industrieunfällen entstammen. Das Ziel dieser Studie war es, den Ursprung der Kontamination sowie eine mögliche Biotransformation zu untersuchen. Die stabile Kohlenstoff-Isotopenanalyse der untersuchten Substanzen ergab, dass am Standort zwei unterschiedliche Quellen von chlorierten Lösungsmitteln präsent sind, die wahrscheinlich unterschiedlichen Produktionslagern

entstammen. Des Weiteren wurden mit Hilfe der stabilen Kohlenstoff-Isotopenanalyse Hinweise auf biologische Abbauprozesse erbracht.

Diese Forschungsarbeit ermöglicht durch die umfassende Anwendung eines integrativen Ansatzes erstmalig eine tiefere Einsicht in die anaerobe Biotransformation von niederchlorierten Kohlenwasserstoffen. Niederchlorierte Kohlenwasserstoffe werden ausschließlich *in situ* abgebaut, wenn die in dieser Arbeit untersuchten wesentlichen hydro-geochemischen und biologischen (Mikroorganismen) Bedingungen vorliegen.

Abbreviation

BTEX	benzene, toluene, ethylbenzene and xylenes
CAH	chlorinated aliphatic hydrocarbons
CAs	chloroethanes = chlorinated ethanes
CBs	chlorinated benzenes = chlorobenzenes
CEs	chloroethenes = chlorinated ethenes
CF	chloroform
CSIA	compound specific isotope analysis
CW	constructed wetland
DCA	dichloroethane
DCB	dichlorobenzene
DCE	dichloroethene
DDT	dichlorodiphenyltrichloroethane
DGGE	Denaturing Gradient Gel Electrophoresis
DO	dissolved oxygen
GC-FID	gas chromatography-flame ionisation detector
GC-IRMS	gas chromatography-isotope ratio mass spectrometry
GC-MS	gas chromatography-mass spectrometry
HCH	hexachlorocyclohexane
MCB	monochlorobenzene
OHRB	organo halide respiring bacteria
PAHs	polycyclic aromatic hydrocarbons
PCB	pentachlorobenzene
PCE	pentachloroethene
PCP	pentachlorophenol
PCR	polymerase chain reaction
PeCA	pentachloroethane
PLFAs	phospholipids fatty acids
PVC	polyvinyl chloride
qPCR	quantitative polymerase chain reaction
SIP	stable isotope probing

TCA	trichloroethane
TCB	trichlorobenzene
TCE	trichloroethene
TeCA	tetrachloroethane
TeCB	tetrachlorobenzene
TeCCH	tetrachlorocyclohexane
TRFLP	terminal restriction fragment length polymorphism
U.S.EPA	United States Environmental Protection Agency
VB	vinyl bromide
VC	vinyl chloride
ε	epsilon, isotope enrichment factor

List of Tables and Figures

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Preface

The present dissertation investigates the transformation of two chemical compound classes: chlorinated hydrocarbons and chlorinated aromatic hydrocarbons. The biotransformation of higher chlorinated solvents is well studied. However, there is a lack of knowledge regarding the biotransformation of the lower chlorinated ones, especially monochlorobenzene and vinyl chloride, which tend to accumulate in the environment and may form long term contamination plumes. Thus, not only a scientific interest emerges on understanding how these solvents are biologically produced and more important how they get degraded, but also a responsibility for the society to keep and improve groundwater quality, potentially affected by chlorinated solvent pollution.

Lower chlorinated compounds are more frequently detected in contaminated groundwater, often more toxic than higher chlorinated ones and therefore constitute the most problematic contaminants in the environment, while being at the same time the degradation product or even the dead end product of higher chlorinated hydrocarbons.

The present research focuses on investigating the biotransformation of 1,2-dichloroethane (1,2-DCA), dichloroethene (*cis*-DCE, *trans*-DCE 1,1-DCE), vinyl chloride, monochlorobenzene (MCB) and dichlorobenzene (1,2-DCB, 1,3-DCB and 1,4-DCB) at different scales. The listed chlorinated solvents were detected and analysed in two contaminated field sites: the multi-contaminated aquifer in Bitterfeld/ Wolfen (Germany) (Chapter 2, 5, 6) and Ferrara/Caretti site (Italy) (Chapter 4). They were both analysed for characterising biotransformation pathways of chlorinated aromatic hydrocarbons in groundwater microcosms and constructed treatment wetland systems as well as for applying source apportionment of the chlorinated hydrocarbons and additionally to investigate their fate, respectively. Interestingly, the above mentioned chlorinated ethenes (CEs) and ethanes (CAs) as well as higher chlorinated aromatic hydrocarbons are included in the broad dechlorination spectrum of a previously obtained *Dehalococcoides* enrichment culture obtained from the contaminated field site Bitterfeld (Chapter 2), potentially involved in the microbial transformation of present chlorinated solvents, contributing to the fate of particular contaminants at the field site.

Remediation strategies require the estimation of plumes' natural attenuation potential as well as the determination of responsible polluters in order to address remediation expenses. The origin and potential biodegradation of VC or 1,2-DCA in the aquifers was investigated at the field site Ferrara (Italy) and is presented in Chapter 4.

In the context of bioremediation, laboratory systems and constructed wetlands of different dimension were used for determining geochemical redox conditions and for characterising MCB and DCB degradation pathways *in situ*. Constructed wetlands represent nature-like systems with interfaces of anoxic groundwater and oxic surface water and are known to be good wastewater treatment plants. Moreover the microbial community dominating such systems and potentially being involved in the fate of these groundwater contaminants was characterised (Chapter 5 and 6).

The research and experiments for this thesis were performed from December 2009 to December 2013 at the Helmholtz Centre for Environmental Research, Leipzig in the Department of Isotope biogeochemistry, supervised by Dr. Ivonne Nijenhuis. The academic supervision at the Martin-Luther-University of Halle-Wittenberg in the Institute of Biology/Microbiology was taken by PD Dr. habil. Ute Lechner. The thesis was written in a cumulative form, based on the following four in peer-reviewed journals published articles and on one manuscript in preparation (*) for submission to a scientific journal.

Kaufhold, T., **Schmidt, M.**, Cichocka, D., Nikolausz, M. and Nijenhuis, I. (2013) Dehalogenation of diverse halogenated substrates by a highly enriched *Dehalococcoides*-containing culture derived from the contaminated mega-site in Bitterfeld. *FEMS Microbiology Ecology* 83(1), 176-188.

Schmidt, M., Lege, S. and Nijenhuis, I. (2014) Comparison of 1,2-dichloroethane, dichloroethene and vinyl chloride carbon stable isotope fractionation during dechlorination by two *Dehalococcoides* strains. *Water Research*. 52: 146–154.

Nijenhuis, I., **Schmidt, M**., Pellegatti, E., Paramatti, E., Richnow, HH. and Gargini, A. (2013) A stable isotope approach for source apportionment of chlorinated ethenes plumes at a complex multi-contamination events urban site. *Journal of Contaminant Hydrology.* 153: 92–105.

Schmidt, M., Wolfram, D., Birkigt, J., Ahlheim, J., Paschke, H., Richnow, HH. and Nijenhuis, I. (2014) Iron oxides stimulate microbial monochlorobenzene in situ transformation in constructed wetlands and laboratory systems. *Science of the Total Environment.* 472: 185-193.

Markantonis, M., Imfeld, G., Klein, BS., Richnow, HH. and Nijenhuis, I. Microbial diversity and activity in a monochlorobenzene removing sulfate reducing wetland (*)

Note that text passages and figures in Chapter 7 are partly taken from the original publication without further indication. The abstracts of the original publications were included as Chapters 2-5.

Declaration on the Author Contribution

Kaufhold T, **Schmidt M**, Cichocka D, Nikolausz M & Nijenhuis I (2013) Dehalogenation of diverse halogenated substrates by a highly enriched *Dehalococcoides*-containing culture derived from the contaminated mega-site in Bitterfeld. *FEMS Microbiology Ecology* 83: 176-188.
doi: 10.1111/j.1574-6941.2012.01462.x

Own contribution:

I. Nijenhuis developed the concept of this study. During the experimental realisation I performed and evaluated the taxon-specific q-PCR of the 16S rRNA genes (experimentation: 30 % and q-PCR data analysis and evaluation: 100 %). I was involved in the writing and editing process of a first version of the manuscript (prepared by T. Kaufhold) and finalised together with the coauthors I. Nijenhuis and M. Nikolausz the manuscript for submission. Therein I wrote in the material and methods section method description of real time PCR and statistical evaluation. In the results part I wrote a first version of the chapter 'Growth of *Dehalococcoides*' and was involved in writing sections in the discussion. Finally I prepared Table 2 in the main text and in the Supporting information Table S3, Table S4, and the section qPCR 'calibration curves', 'statistical analysis' and 'Calculation of growth yield per halogen released' (manuscript writing: 30 %).

Nijenhuis I, **Schmidt M**, Pellegatti E, Paramatti E, Richnow HH & Gargini A (2013) A stable isotope approach for source apportionment of chlorinated ethenes plumes at a complex multi-contamination events urban site. *Journal of Contaminant Hydrology*. 153: 92–105.
doi: 10.1016/j.jconhyd.2013.06.004

Own contribution:

I. Nijenhuis, H. Richnow and A. Gargini developed the concept of the study. I was partly involved in the experimentation by supervising and supporting the performance of the isotope analysis and evaluate the isotope data (10 %). During the writing and editing process of the manuscript I wrote the paragraph on chlorine industry and methane extraction in Northern Italy and described the industrial production of chlorinated gases and hydrocarbons in the supporting material (15-20 %).

Schmidt M, Wolfram D, Birkigt J, Ahlheim J, Paschke H, Richnow HH & Nijenhuis I (2014) Iron oxides stimulate microbial monochlorobenzene in situ transformation in constructed wetlands and laboratory systems. *Science of the Total Environment*. 472: 185-193.
doi: 10.1016/j.scitotenv.2013.10.116

Own contribution:

I developed with I. Nijenhuis the concept of the manuscript and I prepared a first, second and third version of it and finalised the whole manuscript for submission after an internal reviewing round by the coauthors (writing: 80 %). The realisation of the experiments and monitoring work was a two-person responsibility, proportionately shared by D. Wolfram and me. We collected,

analysed and evaluated the samples from the wetland. The preparation, sampling and evaluation of the laboratory microcosm were significantly my work (experimentation: 65 %), including the evaluation of data (75 %). Moreover I designed all figures in the main text and for the supporting material. I calculated the Principal component analysis (PCA) with R based on an existing algorithm prepared and modified by G. Imfeld.

Schmidt M, Lege S & Nijenhuis I (2014) Comparison of 1,2-dichloroethane, dichloroethene and vinyl chloride carbon stable isotope fractionation during dechlorination by two *Dehalococcoides* strains. *Water Research.* 52: 146–154
doi: 10.1016/j.watres.2013.12.042.

Own contribution:
The concept of the study originated from I. Nijenhuis. I prepared a first version of the manuscript and finalised the whole manuscript for submission in team work with I. Nijenhuis. I prepared all figures, graphs and tables, as well as the supporting material (writing: 60 %). The fractionation experiments with 1,2-DCA for *Dehalococcoides mccartyi* strain 195 and strain BTF08 were realized by myself (experimentation 50 %). S. Lege carried out the VC, 1,1-DCE and *cis*-DCE fractionation experiment in the framework of his bachelor thesis The analysis, evaluation and synthesis of the all data were significantly my work (75 %).

Markantonis, M., Imfeld, G., Klein, B.S., Richnow, HH. and Nijenhuis, I. Microbial diversity and activity in a monochlorobenzene removing sulfate reducing wetland [In preparation]

Own contribution:
The concept of the manuscript was developed in cooperation with I. Nijenhuis and G. Imfeld. I prepared a first version of the manuscript, including all figures and tables. The manuscript was finalised in accordance with the co-authors (writing: 85 %). The experimental part (experimentation and data evaluation 100 %) was realised with support of B.S. Klein (in preparing a bacterial clone library and wetland sampling), whereas the evaluation and synthesis of all results was mainly performed by me.

Marie Markantonis (born Schmidt)

The declaration on the author contribution was certified by author and co-author.

Marie Markantonis Dr. Ivonne Nijenhuis

Structure of the Thesis

The doctoral thesis is organized in 6 Chapters and the Appendix.

Chapter 1 gives a general introduction into the current knowledge and previous research on the biotransformation of lower chlorinated hydrocarbons, including chemical properties, applications, sources and remediation approaches in the environment, the microbial degradation and the current methodology applied to assess biodegradation of chlorinated solvents and the microbial community. The research objectives of this thesis are pointed out at the end of this Chapter.

Chapter 2 identifies the substrate range of a *Dehalococcoides*-dominated enrichment culture which originated from the contaminated field site in Bitterfeld. The growth of *Dehalococcoides* was proven, applying molecular biological based methods. Additionally the diversity of the bacterial community and reductive halogenase genes present in this bacterial culture was assessed and discussed (now: *Dehalococcoides mccartyi* strain BTF08).

Chapter 3 investigates the reductive dechlorination mechanism of 1,2-DCA, DCE and VC by *Dehalococcoides mccartyi* strain BTF08 in comparison to *Dehalococcoides mccartyi* strain 195 by using compound specific isotope analysis (CSIA) .

Chapter 4 describes the main findings in a multi-contaminated aquifer system field study in Ferrara (Italy), where CSIA was applied to identify sources of a chloroethene plume in a complex contaminates urban site.

Chapter 5 and **6** focus on the anaerobic biotransformation of MCB and DCB at geochemical gradients in constructed wetlands where:

Chapter 5 examines the degradation activity of the groundwater microbial community and elucidates the transformation pathways in anaerobic groundwater microcosms and in pilot-scale constructed wetlands *in situ* and

Chapter 6 combines the hydro geochemical characterisation of a small pilot-scale constructed wetland, including MCB biotransformation parameter, with the investigation of the groundwater microbial community composition. The relationship of spatial distribution of the microbial community pattern in the wetland sediment and its potential for oxidative and reductive dechlorination of MCB and DCB are critically discussed (manuscript in preparation).

Chapter 7 highlights and discusses main results of this thesis with reference to the following Chapters 2-5, which are already published in peer-reviewed journals and to Chapter 6 (manuscript in preparation) and closes with an outlook and future perspectives.

Chapter 1- General Introduction

I. Lower Chlorinated Solvents as Groundwater Contaminants

1. Sources, Pollutants and Risks

Lower chlorinated solvents are groundwater contaminants of great concern due to their large environmental releases, microbial formation, human toxicity, potential carcinogenicity and tendency to persist in and migrate with groundwater to drinking water supplies, exposing health risk to human and groundwater dependent eco-systems (Zogorski *et al.*, 2006).

The lower chlorinated benzenes (CBs), MCB and DCB are cyclic aromatic hydrocarbons (WHO, 2003a, 2004a), DCB exits in the three isomers: 1,2-DCB (*o*-DCB), 1,3-DCB (*m*-DCB) and 1,4-DCB (*p*-DCB). MCB and 1,2-DCB are colourless liquids, which exhibit low water solubility (SW) and a low octanol/water (log K_{OW}) partition coefficient (Table 1). MCB and 1,2-DCB are more volatile than higher CBs, as the vapour pressure (PV) decreases with increasing number of chlorine substituents. Chlorobenzenes (CBs) are listed as priority environmental pollutants (U.S.EPA, 2009), due to their toxicity for aquatic organisms represented by the low maximum contaminant level (MCL) of 0.1 mg/L for CBs in drinking water (U.S.EPA, 2013b). The low water solubility of MCB in water results in its accumulation and persistence in groundwater reservoirs and therefore makes MCB a recalcitrant and ubiquitous groundwater contaminant (U.S.EPA, 2009).

Historically MCB was used as an intermediate in pesticides and solvents production, e.g. as main precursor for phenols and dichlorodiphenyltrichloroethane (DDT). Nowadays MCB is mainly produced during manufacturing of herbicides, drying substances and rubbers (Rossberg, 2006). 1,2-DCB is an insecticide (termites and borers), softener, degreasing agent, and used for making dyes (U.S.EPA, 2013a). 1,4-DCB was applied as pesticide and space deodorant for toilets (U.S.EPA, 2000). Major amounts of MCB and DCBs were produced worldwide, including MCB production of 102 000 t y^{-1} in 1980s in Western Germany and 103 000 t y^{-1} in the US (Weissermel and Arpe, 1988) and DCBs production of around 50 000 t y^{-1} was estimated (Koch, 1995). CBs are released into the environment during manufacture, through accidental spillage, leakage of storage tanks, inadequate usages and disposal practices and thus affect groundwater reservoirs and groundwater related systems, worldwide.

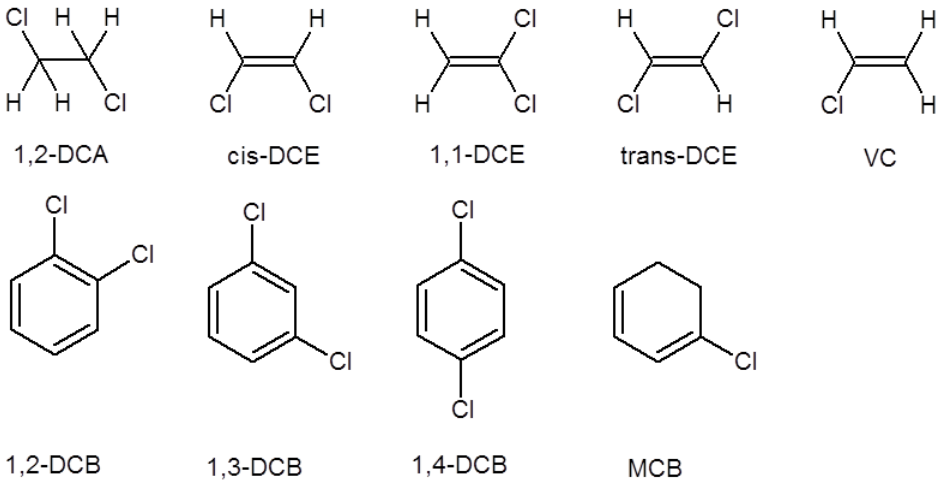

Figure 1.1: *Structural formulas of the model compounds: 1,2-DCA (1,2-dichloroethane),* cis-*DCE (*cis-*dichloroethene) 1,1-DCE (1,1-dichloroethene),* trans-*DCE (*trans-*dichloroethene), VC (vinyl chloride), 1,2-DCB (1,2-dichlorobenzene), 1,3-DCB (1,3-dichlorobenzene), 1,4-DCB (1,4-dichlorobenzene) and MCB (monochlorobenzene)*

Among the lower chlorinated ethenes (CEs) and ethanes (CAs), which are all toxic and suspected to be carcinogenic (EPA, 1980c, a, d, b), in particular vinyl chloride (VC), dichloroethene (DCE) isomers (*cis*-1,2-DCE, *trans*-1,2-DCE and 1,1-DCE) and 1,2-dichloroethane (1,2-DCA) are the most problematic groundwater contaminants due to their low biodegradability in absence of oxygen and consequent accumulation. The carcinogenic gaseous monomer VC, with mild sweet odour, is one of the most used substances worldwide for manufacturing polyvinyl chloride (PVC) (WHO, 2004b). Formerly it was briefly used as refrigerant and inhalational anaesthetic (ATSDR, 1996), but now it is prohibited due to its potential health risk. However VC is still contained in small amounts in furniture and automobile parts (ATSDR, 2006). The two 1,2-dichloroethene isomers, *cis*-DCE and *trans*-DCE, were primarily used as chemical intermediates for the production of other chlorinated compounds (ATSDR, 1996). Additionally, 1,2-DCA, a flammable, colourless and liquid chlorinated alkane with sweet odour, which functions as intermediate in plastic production, was mainly used for the synthesis of other chemicals and also applied as fumigant (ATSDR, 2001).

The limit for chlorinated solvents in drinking water was set by the US EPA 5 µg L^{-1} for 1,2-DCA (3 µg L^{-1} by European Union (EU-EPA)), to 70 µg L^{-1} and 100 µg L^{-1} for *cis*- and *trans*-1,2-DCE, to 0.5 µg L^{-1} for VC, respectively (U.S.EPA, 2012). Historically, more than 100 thousand tons of 1,1-DCE alone, were produced worldwide in the early 1980s (WHO, 2003b), whereas global VC production in 1985 was 13,500,000 t (Ullmann, 1997) in 1977 in Europe 3,500,000 t (Rippen, 1989) and alone in Germany in 1988 1,459,000 t (Rippen, 1991).Over 5000 kilotons of 1,2-DCA were produced in 1977 in the European countries (Atri, 1984). This intense

production of CEs and CAs and their various applications in the chemical industry during the last decades, as well as their inappropriate storage and disposal in the environment, has led to contamination of soils, sediments, groundwater aquifers, and subsurface environments worldwide (WHO, 2003c).

Chlorinated hydrocarbons have a hazardous effect on aquatic environments due to their considerable water solubility which leads to good mobility in soil and water (Koch, 1995). Widespread groundwater contaminations and the formation of long groundwater plumes in aquifer environments are the result of the good mobility of chlorinated solvents in water (Moran *et al.*, 2007; Nijenhuis *et al.*, 2007a), as presented for two contaminated field sites in Chapter 1 Section I.2). Moreover the sorption to organic material and uptake into living organisms impacts the bioavailability, biodegradability and persistence of chlorinated hydrocarbons in soils and sediments (Pignatello and Xing, 1996; Zhang and Bouwer, 1997). Hence sorption of chlorinated volatile organic compounds (VOCs) to soils and sediments does not only cause long-term contaminant sources (Ball and Roberts, 1991) but also reduces bioavailability. Additionally desorption resistant fractions may be formed, which are not or only slowly biodegraded (Lee *et al.*, 2003).

Table 1.1: *Physico-chemical characteristics of the model compounds MCB, 1,2-DCB, 1,4-DCB, VC,* cis*-DCE and 1,2-DCA in their standard state at 25°C. M: molecular mass; ρ: density; PV: vapor pressure; SW: water solubility; log KOW: octanol water partition coefficient; H: Henry's law constant.*

	M [g mol^{-1}]	ρ[g cm^{-3}]	PV [hPa]	SW [mg L^{-1}]	log K_{OW}	H [Pa m^3 mol^{-1}]
MCB	112.6	1.11[4]	16.7[2]	484[1]	2.84[4]	375[2]
1,2-DCB	147.01	1.31[4]	0.19[5]	156[3]	3.43[6]	198[5]
1,4-DCB	147.02	1.25[4]	0.09[5]	91[4]	3.44[4]	160[5]
VC	62.49	0.91 (20 °C)[4]	2300[4]	1100[4]	0.60[4]	2200[3] (20 °C)
cis-DCE	96.95	1.28[4]	200[6]	3500[4]	1.86[4]	1970[4]
1,2-DCA	98.97	1.25[4]	87 (20 °C)[8]	870 (20 °C)[8]	1.86[7]	112[8] (20 °C)

[1] Koch (1995); [2] van Agteren (1998); [3]Montgomery (2007a), [4] Mackay *et al.* (2006); [5] Malcolm *et al.* (2004), [6] Chin (2013), [7] ATSDR (2001), [8] (Montgomery, 2007b)

2. Contaminated Aquifer Field Sites in Europe

Groundwater contamination with chlorinated solvents is a global environmental issue worldwide, indicated in a huge number of contaminated field sites. Their major environmental hazard is the threatening of drinking water resources. As estimated, Europe has about 3 million potentially contaminated field sites (EEA, 2007). Main contaminants affecting soil and groundwater in Europe are heavy metals (37.3 %), mineral oils (33.7 %), polycyclic aromatic hydrocarbons (PAH), aromatic hydrocarbons (BTEX), phenols, chlorinated hydrocarbons and others, whereas chlorinated hydrocarbons are a small part (2.4 %) (EEA, 2007). Germany has over 250'000 contaminated/ potentially contaminated field sites (EEA, 2007). A similar number was estimated in the USA with approximately 294'000 contaminated sites (Bombach *et al.*, 2010a). Two contaminated field sites, Bitterfeld/ Wolfen (Germany) and Ferrara (Emilia-Romagna and Po river region, Italy), which are both related to chemical industry and improper waste disposal, but also from potential current use, were subject of the present thesis.

In Germany one of the biggest contaminated mega sites is located in the area of Bitterfeld/ Wolfen (Saxony-Anhalt, Figure 2A), comprising a contaminated area of 25 km^2 with an aquifer of about 200 million m^3 of groundwater (Weiß *et al.*, 2001), which is heavily polluted mainly with chlorinated aliphatic compounds, including CBs (MCB and 1,2-DCB), hexachlorocyclohexanes (e.g. γ-HCH =Lindane), benzene, toluene, ethylbenzene and xylene (BTEX) and dichlorodiphenyltrichloroethane (DDT) isomers (Thieken, 2002). The aquifer contamination originated from the intense production of up to 5000 different chlorinated substances, like chlorinated solvents, pesticides and dyes by the chloro-organic industry in the 19^{th} century (Tragsdorf *et al.*, 1993; Walkow, 2000). The continuous discharge of contaminants from waste disposal in abandoned open pit mines continues to pollute groundwater (Heidrich *et al.*, 2004). MCB was detected as main contaminant in the runoff of the former production and waste facilities with concentrations up to 23 mg L^{-1} (Heidrich et al., 2004).

Similar to the Bitterfeld field site, also in Ferrara, located in the Emilia-Romagna and Po river region (North Italy) in the urban and residential area of the city Ferrara (Figure 2B), wastes were dumped in open pit mines. North-west of the city of Ferrara, one of the main Italian petrochemical plants (250 ha) is located. It hosts numerous companies, manufacturing various chemicals, including PVC (polyvinyl chloride) and VC, produced from 1953 to 1998 and chloromethanes from 1951 to 1984. Chlorinated aliphatic hydrocarbons (CAH) were detected with concentrations up to 100 mg L^{-1} in the main aquifers, (Pasini *et al.*, 2008).

Plumes with different composition, concentrations and dimensions migrated and spread from local and suspected sources due to inconsistent groundwater regimes, pumping well-

remediation and municipal water-supply activities in the aquifers. The contaminant plume, below the residential areas at the Caretti site (Figure 2B), contains high concentrations of various CEs, CAs and chloromethanes, e.g. PCE (tetrachloroethene), TCE (trichloroethene), DCE, VC, PCA (tetrachloroethane), TCA (trichloroethane), DCA, CF (Chloroform) (Pasini et al., 2008; Gargini *et al.*, 2011). A second plume at Pontelagoscuro, called “Pandora plume” (Figure 2B), mainly contains VC, with maximal concentration of up to 700 µg L^{-1} (Pasini et al., 2008). Primary sources of the CAH contamination in the residential area were assumed to be related to either old dump sites (1950s) like uncontained landfills and open pits or to ‘new’-industrial production (1980s). The severe groundwater contamination by CAH was categorized by potential types of contamination with respect to their origin of either (I) industry or (II) dump-source. Independent from the source, the dumping sites affect residential development areas nowadays, in particular in the case of the Caretti site where houses were built in the area close to previous dump sites.

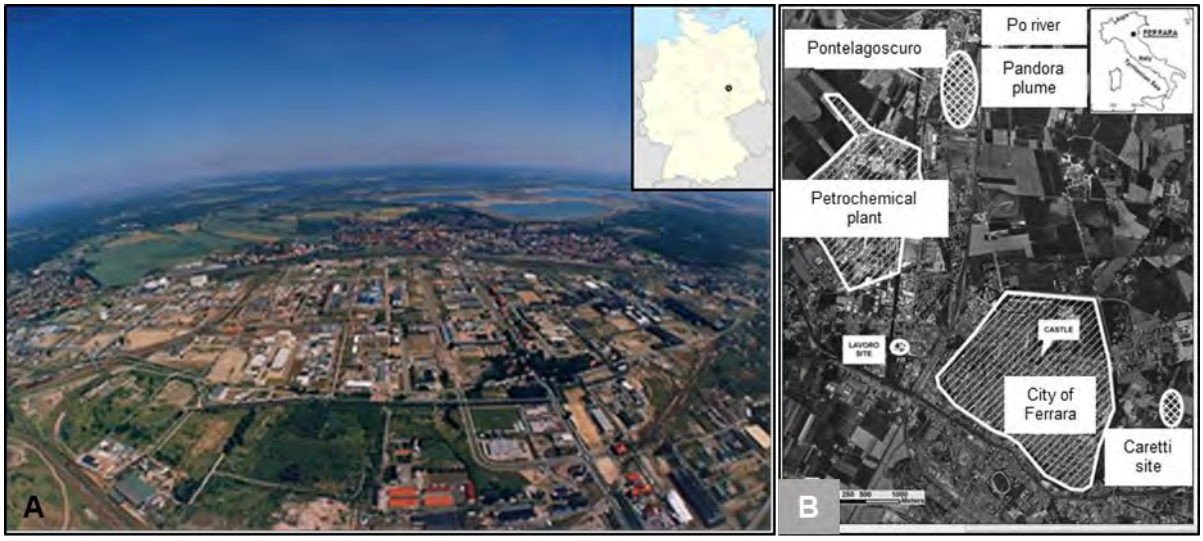

Figure 1.2: *Field sites surveyed in the course of the presented thesis: (A) Aerial image of the field site Bitterfeld/ Wolfen (Weiß et al., 2004) (B): Petrochemical plant and VC contaminated site in the Ferrara area. Adapted from Pasini et al. (2008)*

3. Remediation Options for Chlorinated Hydrocarbons

Groundwater remediation options for chlorinated hydrocarbons are various and can be applied *in situ* or *ex situ* (Farhadian *et al.*, 2008). A general classification of current *in situ* and *ex situ* treatment options is derived from the primary treatment principle, thus one is differentiating between physical, chemical and biological methods. A great number of physical and chemical methods are applicable towards removing chlorinated groundwater contaminants. At the same time biological treatment methods promise a good remediation alternative, which is addressed below. Well-known examples of physical treatment include air sparging, e.g. air stripping for removing volatile chlorinated solvents (Gavaskar *et al.*, 1995); adsorption on activated carbon (AC) (Yu and Chou, 2000) or zeolites, extraction and electro-kinetic separation (USAEC, 1997). Chemical decomposition of contaminants can be achieved by oxidation with ozone or hydrogen peroxide (Bellamy *et al.*, 1991; Gates and Siegrist, 1995; Weir *et al.*, 1996), reduction at reactive walls of zero-valent iron (Phillips *et al.*, 2010) or at Pd metal catalysts (Kopinke *et al.*, 2003; Hildebrand *et al.*, 2009) immobilization, combustion, UV radiation (Roland *et al.*, 2007) and heterogeneous catalysis (Roland *et al.*, 2005). In several so called active *in situ* remediation strategies pump-and-treat technologies are applied, which includes pumping out contaminated water of the aquifer and usually subsequent treatment in above-ground reactors (Shevah and Waldman, 1995). The other namely passive *in-situ* technologies refer to the installation of reactive walls and barriers and *in situ* reactors (funnel & gate) (Starr and Cherry, 1994), containing integrated reactive materials such as aforementioned Fe(0), Zn, Pd, clay or activated carbon. Such technologies enable physical adsorption and subsequent chemical conversion of groundwater contaminants directly in the aquifer or soils.

In general, except biological methods, the above mentioned conventional remediation-techniques still demand high energy and investment costs due to the pumping of large water volumes for long time periods (Travis and Doty, 1990; Weiß et al., 2001). In addition at large-scale contaminated sites (industrial mega sites, USEPA Superfund sites) like the Bitterfeld mega-site (Weiß et al., 2001) introduced in Chapter 1, section I.2, a total remediation of contaminated groundwater is often technically and financially not feasible (Travis and Doty, 1990; Starr and Cherry, 1994; Shevah and Waldman, 1995; McGovern *et al.*, 2002; Birke *et al.*, 2003).

Thus, biological treatment methods have the potential to replace conventional ones due to their lower maintenance costs and energy demands (Sinha *et al.*, 2009) and the use of the natural degradation potential of microorganisms and plants. Bioremediation of chlorinated hydrocarbon contaminated groundwater can be carried out *in situ* by biostimulation and

bioaugmentation (Shevah and Waldman, 1995) as well as in bioreactors (Vogt *et al.*, 2002) or constructed wetlands (CW). CWs have been effective in removing a huge spectrum of organic and inorganic contaminants, like CEs (Mastin *et al.*, 2001; Kassenga and Pardue, 2002; Novak *et al.*, 2002; Lorah and Voytek, 2004; Imfeld *et al.*, 2008a; Tawney *et al.*, 2008), benzene (Rakoczy *et al.*, 2011), MCB (Braeckevelt *et al.*, 2007a; Braeckevelt *et al.*, 2007b), pesticides e.g. hexachlorocyclohexane (Brunke and Gonser, 1997; Bhatt *et al.*, 2009) or ammonium (Zeb *et al.*, 2013) and metalloids e.g. arsenic (Lizama *et al.*, 2011).

The concept of biological treatment, includes the term Natural attenuation, which applies to a multitude of *in situ* processes including decay (radioactive), biodegradation, chemical or biological stabilization, destruction, dilution, dispersion, sorption, transformation or volatilisation. Natural attenuation processes typically occur at most contaminated sites but its effectiveness is controlled by the contaminants of concern, and the physical, chemical, biological and hydrogeological properties of the soil and groundwater. Natural attenuation processes are largely complex oxidation and reduction processes and under favourable conditions they can reduce the risks to human health and the environment (U.S.EPA, 1997). Fundamental requirements for the successful application of natural attenuation (NA) are: (i) reasonable biodegradability of the pollutants, (ii) availability of microorganisms capable to metabolise the present pollutants, and (iii) convenient geochemical conditions (e.g. electron donors/acceptors, redox potential (Eh), temperature (T), nutrients) for microbial degradation, which are operationalized in the concept of MNA (monitored natural attenuation)(Scow and Hicks, 2005). These three conditions are summarizing the three lines of evidence that need to be proven for a successful description of *in situ* natural attenuation of contaminants.

However, biodegradation processes for lower chlorinated solvents under anoxic condition, as present in groundwater aquifers, are reported to generally proceed slowly, due to a lack of appropriate electron acceptors for respiration (Suthersan, 1999). Therefore, a more thorough understanding of anaerobic degradation processes of lower chlorinated hydrocarbons will allow assessing and enhancing their natural (biological) remediation potential *in situ*. In consequence risks are mitigated and cost–effective remediation of contaminated soil and groundwater can replace cost and time consuming pumping and treatment techniques.

II. Microbial Transformation of Chlorinated Solvents

Microbial transformation of chlorinated solvents is an option for the biological treatment and natural remediation of contaminated waste water and groundwater, whereas VC and MCB are the most problematic ones. Thus it is of great importance to investigate biochemical and environmental conditions and their requirements towards an efficient microbial degradation.

The microbial transformation of chlorinated solvents can be classified in two main reaction types: (1) oxidative dechlorination (aerobic or anaerobic) and (2) anaerobic reductive dechlorination known as organohalide respiration (McCarty, 1997). In the first reaction type (1), chlorinated compounds function as electron donor and/or carbon source for bacterial growth. In response to these reactions, the compounds become mineralised utilizing oxygen as most energetically favourable electron acceptor or nitrate, iron (III), sulphate and carbon dioxide (Schlegel, 1992), whereas the reaction may be metabolically or cometabolically (Field and Sierra-Alvarez, 2004).

During anaerobic reductive dechlorination (2) chlorinated solvents function as electron acceptor, while chlorine atoms are cleaved and replaced by hydrogen atoms, however one has to differentiate between cometabolic and metabolic reactions. In this context, Gibbs free energies and redox potentials were estimated for a variety of halogenated aromatic and aliphatic compounds. The estimates suggest that reductive dechlorination yields the considerable energy amount of −130 and −180 kJ/mol per chlorine removed (Dolfing and Harrison, 1992; Dolfing and Janssen, 1994). The first described organohalide-respiring bacterium (OHRB) was the on 3-chlorobenzoate growing *Desulfomonile tiedjei* str. DCB-1 (Suflita *et al.*, 1982; Shelton and Tiedje, 1984), a δ- Proteobacterium, which couples the reduction of organohalide compounds to energy gain (Holliger *et al.*, 1998a; Smidt and de Vos, 2004). These findings changed the former concept of microbial metabolism, added CEs as primary substrates for energy metabolism, and turned the previous assumption of cometabolic to metabolic CE degradation. In principle, the tendency for a reductive dechlorination reaction increases for compounds with increasing chlorine atom numbers and thus highly oxidised compounds (e.g. PCE or higher CBs/ pentachlorobenzene (PCB)), (Vogel *et al.*, 1987; Vogel, 1994). In contrary lower chlorinated solvents such as VC, MCB and DCB tend to be more susceptible to oxidation.

Taking both reaction types (1) and (2) into consideration, a combination of microbial reductive dechlorination of higher chlorinated solvents under anaerobic conditions to lower-chlorinated products and their subsequent microbial oxidation to nontoxic CO_2 under both, anaerobic and aerobic conditions, would allow complete removal of chlorinated soil and groundwater contaminants (Coleman *et al.*, 2002b). Such process could be applied in

groundwater-surface water interfaces and wetlands characterized by geochemical conditions (McClain *et al.*, 2003; Imfeld *et al.*, 2009; Kadlec *et al.*, 2012). In such case, both reductive and oxidative degradation processes may proceed simultaneously or sequentially.

1. Microbial Transformation of MCB and DCB

Besides being primary contaminants, MCB and DCB are reductive dechlorination products of higher CBs (CBs) (van Agteren, 1998) such as trichlorobenzene (TCB) (Adrian *et al.*, 2000) or cyclic chlorohexanes like lindane (γ-HCH, e.g. review of Lal *et al.* (2010)). The reductive dechlorination of higher chlorinated CBs (Figure 3) (e.g. HCB, PCB, TeCB, TCB and DCB) was reported in various field and laboratory studies (Bosma *et al.*, 1988; Fathepure *et al.*, 1988; Holliger *et al.*, 1992; Ramanand *et al.*, 1993; Masunaga *et al.*, 1996; Nowak *et al.*, 1996; Rosenbrock *et al.*, 1997; Susarla *et al.*, 1998; van Agteren, 1998; Fung *et al.*, 2009; Stelzer *et al.*, 2009). However, the reaction often stops *in situ* at MCB (Holliger et al., 1992), which therefore frequently accumulates in anoxic environments due to the lower biodegradability compared to higher chlorinated CBs (Bosma et al., 1988; Fathepure et al., 1988; Ramanand et al., 1993; Masunaga et al., 1996; Susarla et al., 1998; Field and Sierra-Alvarez, 2008; Stelzer et al., 2009). The aerobic microbial degradation process of MCB and DCB is mainly characterized by the dioxygenase-catalysed degradation pathway via chlorocatechol intermediates. The latter was found in different genera of microorganism that are ubiquitous in soil, water and groundwater environments, with *Pseudomonas* and *Burkholderia* strains to name prominent representatives (Reineke and Knackmuss, 1984; Schraa *et al.*, 1986; Spain and Nishino, 1987; Haigler *et al.*, 1992; Müller *et al.*, 1996; van Agteren, 1998; Field and Sierra-Alvarez, 2008). In this case, the ring cleavage proceeds either via the ortho- (Rojo *et al.*, 1987) or meta- pathway (Mars *et al.*, 1997).

In contrast, all DCB isomers can be reductively dechlorinated (Bosma et al., 1988). First indications for reductive dechlorination of MCB to benzene were reported in a methanogenic mixed culture from the river sediment of the Saale River and in an anaerobic contaminated aquifer (Nowak et al., 1996; Kaschl *et al.*, 2005). Reductive dehalogenation of DCB isomers (1,2-DCB; 1,3-DCB and 1,4-DCB) and MCB to benzene was observed in microcosm studies and *Dehalobacter* sp. was identified as involved bacteria (Fung et al., 2009; Liang *et al.*, 2011; Nelson *et al.*, 2011). If benzene is the product of DCB and MCB dechlorination, it may then become oxidised under various terminal-electron-accepting processes (e.g. (Lovley, 2000; Chakraborty and Coates, 2004; Kleinsteuber *et al.*, 2012).

Benzene degradation was reported under nitrate-reducing (e.g. (Burland and Edwards, 1999; Coates *et al.*, 2001; Chakraborty and Coates, 2005), sulphate reducing (e.g. (Phelps *et al.*, 1996; Vogt *et al.*, 2007; Herrmann *et al.*, 2010), iron reducing (e.g. (Lovley, 1995; Jahn *et al.*, 2005; Botton and Parsons, 2006), and even under methanogenic (e.g. (Kazumi *et al.*, 1997; Weiner and Lovley, 1998; Masumoto *et al.*, 2012) conditions in lab studies. So far there are only a few studies, demonstrating MCB oxidation under different terminal-electron-accepting processes (excluding oxygen).

MCB transformation related to water treatment systems was already reported for subsurface flow constructed wetlands (Braeckevelt et al., 2007a; Braeckevelt et al., 2007b) and in MCB *in situ* and *ex situ* microcosm studies (Nijenhuis et al., 2007a; Stelzer et al., 2009). However, the conditions that the reactions require were not identified. Denitrification as well as ferric iron reduction, that are coupled to anaerobic oxidation of benzene (Dermietzel and Vieth, 1999; Vogt *et al.*, 2004), may be also potential requirements for anoxic MCB and DCB mineralisation *in situ*. Considering thermodynamics, even lower chlorinated CBs may, compared to nitrate and iron reduction, function as suitable electron acceptors exhibiting a standard redox potential ranging from +310 mV for MCB to +478 mV for HCB (Dolfing and Harrison, 1992). If reductive dechlorination occurs under anoxic *in situ* conditions it is depending on various factors, such as reaction kinetics (Dolfing and Harrison, 1992), type and availability of the alternative electron acceptors, temperature, substrate bioavailability, and an active community of dechlorinating bacteria (Adriaens and Vogel, 1995; Sulfita and Townsend, 1995; Wiedemeier, 1998).

To current knowledge, conversion rates of MCB in anoxic environments are very low and it is still unclear if the observed reductive dechlorination processes are metabolic or cometabolic (Adrian and Görisch, 2002). To date, degradation pathways are not yet thoroughly described (Nijenhuis et al., 2007a). However *Dehalobacter was* identified being capable for the reductive dechlorination of MCB and DCB (Nelson et al., 2011). Moreover, in the contaminated Bitterfeld groundwater it was initially indicated, that an unclassified betaproteobacterium may be involved in the biotransformation of MCB at the field site (Alfreider *et al.*, 2002; Martinez-Lavanchy *et al.*, 2011).

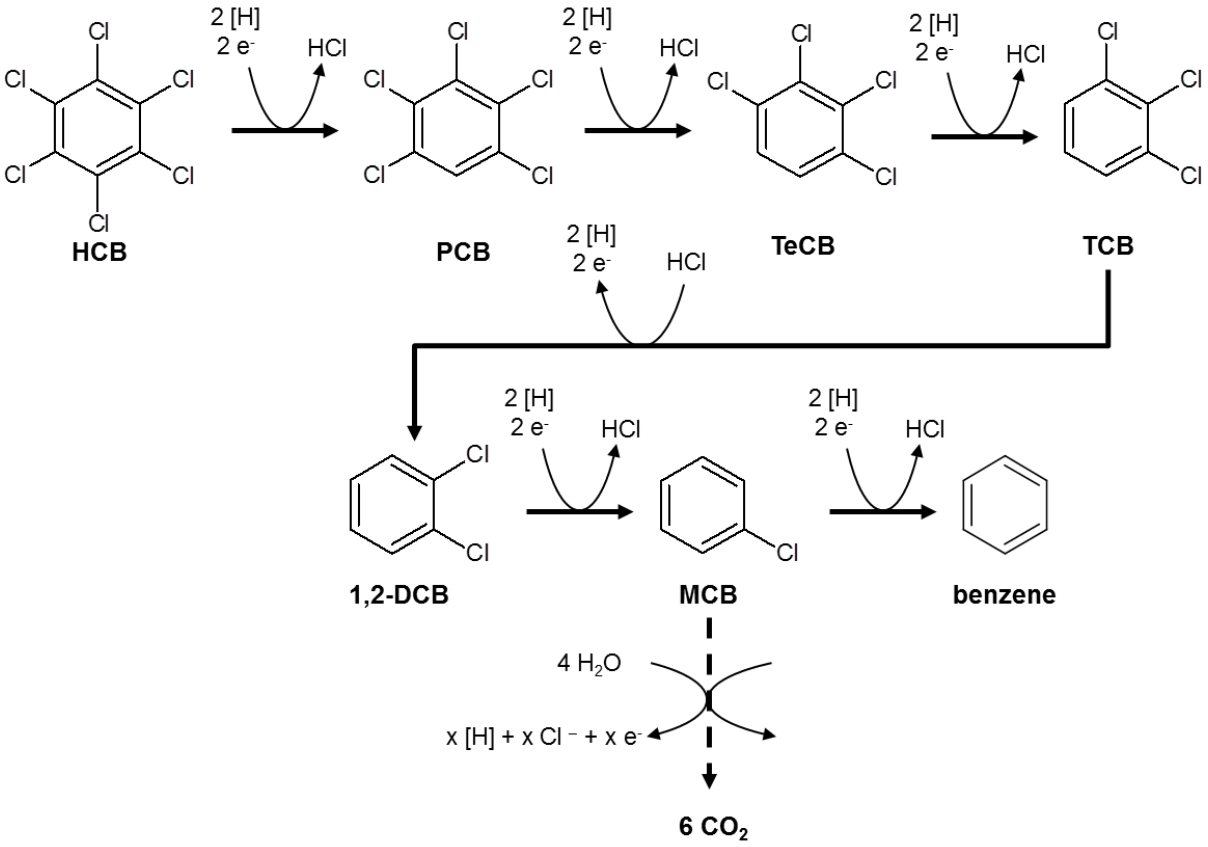

Figure 1.3: *Possible degradation pathways of chlorobenzenes (CBs) including dehalogenase catalyzed anaerobic microbial reductive dechlorination of higher CBs like hexachlorobenzene (HCB) via pentachlorobenzene (PCB) to Tetrachlorobenzene (TeCB), trichlorobenzene (TCB) to dichlorobenzene (DCB) and monochlorobenzene (MCB) to benzene (modified from van Agteren (1998)); and potential anaerobic oxidation of MCB to CO_2. Further benzene degradation under anaerobic condition is not indicated.*

2. Microbial Transformation of Chloroethanes and Chloroethenes

Chloroethanes (CAs) and chloroethenes (CEs) can be oxidised under aerobic and anaerobic conditions (Figure 3). The aerobic oxidation proceeds via a monooxygenase (or dioxygenase) catalysed reaction, where one (or two) oxygen atoms are inserted and resulting intermediates are converted rapidly into aldehydes or organic acids (Field and Sierra-Alvarez, 2004). This oxygenase reaction is catalysed by a large number of microbial species (Janssen *et al.*, 1988; Alvarez-Cohen and Speitel, 2001; Coleman et al., 2002b). Lower chlorinated CEs, such as dichloroethenes and VC can be oxidised under aerobic and other terminal electron accepting conditions like methanogenic, iron- and manganese reducing conditions (Bradley and Chapelle, 1997, 1998). Such conditions are found in wetland and aquifer sediment environments. The aerobic oxidation of 1,2-DCA, cis-DCE and VC is coupled to bacterial growth (Stucki *et al.*, 1992; Coleman et al., 2002b, a; Aulenta *et al.*, 2003). In contrast, aerobic oxidation of higher chlorinated CEs, such as PCE and TCE, is not an energy yielding and growth supporting metabolic reaction (Ryoo *et al.*, 2000). However several detected bacterial strains belonging to the genera of *Pseudomonas*, *Xanthobacter,* and *Ancylobacter* oxidize 1,2-DCA to CO_2 under aerobic conditions, (Stucki *et al.*, 1983; Janssen *et al.*, 1984; van den Wijngaard *et al.*, 1992; Hage and Hartmans, 1999). Two aerobic degradation pathways, whose initial steps differ, have been identified: (A) hydrolytic dehalogenation and (B) oxidation reaction. However, both pathways yield the common intermediate chloroethanol, subsequently oxidised to monochloroacetate, which is eventually mineralised (Hage and Hartmans, 1999). In contrast under anaerobic conditions 1,2-DCA oxidation may be coupled to nitrate reduction (Dinglasan-Panlilio *et al.*, 2006). Reductive dechlorination was illustrated for a variety of CAs and CEs for bacteria of several phylogenetic groups, including 1,2-DCA (*Dehalococcoides* sp., *Desulfitobacterium* sp.), 1,1,1-TCA (*Dehalobacter* sp.), and hexachloroethane (HCA), PeCA TeCA and (*Desulfitobacterium* sp.), and PCE, TCE, *cis*-DCE, 1,1-DCE, *trans*-DCE and VC (*Dehalococcoides* sp.) (Magnuson *et al.*, 1998; Maymo-Gatell *et al.*, 1999; Suyama *et al.*, 2001; Sun *et al.*, 2002; Cupples *et al.*, 2003; He *et al.*, 2003b; Sung *et al.*, 2003). Phylogenetically diverse bacteria dechlorinate PCE and TCE to *cis*-DCE (Smidt and de Vos, 2004), but so far only *Dehalococcoides* strains reduce DCE and even the toxic VC. *Dehalococcoides mccartyi* strain 195 can reduce VC cometabolically to ethene during reduction of PCE (Maymo-Gatell *et al.*, 2001).

In contrast metabolic VC reductive dechlorination to ethene was reported for the *Dehalococcoides mccartyi* strains BAV1, GT, VS (Cupples et al., 2003; He *et al.*, 2003a; He et al., 2003b; Sung *et al.*, 2006a), *Dehalococcoides* containing consortia (ANAS and KB-1) and *Dehalococcoides* enrichment culture BTF08 (Cichocka *et al.*, 2010).

Under anaerobic conditions, reductive dihaloelimination of 1,2-DCA to ethene has also been observed in pure and mixed cultures containing *Dehalococoides mccartyi 195* (Maymo-Gatell et al., 1999; He et al., 2003a). It is reported to emerge cometabolically in sulfate reducing and methanogenic cultures (Egli *et al.*, 1987; van den Wijngaard et al., 1992; Klecka *et al.*, 1998). Additionally, some pure cultures of methanogens such as *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* cometabolically dechlorinate 1,2-DCA to ethane (Egli et al., 1987). An anaerobic dehalorespiring organism *Desulfitobacterium dichloroeliminans* DCA 1, which is capable of reductive 1,2-DCA dihaloelimination was recently isolated (De Wildeman *et al.*, 2003; De Wildeman *et al.*, 2004).

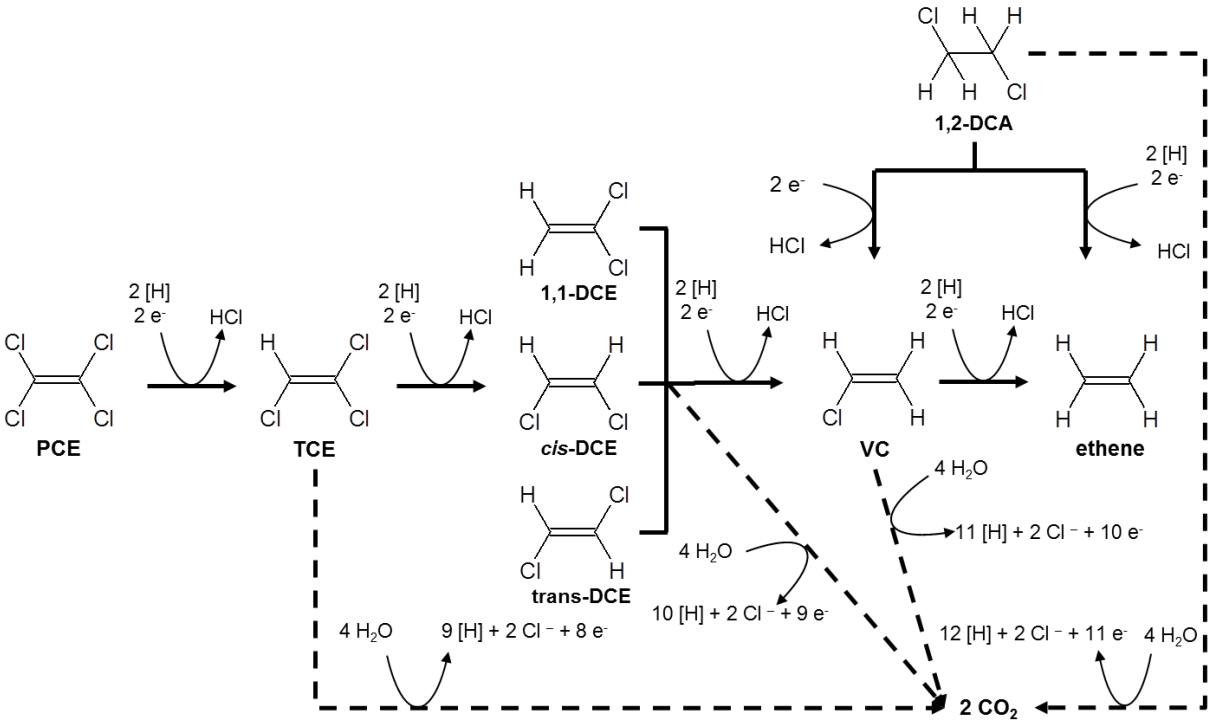

Figure 1.4: *Reductive (adapted after Middeldorp et al. (1999)) and oxidative dechlorination (Bradley, 2003) pathways of tetrachlorethene (PCE), trichloroethene (TCE), 1,1-dichloroethen (1,1-DCE), cis-dichloroethene (cis-DCE), trans-dichloroethene (trans-DCE), vinyl chloride (VC) under anaerobic condition.*

3. Diversity of Organohalide Respiring Bacteria

The variety of to date characterised organohalide respiring bacteria (OHRB), using various organohalides as terminal electron acceptors like CEs (PCE, TCE, DCEs and VC), CAs (PeCA, TeCA, TCA and DCA) and CBs (HCB, PeCB, TeCB and TCB) has increased since the initial findings. OHRB guild members have been discovered and isolated from different organohalide-contaminated environments in a large variety of phyla, including *Chloroflexi*, *Firmicutes* and ε-, γ-, δ-Proteobacteria (Table 2). However, there is still no evidence of a connection between OHRB phylogenetic affiliation and their organohalide terminal electron acceptor (Smidt and de Vos, 2004). The later indicates organohalide respiration as possibly common feature among bacteria, obtained through lateral gene transfer most probably. OHRB are obligate or facultative OHRB, which also use non organohalide electron acceptors. Most OHRB are facultative organohalide respiring bacteria, consisting of the genera *Desulfitobacterium*, *Anaeromyxobacter, Desulfomonile*, *Geobacter* and *Sulfurospirillum*, which are capable of dehalogenating a limited number of halogenated compounds, as part of a versatile metabolism and also use other electron acceptors (Hiraishi, 2008).

The δ-Proteobacterium *Desulfomonile tiedjei* belongs to this group of facultative OHRB, dechlorinating 3-chlorobenzoate (Shelton and Tiedje, 1984), PCE and TCE (Fathepure *et al.*, 1987). Other isolates of the δ-Proteobacteria, including the strains *Desulfuromonas michiganenis*, *Desulfuromonas chloroethenica* (Krumholz, 1997; Sung et al., 2003) and *Geobacter lovleyi* strain SZ reductively dechlorinate PCE and TCE to *cis*-DCE (Sung *et al.*, 2006b). In addition, the δ-Proteobacteria *Sulfurospirillum multivorans* and S. *halorespirans* reduce PCE to *cis*-DCE (Scholz-Muramatsu *et al.*, 1995; Luijten *et al.*, 2003), as well as *S. multivorans* can grow in PCB-dechlorinating cultures (He *et al.*, 2006). Furthermore *Desulfitobacterium* sp., affiliated in the phylum Firmicutes, was reported to reductively dechlorinate a wide range of organohalides, including CEs and CAs, as well as chloro-, bromo-, and fluorophenols. For selected *Desulfitobacterium* strains the specific substrate spectrum is presented in Table 2, whereas all strains reduce PCE to TCE (Miller *et al.*, 1997; Gerritse *et al.*, 1999; Suyama et al., 2001), or even to *cis*-DCE (*Desulfitobacterium frapperi* TCE1, Gerritse et al. (1999)) (see Villemur *et al.* (2006) for a complete review of the genus *Desulfitobacterium*).

In contrast to facultative OHRB, obligate OHRB consist of species, which strictly require an organohalide molecule as terminal electron acceptor for their growth. Among the obligate OHRB, *Dehalobacter* sp. (Firmicutes) has first been reported for PCE and TCE reductive dechlorination to *cis*-DCE (Holliger *et al.*, 1998b), and DCB and MCB reductive dechlorination to

benzene (Fung et al., 2009; Nelson et al., 2011), whereas other representatives of this genus were shown to grow on CAs and brominated organics (Sun et al., 2002).

The *Chloroflexi* represent the phylum with the highest number of isolates of obligate OHRB. The genus *Dehalococcoides mccartyi,* affiliated in the *Chloroflexi* phylum, was intensively studied. *Dehalococcoides mccartyi* uses a broad organohalide spectra, ranging from polychlorinated biphenyls, dioxins, chlorophenols, CBs, CAs, to CEs (for a review, please refer to Taş *et al.* (2010) and Yoshida and Katayama (2010). So far, *Dehalococcoides mccartyi* strains were reported to be the only bacteria that catalyse the last dechlorination steps of the CEs to ethene (Maymo-Gatell *et al.*, 1997; Cupples et al., 2003; He et al., 2003b), while *Dehalococcoides mccartyi* strain 195 and BTF08 (this study, (Kaufhold *et al.*, 2013)) are the only up-to-date bacteria known to completely dechlorinate PCE to ethene. Although the last dechlorination step to ethene is cometabolic for 195, BTF08 dechlorinates VC metabolically (this study, Chapter 2). Recently, *Dehalogenimonas lykanthroporepellens*, which is closely related to *Dehalococcoides* sp., has been shown to reductively dechlorinate chloropropanes and CAs (Moe *et al.*, 2009). In addition, reductive dechlorination of CEs has been reported for uncultured *Chloroflexi*, with dechlorination of PCE to *cis*-DCE (Kittelmann and Friedrich, 2008a) in river sediments microcosms, and to *trans*-DCE (Kittelmann and Friedrich, 2008b). Besides CEs, *Dehalococcoides* reductively dechlorinates other chlorinated compounds, e.g. CBs, naphthalenes, biphenyls and dioxins. *D*. *mccartyi* strain 195 reductively dechlorinates PCE, TCE, cis-DCE, 1,1-DCE, 1,2-DCA and VC. Additionally strain 195 dechlorinates HCB to 1,2,3,5-TeCB and 1,3,5-TCB. In contrast *D*. *mccartyi* strain CBDB1 is an isolate capable to reductively dechlorinate TCB and TeCB to DCB(Adrian et al., 2000), and HCB and PCB but only in crystalline form (Jayachandran *et al.*, 2003) and even dechlorinates chlorinated dioxins (Bunge *et al.*, 2003) and chlorinated phenols (Adrian *et al.*, 2007).

The availability of electron donors is adjusted in laboratory cultures according to the specific research purpose. However, under field conditions the activity and growth of the single OHRB is highly dependent on syntrophic interactions with other non dechlorinating bacteria, such as fermenting and syntrophic bacteria providing the hydrogen, by hydrolysing and fermenting organic compounds (D'Angelo and Nunez, 2010; Heimann *et al.*, 2010) or acetate (Smidt and de Vos, 2004). The syntrophic interaction of bacteria is postulated and proven for complete degradation and mineralisation of organohalides (Becker *et al.*, 2005) as well as for anaerobic benzene degradation (Kleinsteuber et al., 2012) and non-chlorinated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAH).

Table 1.2: *Overview about selected bacterial isolates capable of reductive dechlorination of chlorinated benzenes, chlorinated ethenes and chlorinated ethanes. Parts of content taken from Nijenhuis* et *al. 2002)*

Phylogenetic group	**Electron donors**	**Chlorinated hydrocarbons as electron acceptors and performed dechlorination steps**			**Reference**
Species		Chloroethenes and Chloroethanes	Chlorobenzenes	Other electron acceptors	
Firmicutes					
Desulfitobacterium sp. strain PCE1	formate, butyrate lactate, pyruvate succinate, ethanol	PCE to TCE		mono-,di-, tri chlorophenols, thiosulfate, sulfite fumarate, ClOHPA	Gerritse *et al.* (1996)
Desulfitobacterium frapperi TCE1	butyrate, crotonate, ethanol, formate, hydrogen, lactate	PCE and TCE to cis-1,2-DCE		sulfite and thiosulfate, nitrate, fumarate, phenylacetate	Gerritse et al. (1999)
Desulfitobacterium hafniense TCE1		PCE and TCE			
Desulfitobacterium sp. strain Viet1	hydrogen	PCE to TCE			
Desulfitobacterium sp. PCE-S	Formate, hydrogen, pyruvate, yeast	PCE to TCE		trichlorophenol, pentachlorophenol, fumarate, sulfite	Miller et al. (1997)
Desulfitobacterium hafniense strain Y51		PCE to TCE, hexa-,penta- and tetrachloroethanes			Suyama et al. (2001)
Dehalobacter restrictus	Hydrogen	PCE to *cis*-DCE TCE to *cis*-DCE			Holliger et al. (1998b)
Dehalobacter sp. TEA	Hydrogen	PCE to *cis*-DCE TCE to *cis*-DCE			Wild *et al.* (1997)
Dehalobacter sp. (enrichment culture)			DCB and MCB to benzene		Nelson et al. (2011)
Chlostridium bifermentans strain DPH1	Hydrogen	PCE to *cis*-DCE TCE to *cis*-DCE			Chang *et al.* (2000)
δ-Proteobacteria					
Desulfomonile tiedjei	formate, hydrogen, pyruvate	Comet. PCE to cis-DCE TCE to *cis*-DCE		3-chlorobenzoate, pentachlorophenol, sulfate, sulfite, thiosulfate	(Deweerd and Suflita, 1990; Cole *et al.*, 1995)
Desulfuromonas Chloroethenica	acetate, pyruvate, polysulfide	PCE to *cis*-DCE TCE to *cis*-DCE		fumarate, Fe(III)	Krumholz (1997)
Desulfuromonas Michiganensis	acetate , lactate, pyruvate, succinate, malate, fumarate	PCE to *cis*-DCE TCE to *cis*-DCE		malate, fumarate, Fe(III), sulfur	Sung et al. (2003)

Geobacter lovleyi strain SZ	acetate, hydrogen, pyruvate	PCE to *cis*-DCE TCE to *cis*-DCE		nitrate, fumarate, Fe(III), malate, Mn(IV), U(VI), sulfur	Sung et al. (2006b)
ε-Proteobacteria					
Sulfurospirillum multivorans	ethanol, formate, hydrogen, lactate, pyruvate	PCE to *cis*-DCE TCE to *cis*-DCE		arsenate, fumarate, nitrate, selenate	Scholz-Muramatsu et al. (1995)
Sulfurospirillum halorespirans	lactate, hydrogen, formate, pyruvate	PCE to *cis*-DCE TCE to *cis*-DCE		sulfur, arsenate, selenate, nitrate, nitrite, fumarate	Luijten et al. (2003)
γ-Proteobacteria					
Enterobacter MS-1	Acetate, amino acids, formate, glucose, lactate, pyruvate, yeast extract	PCE to *cis*-DCE TCE to *cis*-DCE		Nitrate, oxygen	Sharma and McCarty (1996)
Chloroflexi					
Dehalococcoides mccartyi strain 195	Hydrogen	PCE, TCE, *cis*-DCE and 1,1-DCE to ethene, (VC to ethene comet.) 1,2-DCA to VC and ethene	HCB to 1,2,3,5-TeCB and1,3,5-TCB	chloronaphthalenes, dioxins, polychloro-biphenyls, chlorophenyls	Maymo-Gatell et al. (1997); Maymo-Gatell et al. (1999); Fennell *et al.* (2004); (Adrian et al., 2007)
Dehalococcoides mccartyi strain BAV1	Hydrogen	cis-DCE, trans-DCE, 1,1-DCE to ethene, VC to ethene (PCE and TCE comet.); 1,2-DCA to ethene			He et al. (2003a), He et al. (2003b)
Dehalococcoides mccartyi strain GT	Hydrogen	TCE, cis-DCE, 1,1-DCE, VC to ethene			Sung et al. (2006a)
Dehalococcoides mccartyi strain FL2	Hydrogen	TCE, cis-DCE and trans-DCE, 1,1-DCE to VC and ethene comet. (PCE and VC comet.)			He *et al.* (2005)
Dehalococcoides mccartyi strain MB	Hydrogen	PCE and TCE to *trans*-DCE and *cis*-DCE			Cheng and He (2009)
Dehalococcoides mccartyi strain VS		TCE, cis-DCE, 1,1-DCE, VC to ethene			Cupples et al. (2003)
Dehalococcoides mccartyi strain CBDB1	Hydrogen	PCE to trans-DCE comet.	HCB (in crystalline form) to PCB an furher to 1,2,3,5-TeCB and 1,2,4,5-TeCB; 1,2,3-TCB; 1,2,4-TCB; 1,2,3,4-TeCB; 1,2,3,5-TeCB;		Adrian et al. (2007), Adrian et al. (2000), Bunge et al. (2003)

			1,2,4,5-TeCB to DCBs or 1,3,5-TCB		
Dehalococcoides mccartyi strain DCMB5 Substrate spectra for former enrichment culture	Hydrogen	PCE to *trans*-DCE comet.	1,2,3-TCB to 1,3-DCB	1,2,4-TrCDD	Pöritz *et al.* (2013), Bunge *et al.* (2008)
***Dehalococcoides mccartyi* strain BTF08**	**Hydrogen**	**PCE, VC, (and VB) to ethene, 1,2-DCA to ethene, γ-HCH to [TeCCH abiotically], MCB**	**PeCB and HCB to 1,2,3,5-TeCB and 1,3,5-TCB; 1,2,3,4-TeCB to 1,2,4-TCB; 1,2,3,5 to 1,3,5-TCB**		**Kaufhold et al. (2013); Pöritz et al. (2013)**
Dehalococcoides mccartyi strain 11a5	Hydrogen	TCE, trans-DCE, cis-DCE, 1,1-DCE to VC (to ethene comet.)			Lee *et al.* (2013)
Dehalococcoides mccartyi 11a	Hydrogen	TCE, trans-DCE, cis-DCE, 1,1-DCE, VC, 1,2-DCA to ethene			Lee et al. (2013)

Comet: cometabolic, new information obtained as result from data presented in this thesis is shown in bold

III. Assessing Biotransformation of Chlorinated Solvents

The spectrum of chemical-analytical, microbial and bio-molecular methods is wide and diverse and offers broad possibilities in investigating and assessing microbial transformation of chlorinated solvents in different scales and under various research approaches. The assessment of chlorinated solvents' biotransformation addresses the three lines of evidence to be fulfilled for assessing natural attenuation *in situ* (see Chapter 1.3.), including: beneficial geochemical conditions, biodegradability of the pollutants and availability of degrading microorganisms.

1. Biotransformation at Different Scales

The investigation of contaminant biotransformation can be carried out in diverse natural and engineered systems of different scales, including laboratory microcosms, pilot scale constructed bioreactors and/or wetlands, contaminated ecosystems, soils and groundwater aquifers in catchment or macro (field site) scale. Thereby the underlying biochemical pathways, basic reaction principles, the microbial community involved in the contaminant degradation and required bio-geochemical conditions for biotransformation processes of chlorinated groundwater contaminants can be revealed.

Following the aforementioned order of scales, laboratory microcosms, which are anaerobic serum bottles filled with material (either with sediment/aquifer material e.g., Kleikemper *et al.* (2002)) and/or groundwater (e.g., Kasai *et al.* (2006)) of a contaminated field site are applied to assess microbial *in situ* degradation processes (for review, see Madsen (2005), Strevett *et al.* (2002)). In this closed microcosm system the factors influencing microbial degradation, relevant degradation processes (Coates *et al.*, 1996; Brauner *et al.*, 2002), and biological degradation potential and rates can be easier identified (Madsen, 1991; Wilson and Wilson, 1997; Wiedemeier, 1998). The advantage of microcosm studies is the rapid and easy preparation of numerous replicates under various redox conditions. The amount of nutrient, electron acceptor and electron donor amendment (Pritchard and Bourquin, 1984; Corseuil *et al.*, 1998; Wiedemeier, 1998) is adjustable. This allows also testing of various biological remediation options by adjusting and mixing the aforementioned parameters. The use of stable isotopes (e.g. ^{13}C) in ^{13}C-labelled chlorinated contaminants allows tracing its transformation into unknown metabolites and/or even its complete mineralisation, which can be directly quantified in the microcosm. Additionally, a bacterial enrichment culture that efficiently degrades the respective contaminant may result from a long term microcosm cultivation experiment. The drawback of anaerobic laboratory microcosms is the long cultivation procedure, including potentially negative

results for the microbial degradation activity for the starting material. In this thesis the microcosm approach was applied to investigate the biogeochemical conditions enhancing the microbial transformation of MCB and DCB and is part of the studies presented in Chapter 5 and 6. Information gained from these laboratory systems, e.g. favoured electron donor/ acceptor requirements and estimated transformation rates may be implemented in up-scaled investigation at a pilot system or at field site dimensions in order to promote the natural attenuation of the chlorinated solvent.

Within the field of waste water remediation natural (Kadlec *et al.*, 1979) and engineered, so called constructed wetlands (e.g., Kadelec and Knight (1996), (Cooper *et al.* (1996); Haberl *et al.* (2003)). have been used since decades, due to their complexity of diverse redox conditions and steep gradients in numerous physicochemical parameters, e.g in oxygen. Additionally, these pilot scale constructed wetlands have been reported to effectively remove various chlorinated contaminants from polluted groundwater, PCE (Wang *et al.*, 2004), CEs and CAs (Mastin et al., 2001; Lorah and Voytek, 2004; Imfeld et al., 2008a; Tawney et al., 2008) and lower CBs (Lee et al., 2003). The constructed wetlands in a flow-through mode consist typically of four main compartments: plants, sediment and soil, microbial biomass and an aqueous phase loaded with the chlorinated contaminants (Imfeld et al., 2009). The bioremediation potential of wetlands, both natural and constructed ones, basically results from the plant rhizosphere associated microorganisms which are able to metabolize hazardous substances, using them directly as carbon or energy source to support growth, or as secondary substrates in cometabolic pathways (Kadelec and Wallace, 2008), as well as from the geochemical gradients between the different redox zones in the sediment. Several elimination pathways may occur in a complex constructed wetland system. Apart from biological degradation other physical and chemical processes affect the contaminant load, such as volatilisation, photochemical oxidation, sedimentation and sorption (Kadlec, 1992). The gained knowledge on contaminant removal processes in monitored constructed wetlands can be transferred to a natural wetland ecosystem, where groundwater enters surface water.

Regarding the field site scale, in contaminated aquifers the assessment of biotransformation of chlorinated contaminant becomes highly complex, and often involves the aforementioned approaches in order to verify if a degradation potential for the respective contaminant is present. Moreover, aquifers are extremely heterogeneous (Wilson *et al.*, 2004), even at small scale, and are dynamic microbial ecosystems, with complex interactions between physical, chemical and biotic components (Haack *et al.*, 2004). Depending on the dimension of the field site, numerous monitoring wells or points have to be set up in order to take samples for

evaluating hydrological and geochemical conditions (Chapter 3.2) in aquifers over space and time and for determining a reduction of the contaminant load.

2. Hydro-geochemical, Contaminant and Metabolite Analysis

The combined evaluation of biogeochemical conditions and the variation in contaminant and metabolites concentrations in groundwater and soil systems is a mainstream process in understanding the behaviour of a contaminant at a field site. The hydro-geochemical analysis of water or sediment samples includes the determination of contaminant concentration and of naturally available and dissolved electron acceptors, e.g. O_2, NO_3^-, Mn(IV), Fe(III), SO_4^{2-} and CO_2. (Chapelle and Lovley, 1992; McMahon and Chapelle, 2008). Based on these redox-sensitive parameters, like detection of Fe(II), HS^-/S^{2-} and CH_4 as well as physical parameters (redox potential, pH and electric conductivity), geochemical processes can be characterized. However, only a monitoring of the above-listed parameters over space and time, including microbial analysis (Chapter 3.3), results in collecting enough indications for characterising the biogeochemical processes. These processes, potentially affect the reduction of the contaminant load and may lead to the conclusion of natural attenuation potential (Reddy and DAngelo, 1997) at the field site. However, biodegradation at the field site frequently terminates due to one or more factors, such as transfer, availability of electron donors and acceptors, the thermodynamic characteristics of the processes, and microbial kinetics limit the redox activity (Christensen *et al.*, 2000).

Groundwater systems are generally anoxic, as O_2 tends to be consumed along aquifer flow paths, due to the isolation of groundwater from the atmosphere and presence of the organic contaminant. Therefore terminal electron-accepting processes follow an order of electron acceptor utilization that is driven by the next most energetically favourable naturally available electron acceptor NO_3^- > Mn(IV) > Fe(III) > SO_4^{2-} > CO_2, which may then lead to the formation of hydrochemical gradients over both spatial and temporal scales.

Redox heterogeneity, possibly affecting the fate and transport of natural and anthropogenic contaminants may also be detected in water samples indicating mixed redox processes (McMahon and Chapelle, 2008). Consequently, the interpretation of contaminant degradation processes in geochemically heterogeneous aquifers may be only an approximation.

3. Microbial Analysis

In general, microbial analyses involve a broad spectrum of methods, mainly classified into cultivation-dependent and cultivation-independent approaches. Both techniques offer considerable advantages and disadvantages with regard to answering the research question if and how biotransformation of chlorinated contaminants proceeds. The challenging task to enrich and cultivate potential contaminant-degrading bacteria and bacterial consortia, by inoculating basal microbial media with sediment or water samples from a contaminated field site to create a laboratory microcosm, is a commonly applied procedure in order to assess the degradation potential of the indigenous microbial community (Aulenta *et al.*, 2005; Nijenhuis et al., 2007a). The low cost but most usual time consuming search for appropriate conditions allows in small scale experiments controlled amendment and adjustment of vitamins, electron donors and acceptor in order to assess the potential degradation pathways for the respective contaminant. Moreover, physical processes, such as sorption, dilution and evaporation of the contaminants, impacting results in field investigations will not interfere (Meckenstock *et al.*, 2004). Additionally, the preparation of microcosms allows the quantification of underlying degradation reaction by calculating degradation rates in closed systems without losses. The enrichment of specific degrading bacteria may eventually result in the isolation, physiological and genome characterisation of the pure strain. The strain, if transferrable into *in situ* conditions, e.g. contaminated soil and aquifer, enhances the bioremediation at the field site in a bio-augmented approach. However, the cultivation of microbes from environmental samples is quite challenging since less than 1 % of bacteria is cultivatable (Amann *et al.*, 1995; Ferrari and Hollibaugh, 1999).

In parallel, the rapid development of time- and cost-efficient molecular techniques for microbial identification provides cultivation independent methods to identify microbial species, enzymes and community structures of microorganisms and is not restricted by the proportion of microorganism which is challenging and time consuming to cultivate (Richardson *et al.*, 2002). Such methods include nucleic acid techniques, e.g. sequencing of DNA and RNA fragments for microbial and specific gene identification, quantification and detection of specific gene sequences, including taxonomical or catabolic gene sequence by using polymerase chain reaction (PCR), and molecular fingerprinting methods, e.g. DGGE and TRFLP to determine the relative abundance and diversity of the microbial community. The picture of phylogenetic diversity, composition and structure of the indigenous microbial community may allow assumptions about the microbial ecology and functions in contaminated ecosystems. Furthermore, the identification of bacteria affiliated to those, playing key roles in organohalide respiration (presented in Chapter 2.3), such as *Dehalobacter* spp., *Desulfitobacterium* spp.,

Dehalococcoides or *Geobacter* spp. supports predicting the potential for dechlorination activity *in situ* (Löffler *et al.*, 2000; Hendrickson *et al.*, 2002; Duhamel and Edwards, 2006). Nevertheless, the correlation between concrete functions of identified bacterial taxa in regard to contaminant degradation in the specific ecosystem remains a challenging issue.

Recently developed new technologies will allow distinguishing the actively involved bacteria in the microbial consortia, by using isotope labelled chlorinated substrates in tracer experiments or stable isotope probing techniques (DNA- or RNA, Protein SIP)(Boschker *et al.*, 1998; Manefield *et al.*, 2002; Jehmlich *et al.*, 2008; Bombach *et al.*, 2010b; Jehmlich *et al.*, 2010). Besides using radioactive isotopes (^{14}C), stable isotopes (e.g. ^{13}C) are easy to apply in tracer studies, e.g. microcosm and BACTRAPS® (section 3.4) (Bombach et al., 2010a). In this case not only mineralisation and transformation of the chlorinated compound can be traced when ^{13}C labelled degradation metabolites are detected, but also the ^{13}C incorporation into bacterial biomarker molecules, e.g. nucleic acids (DNA and RNA), amino acids or in phospholipids fatty acids (PLFAs). In order to identify the bacterial contaminant degraders, these methods are restricted to the incorporation of ^{13}C-carbon into bacterial biomass, present when the labelled compound is the carbon source. However, they do not apply for reductive dechlorination reactions, as the ^{13}C-compound is used as electron acceptor to gain energy by microbial respiration, but not as carbon source.

1. Stable Isotope Analysis

Stable isotope approaches can be used to trace biotransformation of groundwater contaminants *in situ* (Hunkeler *et al.*, 1999; Sherwood Lollar *et al.*, 2001; Meckenstock et al., 2004; Hunkeler and Morasch, 2010; Hunkeler *et al.*, 2011). Compound specific isotope analysis (CSIA) has been widely used as an effective tool to confirm, assess and/or quantify *in situ* biodegradation of various types of organic chemicals such as CEs, BTEX or fuel oxygenates based on carbon ($^{13}C/^{12}C$) and/or hydrogen ($^{2}H/^{1}H$) isotopic analyses (Hunkeler *et al.*, 2002; Mancini *et al.*, 2003; Richnow *et al.*, 2003; Fischer *et al.*, 2007; Rosell *et al.*, 2007).

The isotope composition of organic compounds provides additional and unique information for identifying their sources, sinks and transformation processes, which cannot be easily deduced from concentration data. Stable isotope analysis of an element within an organic molecule involves measurement of the relative abundance of the heavy isotope against the light (i.e. isotope ratio, for example $^{13}C/^{12}C$ for carbon stable isotopes) (EPA, 2009). The two elements in organic contaminants more used to date in environmental applications are carbon and hydrogen (EPA, 2009). Carbon and hydrogen exist in two stable isotopic forms, $^{13}C/\ ^{12}C$, with natural abundance of 1.11/ 98.89 %, and $^{2}H/\ ^{1}H$ with a natural abundance of 0.02/ 99.98 %, respectively.

Two main approaches are applied to use stable isotope analysis for biotransformation investigations: (1) stable isotope probing, SIP (so called stable isotope tracer), using [$^{13}C_6$]-isotope enriched substrates and (2) compound specific isotope analysis (CSIA) of organic compounds at natural isotope abundance. Stable isotope tracer experiments used [$^{13}C_6$]-isotope enriched substrates to determine biotransformation products, metabolites or biomass production from chlorinated compounds or to investigate the isotope fractionation of the substrate to identify the reaction mechanism, wherein the $^{13}C/^{12}C$ ratio of the degraded compound get enriched in and products get depleted in ^{13}C. Both techniques can be applied in laboratory and field investigations.

(1) In stable isotope tracer experiments artificially produced [$^{13}C_6$]-enriched chlorinated compounds are used. When applied in laboratory microcosm, in *in situ* or *ex situ* systems, like BACTRAPS® (for review see (Bombach et al., 2010a)) the degradation activity of the indigenous bacterial community can be investigate. Since ^{13}C is rare in nature the labeled compound can be readily differentiated, as well as the metabolites or products. The analysis of ^{13}C-labeled substances, including substrate, metabolites and products can be done both by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) (Blessing *et al.*, 2008) and by gas chromatography-mass spectrometry (GC-MS). The amount of substrate degraded and product

formed can be quantified using the measured $^{13}C/^{12}C$ ratio (Richnow *et al.*, 1999) and was applied in Chapter 5.

In particular, the incorporation of ^{13}C into biomass demonstrates the anaerobic biodegradation of the parent compound and can be detected by analysing fatty and amino acids, nucleic acids and proteins (Chapter 3.3). The concept of stable carbon (^{13}C) isotope tracer experiments has been successfully reported for different substance classes, e.g. BTEX compounds (Geyer *et al.*, 2005; Kästner *et al.*, 2006; Stelzer *et al.*, 2006) and including MCB (Nijenhuis et al., 2007a; Stelzer et al., 2009).

(2) Applying compound stable isotope analysis (CSIA) with substrates at natural abundances, several laboratory studies have shown that biological degradation of chlorinated solvents can involve reproducible kinetic isotope effects, producing systematic changes in the $^{13}C/^{12}C$ ratio of the residual contaminant fraction (Bloom *et al.*, 2000; Slater *et al.*, 2000; Sherwood Lollar et al., 2001; Barth *et al.*, 2002; Hunkeler and Aravena, 2002; Kaschl et al., 2005; Nijenhuis et al., 2007a). The method is based on the different reaction rates for molecules with light (e.g. ^{1}H, ^{12}C) and heavy (e.g. ^{2}H, ^{13}C) isotopes, resulting from a lower activation energy for the chemical bond cleavage of lighter stable isotopes (Bigeleisen and Wolfsberg, 1958) which may generate an isotope shift in the non-degraded residual substrate fraction of contaminant and/or in the corresponding degradation products, resulting in an enrichment and depletion of ^{13}C in isotope ratio $^{13}C/^{12}C$, of the parent and product compound, respectively (Meckenstock et al., 2004). The isotope composition of molecules at natural abundance can be only measure by GC-IRMS due to the high precision required at the mass spectrometer to measure the heavy isotopes (^{13}C) at the very low natural abundance.

This isotope ratio of the respective substance is given as the difference in delta per mill (δ-notation [‰]) (Hoefs, 1997) to an international isotope standard, in case of carbon isotope compositions the Vienna-PeeDee-Belemnite standard (V-PDB) (Coplen *et al.*, 2006) (equation 1).

$$\delta^{13}C[‰] = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \right) \times 1000 \qquad \text{Eq. 1}$$

The difference in the reaction (i.e. change in $^{13}C/^{12}C$ ratio) during reductive dechlorination or aerobic degradation of chlorinated hydrocarbons, like VC or MCB, produces an isotope fractionation pattern typical for a specific reaction mechanism which can be expressed as the enrichment factor ε. The enrichment factor ε relates the decrease of the substrate concentration directly to changes in the isotope ratios of the parent compound during a transformation process, which is presented in the Rayleigh equation (equation 2) (Mariotti *et al.*, 1981), where R = 1000 + $\delta^{13}C$ and C represent the carbon stable isotope composition and concentration, respectively, of the substrate at time 0 and time t.

$$\left(\frac{\varepsilon}{1000}\right)\times\ln\left(\frac{C_t}{C_0}\right)=\ln\left(\frac{R_t}{R_0}\right) \qquad \text{Eq.2}$$

Specific enrichment factors (ε), derived from degradation experiments of a target compound under controlled microcosm conditions or in bacterial pure cultures, can provide information about the reaction mechanism and to differentiate aerobic from anaerobic degradation of chlorinated hydrocarbon, as for example shown for the microbial degradation of 1,2-DCA. Aerobic microbial degradation of 1,2-DCA via a monooxygenase reaction can be clearly distinguished (ε= -3.0 ‰, Hirschorn *et al.* (2004) from a hydrolytic dehalogenation under nitrate reducing conditions(ε= -25.8 ‰, (Hirschorn *et al.*, 2007), which exhibits a stronger fractionation. Thus far, 1,2-DCA fractionation was not reported for reductive dehalogenation by *Dehalococcoides*, and was investigated in the present study (Chapter 3). Implementing the knowledge about reaction mechanism obtained from laboratory cultures and applying CSIA under field conditions (contaminated aquifers, wetlands) allows the identification of ongoing biotransformation processes, mechanisms and source identification (Chapter 4).

A significant shift in the isotope ratio for carbon enables distinguishing a biological (enzymatic) and chemical reactions from non-destructive, physical processes which also lead to a contaminant load reduction by dilution, volatilisation or sorption of the contaminant in the aquifer (Vieth *et al.*, 2003; Griebler *et al.*, 2004).

Research Objectives

The research presented in this thesis aims at characterizing the anaerobic biotransformation of lower chlorinated hydrocarbons, by (1) understanding the bio-geochemical processes in laboratory studies and by (2) implementing an integrative approach to evaluate the *in situ* biotransformation.

In this context, different technical approaches were applied, including small (laboratory) as well as large (field) scale, whereas the succeeding specific objectives were addressed in the following chapters 2-6.

(1) Description of the dechlorination substrate spectrum of a *Dehalococcoides* highly enriched culture, namely *Dehalococcoides mccartyi* strain BTF08, obtained from the contaminated field site Bitterfeld

(2) Characterisation of the 1,2-DCA, VC and DCE reductive dechlorination mechanism of two *Dehalococcoides mccartyi* strains in laboratory cultures

(3) Identification and description of potential degradation pathways of MCB and DCB in laboratory microcosm

(4) Investigation of the relationships between the microbial community and ambient hydro geochemical conditions in constructed wetlands

(5) Characterisation of the biogeochemical development of constructed model wetland systems of various dimensions treating MCB and DCB-contaminated groundwater

(6) Demonstration of *in situ* biotransformation of MCB and DCB in a constructed model wetland

(7) Implementation of an integrative approach, including CSIA, for source apportionment and biodegradation of chlorinated hydrocarbons in a multi contaminated aquifer system

Chapter 2:

Dehalogenation of diverse halogenated substrates by a highly enriched *Dehalococcoides*-containing culture derived from the contaminated mega-site in Bitterfeld

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Abstract

An enrichment culture dominated by one type of *Dehalococcoides* sp. (83% of clones) as characterised. This culture, originally derived from contaminated groundwater from the area of Bitterfeld-Wolfen (Saxony-Anhalt, Germany), dehalogenates chlorinated ethenes to ethene. Further, the culture also dehalogenated vinyl bromide (VB) and 1,2-dichloroethane (DCA) to ethene, 1,2,3,4- and 1,2,3,5-tetrachlorobenzene (TeCB), penta- and hexachlorobenzene (PeCB and HCB) to trichlorobenzenes (TCB), lindane to monochlorobenzene (MCB) and pentachlorophenol (PCP) to 2,3,4,6-tetrachlorophenol (TeCP). Growth was proven by quantitative PCR for all active cultures, except for those with TeCB, lindane and PCP. The growth yields obtained ranged from (2.9 ± 0.7) x 10^7 cells per µmol Br^- released on VB to (34.8 ± 5.4) x10^7 cells per µmol Cl^- released on VC Genes coding for nine putative reductive dehalogenases, the enzymes that sequence.

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Chapter 3:

Comparison of 1,2-dichloroethane, dichloroethene and vinyl chloride carbon stable isotope fractionation during dechlorination by two *Dehalococcoides* strains

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Abstract

Carbon stable isotope fractionation during 1,2-dichloroethane (1,2-DCA), dichloroethene (DCE) and vinyl chloride (VC) dechlorination was analysed for two *Dehalococcoides* strains, *Dehalococcoides mccartyi* strain 195 (formerly D. ethenogenes strain 195) and *Dehalococcoides mccartyi* strain BTF08, and used to characterise the reaction. The isotope enrichment factors (εC) determined for 1,2-DCA were -30.8 ± 1.3 ‰ and -29.0 ± 3.0 ‰ for *Dehalococcoides mccartyi* strain BTF08 and *Dehalococcoides mccartyi* strain 195, respectively. Enrichment factors (εC) determined for chlorinated ethenes with strain BTF08 were -28.8 ± 1.5 ‰ (VC), -30.5 ± 1.5 ‰ (*cis*-DCE) and -12.4 ± 1.1 ‰ (1,1-DCE). Product, ethene, related enrichment factors (εC1,2-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 (εC1,2-DCA) supporting 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.

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Chapter 4:

A stable isotope approach for source apportionment of chlorinated ethenes plumes at a complex multi-contamination events urban site

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Abstract

The stable carbon isotope composition of chlorinated aliphatic compounds such as chlorinated methanes, ethanes and ethenes was examined as an intrinsic fingerprint for apportionment of sources. A complex field site located in Ferrara (Italy), with more than 50 years history of use of chlorinated aliphatic compounds, was investigated in order to assess contamination sources. Several contamination plumes were found in a complex alluvial sandy multi-aquifer system close to the river Po; sources are represented by uncontained former industrial and municipal dump sites as well as by spills at industrial areas.

The carbon stable isotope signature allowed distinguishing 2 major sources of contaminants. One source of chlorinated aliphatic contaminants was strongly depleted in ^{13}C (<-60 ‰) suggesting production lines which have used depleted methane for synthesis. The other source had typical carbon isotope compositions of > -40 ‰ which is commonly observed in recent production of chlorinated solvents. The degradation processes in the plumes could be traced interpreting the isotope enrichment and depletion of parent and daughter compounds, respectively. We demonstrate that, under specific production conditions, namely when highly chlorinated ethenes are produced as by-product during chloromethanes production, ^{13}C depleted fingerprinting of contaminants can be obtained and this can be used to track sources and address the responsible party of the pollution in urban areas.

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Chapter 5:

Iron oxides stimulate microbial monochlorobenzene in situ transformation in constructed wetlands and laboratory systems

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Abstract

Natural wetlands are transition zones between anoxic ground and oxic surface water which may enhance the (bio)transformation potential for recalcitrant chloro-organic contaminants due to the unique geochemical conditions and gradients. Monochlorobenzene (MCB) is a frequently detected groundwater contaminant which is toxic and was thought to be persistent under anoxic conditions. Furthermore, to date, no degradation pathways for anoxic MCB removal have been proven in the field. Hence, it is important to investigate MCB biodegradation in the environment, as groundwater is an important drinking water source in many European countries. Therefore, two pilot-scale horizontal subsurface-flow constructed wetlands, planted and unplanted, were used to investigate the processes in situ contributing to the biotransformation of MCB in these gradient systems. The wetlands were fed with anoxic MCB-contaminated groundwater from a nearby aquifer in Bitterfeld, Germany. An overall MCB removal was observed in both wetlands, whereas just 10 % of the original MCB inflow concentration was detected in the ponds. In particular in the gravel bed of the planted wetland, MCB removal was highest in summer season with 73 ± 9 % compared to the unplanted one with 40 ± 5 %. Whereas the MCB concentrations rapidly decreased in the transition zone of unplanted gravel to the pond, a significant MCB removal was already determined in the anoxic gravel bed of the planted system. The investigation of hydro-geochemical parameters revealed that iron and sulphate reduction were relevant redox processes in both wetlands. In parallel, the addition of ferric iron or nitrate stimulated the mineralisation of MCB in laboratory microcosms with anoxic groundwater from the same source, indicating that the potential for anaerobic microbial degradation of MCB is present at the field site.

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Chapter 6:

Microbial diversity and activity in a monochlorobenzene removing sulphate reducing wetland

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(Manuscript in preparation, see Appendix A5, A6)

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Abstract

The diversity and activity of microbial communities in the sediment compartment of a planted model wetland was characterised, which effectively removed monochlorobenzene (MCB) from the supplied contaminated groundwater (dichlorobenzene (DCB), MCB and benzene) from a regional contaminated aquifer. TRFLP analysis of sediment samples obtained from different parts of the wetland revealed that the local distribution of distinct bacterial communities coincided with the hydro-geochemical conditions in the wetland, whereas two distinct community clusters were present, correlating with anoxic or micro to oxic zone. DCB, MCB and benzene were effectively removed in the anoxic aqueous phase of the sediment and iron (III) was thought to play a role as terminal electron acceptor in complete mineralisation of chlorobenzenes. An increase in benzene, as potential product of reductive dehalogenation, was not observed, however, benzene may have been further degraded.
The microbial activity with respect to the observed contaminant removal in the wetland was investigated in selected sets of anaerobic wetland sediment microcosms. MCB was a major product from DCB and further dehalogenation to benzene, being mineralised was elucidated. Additionally, mineralisation of MCB was observed similar to previous findings using only the groundwater instead of sediment. 16S rRNA gene libraries of the supplied contaminated groundwater revealed the presence of a diverse community which could be linked to methanogenic, sulphate or iron reducing activity, whereas 36 % of the 16S rRNA gene sequences were related to sulphate reducers. Additionally representatives (3 %) of the phylum *Chloroflexi* related to *Dehalogenimonas* which are potentially involved in the dehalogenation of chlorinated contaminants were detected. Based on a multiple-method approach, this study illustrates the linkage between microbial community composition and activity with hydro-geochemical conditions and processes of MCB biotransformation in a wetland system.

Chapter 7: General Discussion

Degradation of Lower Chlorinated Hydrocarbons in Contaminated Groundwater

I. Characterisation of Microbial Degradation Pathways

Towards an efficient management of lower chlorinated groundwater contamination it is necessary to determine the prevalent degradation pathway and the hydro-geochemical conditions that lead to a sustainable reduction of the contaminant at the contaminated field site or in water treatment systems, such as constructed wetlands. In particular, the knowledge about the reaction mechanism, oxidative or reductive degradation, and which factors, e.g. bioavailability of electron donor and acceptor, pH and redox condition, are influencing the reaction will facilitate the application of a successful remediation strategy. In order to reduce the complexity of the larger and rather heterogeneous groundwater or engineered systems, the elucidation of the anaerobic microbial degradation pathways of lower chlorinated hydrocarbons in bacterial cultures, obtained from the contaminated site or treatment system after long term enrichment, is the initial step in such research.

Following this approach the reductive dechlorination of DCEs, VC and DCA was characterised in a *Dehalococcoides* culture (*D. mccartyi* strain BTF08, Chapter 3) and its dehalogenation substrate spectrum was identified (Chapter 2). With *D. mccartyi* strain BTF08 a new *Dehalococcoides* isolate was described, which is time wise the first isolated bacterium after strain 195 dechlorinating PCE to ethene, in which each step including VC to ethene is coupled to energy conservation (Chapter 2), whereas in strain 195 the reduction of VC to ethene is only cometabolic (Maymo-Gatell et al., 1997).

In addition to CEs and CAs, potential anaerobic MCB transformation pathways were evaluated in laboratory microcosms, obtained from a model wetland system and from the field site, where *D. mccartyi* strain BTF08 originates from (Chapter 5). For the first time, the mineralisation of MCB coupled to nitrate and iron reduction was demonstrated. Moreover, in agreement to previous studies (Burland and Edwards, 1999; Lovley, 2000; Vogt et al., 2007) the mineralisation of benzene coupled to nitrate, iron and sulphate reduction was shown (Chapter 5). Further, a sediment microcosm study (Chapter 6), derived from a small-scale wetland sediment, indicated the capability of the settled groundwater microbial community to reductively dechlorinate in addition to 1,2-DCB to MCB also MCB to benzene, followed by a further mineralisation. Details on such processes are provided in the next sections.

1. Dechlorination Spectrum of *Dehalococcoides mccartyi* strain BTF08

The former *Dehalococcoides* containing enrichment culture, assigned as *Dehalococcoides mccartyi* strain BTF08 (chapter 2, Kaufhold et al. (2013)), was obtained from the contaminated Bitterfeld field site (Cichocka et al., 2010). Among the thus far isolated OHRB belonging to different phyla (Chapter 1, section II.3), *Dehalococcoides mccartyi*, affiliated in the *Chloroflexi* phylum, is the only bacterium known to complete reductively dechlorinate CEs to ethene.

The isolated strain BTF08 reductively dechlorinates PCE completely to ethene (Cichocka et al., 2010), which was reported thus far only for *D*. *mccartyi* strain 195 (Maymo-Gatell et al., 1997; Maymo-Gatell et al., 1999). However, in strain 195 the last dechlorination step from VC to ethene is cometabolic and significantly slower (Maymo-Gatell et al., 1999; Löffler *et al.*, 2013) in comparison to strain BTF08. Therefore, BTF08 is the first isolated *Dehalococcides mccartyi* strain, which metabolically dechlorinates each step from PCE to ethene, which was confirmed by identifying the corresponding genes *pceA*, *tceA* and *vcrA* in the genome (Pöritz et al., 2013). Among lower chlorinated substrates, e.g. DCE, VC, vinyl bromide and 1,2-DCA, BTF08 was revealed to use various higher chlorinated hydrocarbons, e.g. TeCB, PeCB, HCB, lindane (α-HCH) and pentachlorophenol (PCP) as terminal electron acceptors during organohalide respiration (Chapter 2), in which all dehalogenation steps were found to be metabolic except the dechlorination of TeCB, PCP and lindane.

The ability of BTF08 to metabolically detoxify VC to ethene (Chapter 2) is a special trait among *Dehalococcoides mccartyi* isolates. Only a small number of *Dehalococcoides mccartyi* isolates, namely strain 11a (Lee et al., 2013), GT (Sung et al., 2006a), BAV1 (He et al., 2003a; He et al., 2003b) and VS (Cupples et al., 2003), metabolically dechlorinate VC and other lower CEs, including the DCE isomers (*cis*, *trans* and 1,1), whereas others reduce VC to ethene only cometabolically, e.g. strain FL2 (He et al., 2005), strain 11a5 (Lee et al., 2013) and strain 195 (Maymo-Gatell et al., 1999). Comparing the substrate spectrum of strain BTF08 to those of other *Dehalococcoides* spp., the novel strain comprises the unique ability to grow with VB and to reduce lindane to MCB via tetrachlorocyclohexane (TeCCH) (Chapter 2), which suggests high flexibility in its metabolism in the environment, most likely gained due to its living at a multi-contaminated field site in Bitterfeld (Heidrich et al., 2004). Recently, also strain 195 was shown to dechlorinate lindane to MCB (unpublished results), which confirms strong similarities in the organohalide substrate range of both *Dehalococcoides* spp., except the already mentioned differences.

The so far identified spectra of used terminal electron acceptors of the known *Dehalococcoides* isolates are quite uniform with respect to different compound classes, e.g. strain BAV1 (He et al., 2003a; He et al., 2003b), GT (Sung et al., 2006a), MB (Cheng and He, 2009), VS (Cupples et al., 2003), 11a and 11a5 (Lee et al., 2013), that are specialized on CEs. Whereas the novel strain BTF08 represents together with strain CBDB1(Adrian et al., 2000; Bunge et al., 2003; Adrian et al., 2007), DCMB5 (Bunge et al., 2008; Pöritz et al., 2013) and 195 (Maymo-Gatell et al., 1997; Maymo-Gatell et al., 1999; Maymo-Gatell et al., 2001; Fennell et al., 2004; Adrian et al., 2007) a group of *Dehalococcoides* isolates comprising a highly versatile dechlorination spectrum, including CBs, dioxins, chlorophenyls, HCH, CEs and DCA. The versatility of dehalogenation substrates indicates that BTF08 plays an important role as an active OHRB at the field site and also in groundwater treatment systems, such as demonstrated for *cis*-DCE degradation in a constructed wetlands (Imfeld et al., 2008a). However, the capability of BTF08 to convert lindane to MCB might have resulted in the observed increase in the MCB concentration under anoxic aquifer conditions (Kaschl et al., 2005).

As above mentioned, DCB and MCB can be a product of the dechlorination activity of *Dehalococcoides mccartyi* and strain BTF08 is not capable to dechlorinate DCB and MCB, which in consequence may drive the MCB accumulation in the environment, usually more persistent under anoxic than oxic conditions.

2. Reductive Dechlorination of Dichloroethenes, Vinyl chloride and Dichloroethane by *Dehalococcoides mccartyi*

The incomplete reductive dechlorination of higher CEs such as PCE and TCE may lead to the formation of *cis*-DCE (Smidt and de Vos, 2004). Several bacterial genera are known to use PCE and TCE as electron acceptor during organohalide respiration, e.g. *Dehalobacter (Holliger et al., 1998b)*, *Sulfurospirillum* (formerly *Dehalospirillum*) (Luijten et al., 2003), *Desulfitobacterium* (Gerritse et al., 1999; Suyama et al., 2001; Finneran *et al.*, 2002) and *Geobacter* (Sung et al., 2006b). In contrast, *Dehalococcoides mccartyi* is the so far only known group of bacteria capable to completely dechlorinate CEs to ethene (Maymo-Gatell et al., 1997; Maymo-Gatell et al., 1999; He et al., 2003a; Nishimura *et al.*, 2008). Just a few isolated *Dehalococcoides* spp., namely strain 11a (Lee et al., 2013), GT (Sung et al., 2006a), BAV1 (He et al., 2003a; He et al., 2003b) and VS (Cupples et al., 2003), metabolically dechlorinate lower CEs, including the DCE isomers (*cis*, *trans* and 1,1) and VC to ethene. Additionally, strain BAV1 and strain 11a were reported to form ethene from 1,2-DCA (Lee et al., 2013).

Moreover, strain BTF08 was shown to reductively dechlorinate all DCE isomers to VC and subsequent to ethene in a reaction mechanism so called stepwise hydrogenolysis (Figure 4).

For the first time it was demonstrated that BTF08 dechlorinates 1,2-DCA to mainly ethene and minor amounts of VC (Chapter 3). The reaction mechanism, revealed by carbon stable isotope analysis, was suggested to be a dichloroelimination resulting in ethene as endproduct, whereas VC was formed only in minor amounts likely as result of a branching dehydrochloroelimination reaction of 1,2-DCA (Chapter 3 and Figure 7-1).

The genome annotation of strain BTF08 revealed gene homologues to known reductive dehalogenase genes (*rdh*), e.g. *pceA*, *tceA* as well as *vcrA*, a vinyl chloride reductase (Chapter 2). The identified genes are highly similar to the *rdh*s of strain 195 (Seshadri *et al.*, 2005; Pöritz et al., 2013), whereas strain 195 does not possess a VC reductase. However, the gene *tceA* of strain 195 encodes for a TceA RDase, responsible for 1,2-DCA dehalogenation to mainly ethene and minor amounts of VC. Additionally, TceA dehalogenates TCE and *cis*-DCE to mainly VC and metabolically to ethene (Magnuson *et al.*, 2000). In contrast, *D. mccartyi* strain BAV1 expresses a vinyl chloride dehalogenase (BvcA) which dehalogenates DCEs, VC and 1,2-DCA to ethene (Magnuson et al., 2000; He et al., 2003a; Tang *et al.*, 2013). BvcA is therefore catalysing different dehalogenation reactions, e.g. of DCE, VC and DCA to ethene. In the genome of strain BTF08 among others the gene homologe *tceA* was identified (Chapter 2, Pöritz et al. (2013)), suggesting that DCE dechlorination to VC and ethene and DCA dechlorination to mainly ethene are potentially catalysed by TceA, as similarly described for strain 195 (Magnuson et al., 2000). The hypothesis that in both *Dehalococcoides* strains the same enzyme catalyse the same reaction is supported by similar 1,2-DCA isotope fractionation patterns determined for both strains (Chapter 3). However, the genome of strain BTF08 additionally contains a *vcrA* gene homologue, suggesting a dechlorination of VC, produced during 1,2-DCA dechlorination in minor amounts in a branching reaction, to ethene.

In conclusion, in the case of absence of VC dehalogenating bacteria VC with imposing health risks may accumulate in the environment due to incomplete dechlorination of PCE, TCE, DCEs and DCA (Hunkeler et al., 2002). Thus, not only the monitoring of the VC concentration at the field site, but also the identification of bacterial marker genes, e.g. taxon or dehalogenase genes for VC reductases, will allow a better remediation and risk assessment.

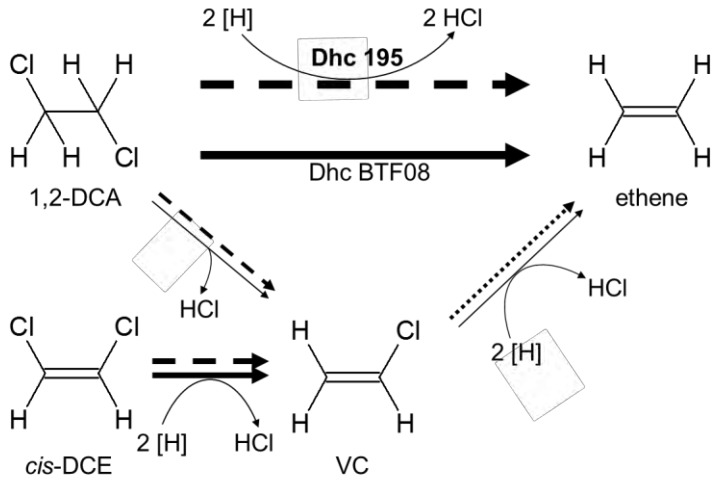

Figure 7-1: *Dehalogenation of 1,2-DCA and* cis*-DCE by* Dehalococcoides mccartyi *strain 195 (Dhc 195,bold dashed lines) and* Dehalococcoides mccartyi *strain BTF08 (Dhc BTF08, lines). Dotted arrow for strain 195 from VC to ethene indicates a co-metabolic reaction (taken from Chapter 2 without editing)*

3. Microbial Transformation of Monochlorobenzene

The accumulation of MCB in anoxic groundwater environments is a global problem. MCB is present in the environment either due to microbial production during degradation of higher CBs (Bosma et al., 1988; Adrian and Görisch, 2002) and cyclohexanes (Ramanand et al., 1993; Lal et al., 2010) under reducing conditions or due to the non-/ intentional introduction of MCB as a parent compound in waste dumps and disposal sites. The aerobic biodegradation of MCB proceeds quite rapid and is well characterized (Field and Sierra-Alvarez, 2008). In contrast, only little is known about the anaerobic biodegradation of MCB. Due to lower availability of energy-yielding alternative electron acceptors, such as nitrate (E_0': ~400 mV) at the field site, e.g. in contaminated groundwater aquifers, degradation may proceed extensively slower. The reductive dechlorination of higher CBs typically stops with MCB or may continue to benzene (van Agteren, 1998). The incomplete degradation constitutes a risk for the ecosystem, as MCB and benzene are more toxic, e.g. for aquatic organisms (U.S.EPA, 1980, 1995).

The presented results of anoxic laboratory microcosms, set up with anoxic MCB contaminated groundwater from the Bitterfeld field site (Chapter 5) and sediment of a small pilot-scale constructed wetland (Chapter 6), treating the field site groundwater over about 1.5 years, indicated the potential for complete degradation of MCB present at the field site. Both, reductive and oxidative degradation pathways may take place in the constructed wetland, whereas MCB is transformed to benzene via reductive dechlorination (Chapter 6), followed by subsequent mineralisation coupled to sulphate and iron reduction.

Alternatively, MCB may undergo a direct mineralisation coupled to iron reduction as shown in Chapter 5. Furthermore, MCB was identified as a dechlorination product of 1,2-DCB in the sediment microcosms containing a 1:1 mixture of 1,2-/1,3-DCB (Chapter 6). The one chlorine atom was preferentially cleaved in *ortho* orientation over the *meta* position and urges for further investigations on the protein level of the involved enzyme, once a pure strain is isolated. An important finding was that 1,2-DCB was dechlorinated to MCB under addition of iron (III) and under methanogenic conditions.

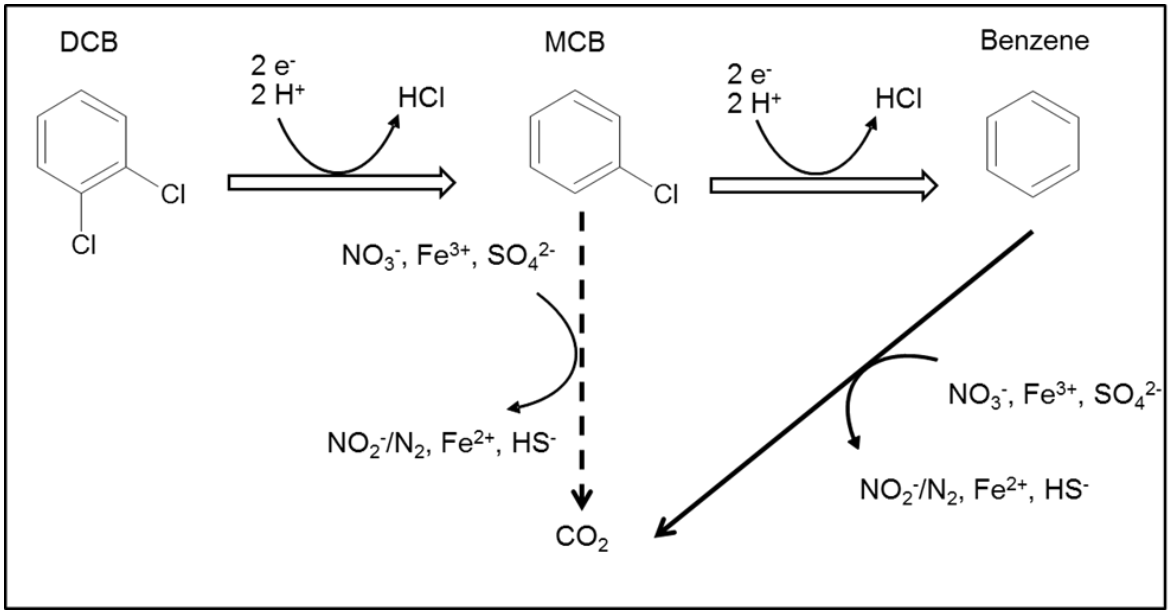

Figure 7-2: *Proposed anaerobic degradation pathways, suggested (dashed line) and published (solid line), for the degradation of dichlorobenzene (DCB), monochlorobenzene (MCB) and benzene. Reductive dechlorination of DCB via MCB to benzene (open arrows) reported by Fung et. al (2009) with hydrogen as electron donor. Anaerobic oxidation of benzene was reported under nitrate (Burland and Edwards 1999), iron (Lovley 2000) and sulphate reducing conditions (Vogt et al., 2007). Direct mineralisation of MCB with NO_3^-, Fe^{3+} or SO_4^{2-} as electron acceptor was suggested as metabolic pathway at corresponding reducing conditions (taken from Chapter 5 without editing).*

MCB dechlorination to benzene with further mineralisation to CO_2 was determined in sediment microcosm amended with iron (III), however, no dechlorination activity was found in bottles amended with lactate and acetate/hydrogen, respectively, as electron donor (Chapter 6). It was assumed that, by addition of iron oxide, iron (III) functions as favoured electron acceptor and MCB gets mineralised, as it was determined in the anaerobic laboratory microcosm, prepared with contaminated groundwater (Chapter 5). Results of this study demonstrated MCB mineralisation in groundwater (Bitterfeld) microcosms ammended with nitrate and goethite as electron acceptor as well as benzene mineralisation coupled to nitrate, sulphate and iron(III) reduction, as already reported (Burland and Edwards, 1999; Lovley, 2000; Vogt et al., 2007).

However, no reductive dechlorination of either DCB or MCB was determined in the groundwater originated microcosms. 1,2-DCB and MCB as electron acceptors reveal a standard redox potential of +380 mV and +310 mV (Dolfing and Harrison, 1992), respectively. However, the observed discrepancy in reductive dechlorination and anaerobic oxidation of MCB in the different microcosm set ups implies that OHRBs need a growth surface (e.g. sediment) to perform a specific reaction, whereas MCB degrading bacteria were enriched in the constructed wetland, which may function as mesocosm. The dependency of benzene degrading bacteria on sediment as a growth substrate has been reported earlier (Vogt et al., 2002) and appears to be essential for the MCB degrading microbial community too.

Recently, reductive dechlorination of DCB isomers to monochlorobenzene (MCB) and benzene was demonstrated in sediment microcosms (Fung et al., 2009), derived from a MCB contaminated site. A *Dehalobacter* species was identified in the resulting enrichment culture, supporting its role in the reductive dechlorination (Nelson et al., 2011). The reductive dechlorination of DCB isomers to MCB was shown in Chapter 6, however, in comparison to the study by Nelson et al. (2011) with much slower transformation. Moreover, *Dehalobacter* spp. was not identified in a bacterial clone library performed with DNA extracted from the field site groundwater as well as in an Illumina library, produced from pore water samples obtained from the contaminated aquifer and from the field site wetland (data not shown). This implies the absence of this key player or the presence in an undetectable amount. In contrast *Dehalogenimonas*-like species were observed, however, a proof for its involvement in DCB dechloriantion could not be obtained, yet.

This study indicated for the first time the dechlorination of MCB to benzene with potential further complete mineralisation to CO_2 (Chapter 6), without having the claimed stimulating effect of higher chlorinated compounds. In a previous study (Nowak et al., 1996) a dechlorination of MCB to benzene (under sulphate reducing and methanogenic conditions) required the presence of TCBs. Moreover, in a recent study two enrichment cultures, in which one (Fung et al., 2009) is shown to reductively dechlorinate MCB to benzene and the other one (Nales *et al.*, 1998; Ulrich and Edwards, 2003) is able to mineralise benzene, were combined for the engineered purpose of a complete MCB degradation in the field, including the aspect of enhanced remediation and bioaugmentation (Liang *et al.*, 2013).

Summarizing, the results of the microcosm studies (Chapter 5 and 6) indicated that the indigenous groundwater microbial community was capable of completely degrading MCB, which promotes the hypothesis that a natural remediation potential for this compound is present at the contaminated field site in Bitterfeld.

II. Implications of an Integrative Approach in Assessing Biotransformation

An integrative approach was applied to assess the *in situ* biotransformation of lower chlorinated hydrocarbons by investigating the transformation in bacterial cultures (Chapter 2 and 3), laboratory microcosms (Chapter 5 and 6), model gradient and groundwater systems (Chapter 4, 5 and 6). Including the gathered results of different scales and diverse analytical methods, a comprehensive characterisation of sources of contaminants, reaction pathways, hydro-geochemical reaction conditions, degradation potential at the field site and the microbial community most likely associated with the contaminant transformation was obtained. Coupling hydro-geochemical methods, CSIA and analysis of the microbial communities in the above listed scales, robust evidences of *in situ* transformation of lower chlorinated hydrocarbons were obtained (Chapter 2-6).

1. *In situ* Biotransformation of Monochlorobenzene and Dichlorobenzene

With respect to the *in situ* transformation of lower CBs, in two model wetlands of different dimension (Chapter 5 and 6), (1,2)-DCB and MCB were effectively removed from the contaminated groundwater. No formation of potential toxic intermediates or products, such as benzene was identified. These findings generally promote the application of engineered model wetlands as a remediation approach (Chapter 1.3) for groundwater contaminated with CBs. In particular, concentrations of up to 5 mg L^{-1} MCB and 0.03 mg L^{-1} DCB were almost completely removed by passing the sediment compartment (99 % reduction of MCB in the small scale greenhouse wetland and up to 88 % of MCB in the planted part of the pilot scale system) (Chapter 5 and 6).

CSIA was applied in order to identify if biotransformation processes were driving the monitored contaminant removal, which would result theoretically in a reaction specific isotope pattern. Microbial degradation will entail an enrichment of ^{13}C in the remaining substrate fraction, whereas degradation products contain a more depleted isotope pattern. However, analysed isotope ratios of MCB did not reveal a significant strong isotope fractionation with decreasing concentration. Though, a trend towards a minor enrichment in ^{13}C of MCB with progressing decrease in concentration was detected (Chapter 6).

Non-destructive removal processes for MCB and DCB, such as evaporation, volatilisation, and evapotranspiration by plants were theoretical considered (Chapter 5), but not investigated in the present study. These processes, reviewed by Imfeld et al. (2009), were already addressed in previous studies with similar model wetlands and were proven to contribute in minor extent to MCB removal (Braeckevelt *et al.*, 2011). However, sorption of DCB and MCB

to brown coal particles present in the groundwater and to organic material, e.g. plant´s rhizosphere may contribute to the observed concentration decrease. Sorption processes would be in steady state though after some time (Imfeld et al., 2009).

Monitoring hydro-geochemical parameters of wetland pore water samples (Chapter 5 and 6), including redox sensitive elements (sulphur and iron) as potential microbial alternative electron acceptors revealed that geochemical gradients developed over time and that redox processes can vary over small distances. The upper horizontal zones and the water pond (surface water) of both wetlands were oxic (DO: up to 4 ppm in average), while the deeper areas revealed anoxic to micro-oxic conditions (DO: up to 0.10 ppm in average). Moreover, prevailing iron (III)- and sulphate- reducing conditions were indicated. In particular, the sulphide (HS^-/S^{2-}) and ferrous iron (Fe^{2+}) concentrations were positive correlated with increasing depth. Hence, high removal of MCB and DCB was observed in sulphate and iron reducing zones. The degradation of these lower chlorinated hydrocarbons in such geochemically heterogeneous systems may occur via reductive dechlorination and/or alternative degradation pathways, e.g. the postulated anoxic oxidation using ferric iron or sulphate (Chapter 5 and 6). Different combinations of electron donor and electron acceptor were tested in groundwater and wetland sediment derived microcosms. Resuming the findings of the microcosm study, the indigenous groundwater microbial community mineralises MCB under addition of nitrate and iron (III), but no indications for reductive dechlorination of MCB to benzene with subsequent mineralisation of benzene were found in groundwater microcosm (Chapter 5). However, benzene mineralisation was determined with addition of sulphate, nitrate and iron (III) as potential electron acceptors confirming previous findings (Burland and Edwards, 1999; Lovley, 2000; Vogt et al., 2007). Considering the results obtained in the sediment microcosm (Chapter 6), reductive dechlorination of 1,2-DCB to MCB and MCB to benzene, with subsequent mineralisation are additional contaminant removing reactions. Benzene was present in the groundwater in low concentrations and did not accumulate in the model wetlands due to potential mineralisation coupled to sulphate reduction, as revealed in groundwater microcosm (Chapter 5). A relatively fast mineralisation might be a reason for the absence of benzene as an intermediate during MCB dechlorination in the model wetlands. Additionally, CSIA applied for the dechlorination substrates and products DCB, MCB and benzene as well as CH_4 indicated in preliminary analysis a small fractionation during sequential dechlorination (Chapter 6), supporting the presence of biodegradation. To date only a few studies reported the fractionation pattern of MCB in pure bacterial consortia and pure cultures during reductive dechlorination (ε= 5 ‰, Liang et al. (2013)), during aerobic mineralisation (ε = 0.1 - 0.4 ‰, Kaschl et al. (2005)) as well as under field conditions (ε= -0.5 ‰, Kaschl et al. (2005)). In the present study the estimation of the

isotope enrichment factor ε of the MCB removal in the wetland samples resulted in -0.4 ± 0.7 ‰, however was statistically not significant. This weak enrichment factor is difficult to interpret as MCB might be product and substrate at the same time. Moreover, isotope patterns for anaerobic MCB oxidation with iron (III) have not been reported and could not be determined in the present study yet. Therefore, currently CSIA cannot be used to characterise the transformation pathways in the microcosms.

In reference to the microbial community analysis (Chapter 6), a diverse microbial community of anaerobic bacteria was identified mainly consisting of Proteobacteria as dominant group, whereas the detailed composition was found to be in accordance with previous studies at the field site (Alfreider et al., 2002). Deltaproteobacteria, consisting mainly of sulphate (genera *Desulfobacterium*) and iron reducing (genera *Geobacter*) bacteria, further *Dehalogenimonas* affiliated in the *Chloroflexi* group, as well as aerobic Betaproteobacteria (genera *Burkholderia*) were assigned (Chapter 6). The microbial data were in line with the dominant geochemical conditions determined in the groundwater and wetland system. No affiliation of obtained 16S rRNA gene amplicons to *Dehalobacter* ssp. was found in the groundwater bacterial clone library. Results from taxon-specific amplification of 16S rRNA genes of *Dehalobacter* ssp. in DNA extracts of wetland sediment (Chapter 6) were negative. Nevertheless, gene amplificates of the *bamA* gene, a biomarker for aromate degradation (Kuntze *et al.*, 2011), was identified in the greenhouse model wetland supporting the proposed fast degradation of benzene in the wetland, if formed during DCB and MCB dechlorination. As describe above (I. 3.) *Dehalobacter* ssp. was recently identified to be involved in the reductive dechlorination of DCB to MCB, and further to benzene after dechlorination activity of DCB increased (Nelson et al., 2011). Hence, the observed 1,2-DCB and MCB dechlorination capability of the microbial community settled the wetland sediment and indicates potential new bacterial strains, maybe affiliated with *Dehalococcoides mccartyi* or *Dehalogenimonas*, as observed in the bacterial clone library. To date no *Dehalococcoides* sp. isolate has been reported to dechlorinate lower chlorinated hydrocarbon like DCB and MCB. Although no bacterial candidate for DCB and MCB transformation was isolated in the present study, such model wetlands provide right geochemical conditions for *Dehalococcoides* ssp.. Its presence and dehalogenation activity was indicated in a similar model wetland while investigating the microbial transformation of *cis*-DCE (Imfeld et al., 2008a). The detection of gene sequences assigned to *Dehalococcoides mccartyi* in these model wetlands indicated that also *Dehalococcoides mccartyi* strain BTF08 may habitat such ecosystem and be a good candidate for natural remediation due to the dechlorination spectra of diverse halogenated hydrocarbons of different chemical categories reported in Chapter 2 and discussed above (Chapter 1, I.1). Additional knowledge about the relationship of

environmental factors and *in situ* microbial communities was obtained by combining in a statistical approach microbial community pattern data, retrieved from TRFLP analysis and prevalent hydro-geochemical parameter of the hydro-geochemical long term monitoring (Chapter 6), which are environmental vectors impacting the observed *in situ* contaminant removal (Chapter 5). The distribution and environmental dependencies of the community pattern and the hydro-geochemical variables in the greenhouse model wetland indicated the presence of comprehensive microbial network. The environmental parameter, both oxygen and ferrous iron, seemingly influenced the observed microbial pattern distribution in the wetland sediment. The distribution was depth related. The upper layer of the wetland sediment (more oxic) revealed a distinctly different community pattern compared to the bigger anoxic sediment part (Chapter 6).

The multivariate analysis of data is an established approach in ecological research questions and was introduced to identify microbial processes and to describe the biodegradation potential in the investigated model wetland. General conclusions were drawn about the community distribution that was correlated with prevalent environmental parameters which was depth related. However, the study was limited in identifying clear microbial processes and functionality with respect to *in situ* contaminant degradation. A deeper process understanding will be gained not only by combining microbial and environmental data over space as presented in Chapter 6, but additionally by including the factor time. This means microbial data are gathered after different time points during proceeding contaminant removal in the constructed wetland. Such an approach can show the dynamic of microbial communities over time with respect to the hydro-geochemical evolution of the system, as reported recently in a *cis*-DCE treatment wetland (Imfeld *et al.*, 2010) . Nevertheless, in the present study microbial data were analyzed from sediment samples, containing bacteria of the aqueous and solid phase, which is an enhancement compared to the aforementioned study (Imfeld et al., 2010), which include only the aqueous phase, due to identifying community patterns of the microbes settled in the monitored redox conditions. Despite existing bias caused by sampling and analytical procedures applied the microbial fingerprinting method provided meaningful information about the presence, residence and relative abundance (Chapter 6).

Considering all new findings discussed above, observed removal of DCB, MCB and benzene *in situ* is a microbial driven biotransformation, which is most likely a combined effect of apparently both processes, oxidative and reductive degradation pathways taking place sequentially or in parallel. Besides microbial transformation of lower chlorinated hydrocarbons, evapotranspiration and volatilisation may as well affect the contaminant load, but in a minor extent

(Braeckevelt et al., 2011). The considered removal pathways for the lower chlorinated CBs and the prevalent hydro-geochemical redox conditions were illustrated in a conceptual model (Fig. 7-3).

The various studies performed to investigate the biotransformation pathways of DCB and MCB underlined the necessity of integrated approaches like reflected in this thesis.

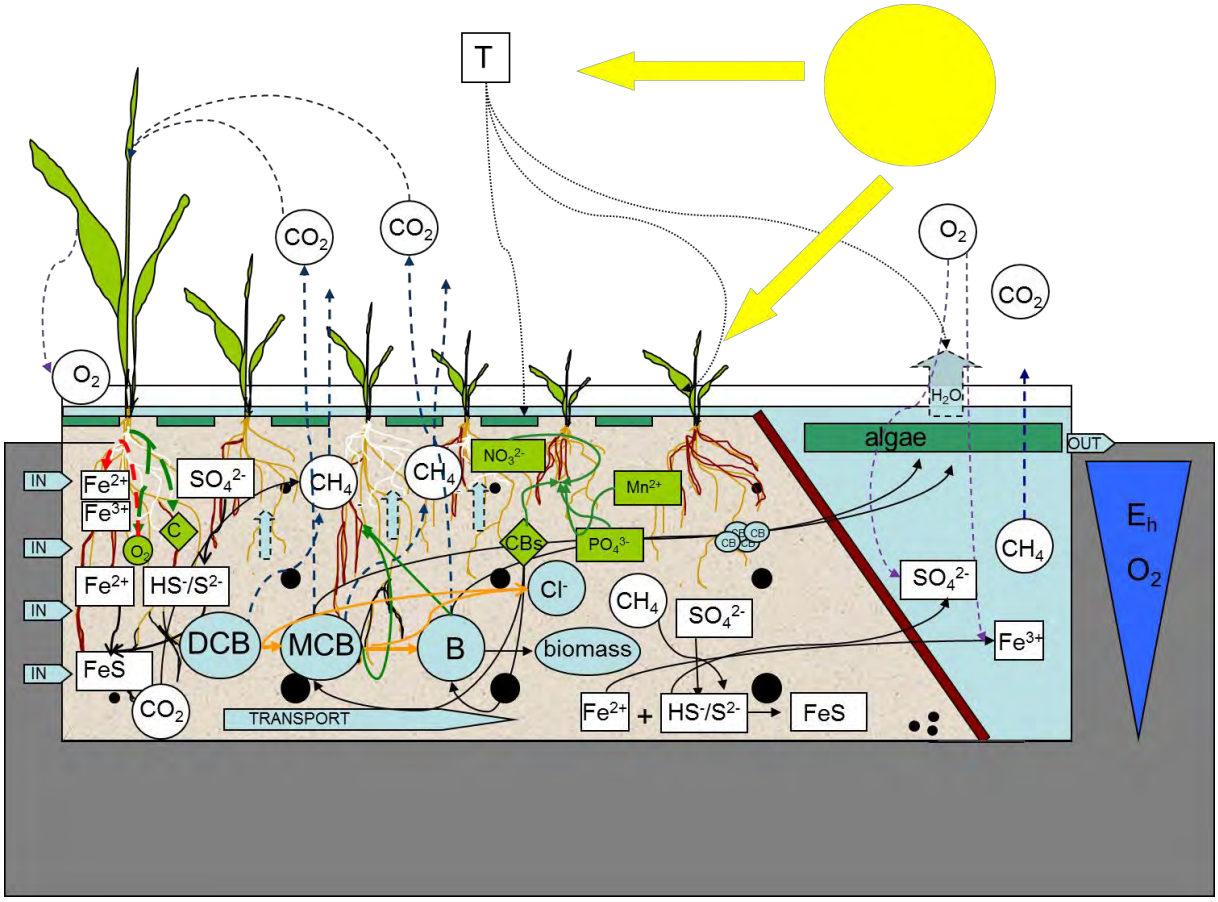

Figure 7-3: *Conceptual model of the DCB and MCB biotransformation in the pilot scale constructed wetland filled with sediment and planted with Juncus effuses* (L.)*. Light blue-solid line arrows indicate the water flow; light blue-dashed line arrows represent the evaporation and evapotranspiration of the wetland water; orange solid line arrows indicated the reductive dechlorination of DCB via MCB to benzene with release of Cl⁻. Dark blue dashed line arrows show the mineralisation of CBs and benzene (to CO_2 or CH_4); green arrows are related to the plant rhizosphere: with dashed arrows indicating oxygen intrusion in form of exudates (oxidised organic acids), solid lines show potential plant uptake of CBs and PO_4^{3-}, NO_3^{2-} and Mn^{2+}; violet dashed arrows indicated atmospheric oxygen intrusion into wetland; black solid arrows show oxidation and reduction reactions of sulphur and iron, whereas red dashed arrow symbolises oxidation reaction of Fe^{2+} at the plant roots. Black dots of increasing volume in greater depth indicate increasing formation of FeS-complexes. Presence of algae is shown in turquoise at wetland areas accessible for light (indicated by yellow sun). The impact of the air temperature (T) is shown as black dotted arrow. Redox potential (Eh) and dissolved oxygen (O_2) are highlighted in the intense blue triangle, indicating lower values with increasing depth.*

2. Application of CSIA in the Contaminated Field

While CSIA was not informative for distinguishing pathways of DCB and MCB degradation *in situ* (Chapter 5 and 6), it was possible to identify sources and biotransformation of CEs at a complex field site in Italy (Chapter 4). CSIA of chlorinated hydrocarbons could be used to distinguish contamination plumes from different production pathways. Different production pathways, by using different raw material with distinct stable isotope signature result in distinct isotope signature in the synthesized product. At the field site (Chapter 4) one group of CEs was identified with isotope signatures in the previously observed range for commercial chemicals of recent production between $\delta^{13}C$ −23.3 and −37.2 ‰ (van Warmerdam *et al.*, 1995). Such range of isotope signatures of recent production was reported for other field sites as well (Nijenhuis *et al.*, 2007b; Imfeld *et al.*, 2008b; Hunkeler *et al.*, 2012). A second group of CEs and chlorinated methanes with lighter carbon stable isotope signatures was determined, where methane with a typical depleted carbon isotope signature was used as starting material. It is well known that formerly chloromethanes were produced via a thermal radicalic chlorination, obtaining CEs and CAs as waste by-products. Methane has a natural light microbial signature (Mattavelli and Novelli, 1988) and was most likely retrieved from natural gas reservoirs (e.g. Po river area). This way of manufacturing produced specific depleted isotope signature, which allows the identification of the origin of these chlorinated contaminants. On basis of the two different detected carbon isotope signals, more enriched or more depleted in ^{13}C, diverse production processes could be distinguished to recent and former production.

Apart from linking ^{13}C depleted and ^{13}C enriched isotope pattern to different chemical production lines, isotope fractionation results in enrichment of ^{13}C in the residual fraction of the dehalogenation substrate and in a depletion of the product during dehalogenation (Chapter 1, III.4), as presented in laboratory experiments for 1,2-DCA, *cis*-DCE, 1,1-DCE and VC dehalogenation by *Dehalococcoides mccartyi* BTF08 (Chapter 3). Similarly during microbial dehalogenation in contaminated aquifers ^{13}C isotope become enriched in the remaining fraction of the substrate and depleted in the product, whilst products can be intermediates for further reactions, getting eventually enriched in ^{13}C in a typical dehalogenation sequence, like DCE→VC→ethene. The dehalogenation of CEs and CAs results in ethene or ethane, which are present in most plumes in Ferrara suggesting active natural attenuation processes. In general, CEs were observed to be ^{13}C enriched in some sampled boreholes and in others the isotope pattern was rather variable (Chapter 3). The average composition of ^{13}C enriched CEs indicated biotransformation, but excludes mineralisation.

Profound knowledge on biotransformation of lower chlorinated hydrocarbons and other organohalides *in situ* are achieved by applying the presented concept of combining the proposed methods; CSIA, hydro-geochemical investigations and molecular biological analysis. The concentration decrease of organo-halide compounds and hydro-geochemical parameters (including redox species of different elements serving as potential electron acceptors, e.g. O_2, NO_3^-, Fe^{3+}, SO_4^{2-}, ect.) can be analytically determined and may explain an observed contaminant reduction due to the respective redox conditions. Excluding physical processes, such as sorption and dilution, impacting the concentration of chlorinated hydrocarbons in the aquifer, CSIA is a reliable analytical tool. Hence, concentration change of chlorinated hydrocarbons (substrate) can be correlated to enrichment in ^{13}C in the residual fraction of the degraded contaminant (Chapter 1, III.4).

Applying this approach in laboratory reference studies, including bacterial or enrichment culture, reaction specific and compound specific isotope enrichment factor (ε) (Chapter 3) are determined. The extent of stable isotope fractionation allows to assess the biodegradation of chlorinated and non-chlorinated hydrocarbons *in situ*, qualitatively and quantitatively as well as to elucidate reaction mechanisms (Fischer et al., 2007; Hofstetter and Berg, 2011; Thullner *et al.*, 2012). Complementing hydro-geochemical and CSIA investigations with molecular microbial data of taxon or other specific marker genes of known organohalide degrading bacteria (Chapter 1, II.3) will foster the characterisation of *in situ* transformation processes for chlorinated hydrocarbons in depth and eventually predictions about natural attenuation scenarios can be made.

The presented integrative monitoring approach complements and enhances engineered remediation methods in the contaminated site management.

III. Outlook and future perspectives

The study demonstrated that CSIA is a valid method in an integrated approach to assess the *in situ* biotransformation activity in field sites contaminated with CEs, CAs (Chapter 4) and CBs. However, the application of CSIA for MCB and DCB in pore water samples of constructed wetlands, revealed only a weak isotope fractionation during MCB mineralisation or reductive dechlorination (Chapter 5 and 6). In sediment microcosms the isotope shift of about 2 ‰ during reductive dechlorination of DCB and of MCB indicated biotransformation (Chapter 6). The enrichment and later the isolation of bacterial key players in the DCB/MCB dechlorination will allow further degradation and isotope fractionation experiments to elucidate the reaction mechanism, as achieved for *Dehalococcoides mccartyi* strain BTF08 (Chapter 2 and 3). Moreover, an isolate or a stable microbial consortium which can dehalogenate DCB and MCB enables the investigation of catalyzing enzymes and encoding genes, which to date are not identified.

Additionally, chlorine isotope analysis has recently gained big advancement (Shouakar-Stash *et al.*, 2003; Shouakar-Stash *et al.*, 2005). As future application CSIA could be used combining both elements, carbon (C) and chlorine (Cl), in a routine method. This approach allows performing dual isotope plots (2D-C/Cl) to better elucidate degradation pathways in the environmental fate of chlorinated substances, as reported recently for CEs (Hunkeler et al., 2011; Wiegert *et al.*, 2012). The application of 2D-C/Cl isotope plots for lower CAHs in different environmental systems (microcosms, wetlands and contaminated aquifers) improves the characterisation of the prevailing degradation pathways with special regard to the differentiation between certain groups of bacteria. This differentiation between bacterial groups and aerobic or anaerobic degradation pathways was previously obtained for non-chlorinated hydrocarbons, such as BTEX and toluene, using 2D-C/H isotope plots.(Fischer et al., 2007; Vogt *et al.*, 2008).

Dechlorination of DCB and MCB as well as mineralisation of MCB coupled to nitrate, iron (III) and sulphate reduction was shown in laboratory experiments (Chapter 5 and 6). However, a better documentation of the transformation mechanism involved in the reactions requires the isolation of the responsible bacteria. CSIA can then be used to investigate the associated isotope fractionation in pure cultures. Further efforts should aim at the implementation of isotopic data from the reference cultures in up-scaled systems, such as constructed wetlands or aquifers. Additionally the reduction of the distances sampling points in the wetland's sediment will result in a deeper understanding of the geochemical processes coupled to *in situ* MCB transformation.

Additional processes like volatilisation, uptake by plants or hydrophobic sorption may significantly contribute to contaminant removal in wetlands or sediment rich aquifers.

Phytoremediation for MCB contaminated groundwater is not covered in this study, however wetland plant related processes, such as potential contaminant uptake and breakdown by plant enzymes, should be included in future investigations to predict contaminant fractions removed by these processes, as suggested by Braeckevelt et al. (2011).

A closer look on the influence of non-destructive processes (e.g. sorption and volatilisation) on the MCB and DCB loads is required, although not significantly affecting chlorinated solvents´ concentration in wetlands, to further develop monitoring concepts of biodegradation in both, wetlands and aquifers. Additionally, the hydrological groundwater flow characterisation will give additional information on observed redox and biotransformation processes (Chapter 5 and 6) in model systems (e.g. wetlands), by analysing environmental and conservative non-reactive tracers

Screening of microbial communities via culture-independent methods, combined with the hydro-geochemical and isotopic assessment of biotransformation gives a meaningful understanding of microbial dynamics, activities and pathways at discrete time periods and as a function of the contaminant concentration (Imfeld et al., 2009; Imfeld et al., 2010). Routine bacterial fingerprinting methods such as TRFLP and DGGE or taxon-specific PCR-analysis are rapid and reliable methods for screening the presence of key players in contaminant degradation. The identification of microbial communities in both, aqueous and solid phase of wetlands will improve the characterisation of their distribution in the ecosystem and helps to discover the bacterial portion attached to the substrate surface (e.g. wetland sediment) and in the pore water. However, collecting serial sediment samples from different locations at discrete time points is difficult and may disturb the stable system in terms of geochemical conditions and microbial contaminant transformation. Sampling techniques have to be developed to collect smaller amounts of sediment in order to apply a less invasive sampling strategy. At the same time collecting pore water samples for microbial analysis in spatial-temporal intervals will allow the characterisation of microbial community structure and dynamic over time. The combined analysis of the sediment-attached and pore water microbial communities together with hydro-geochemical data will give deeper insights into the partitioning of DCB and MCB dechlorinating bacteria and their degradation activity in the aqueous and sediment phase.

Moreover, continuously improved rapid sequencing technologies, e.g. Pyro- and Illumina sequencing allow a high throughput of samples. Biomass samples obtained from hot spot zones of microbial activity, e.g. in the rhizophere and sediment areas of the presented model wetlands in which steep geochemical gradients (e.g. O_2, SO_4^{2-}, Fe^{3+}) are present, will allow an extensive characterisation of the microbial community.

Identifying OHRBs on DNA level (16S rRNA genes), indicates their presence, but does not allow conclusions about their activity in terms of contaminant dechlorination. The identification of metabolic genes on DNA and RNA level, like the reductive dehalogenases (TceA, BvcA, and VcrA), which are responsible for CE (and CA) dehalogenation, will improve the evaluation of the *in situ* biotransformation and can be applied routinely. Despite the routine analysis for identified dehalogenase genes, a lot of enzyme genes are not identified yet, e.g. a putative MCB and DCB dehalogenase gene. Moreover the expression of the reductive dehalogenase genes is still unclear and is lately an active field of research. Therefore, besides CSIA, stable isotope probing (SIP) techniques (DNA-, RNA- and protein-SIP, (Boschker et al., 1998; Manefield et al., 2002; Jehmlich et al., 2008; Bombach et al., 2010b; Jehmlich et al., 2010)) offers a relevant approach to determine the metabolically active microbial community members, using ^{13}C-labelled electron chlorinated solvents in microcosm and model system experiments.

Geochemical data sets can be explored and interpreted in relation to biological information (i.e. diversity pattern of microbial community) through statistical multivariate analysis and modelling approaches. These numerical approaches will contribute to the identification of the main factors driving contaminant removal and place the basis for scenario predictions in contaminant plumes in aquifers, providing the basis for better management of engineered systems like constructed wetlands. Even if constructed wetlands represent one of the most studied models to investigate natural processes, this study provides new opportunities to optimise microbial contaminant treatment conditions, like the addition of electron acceptors, e.g. an iron oxide source for MCB mineralisation (Chapter 5 and 6).

Given the outcome of this study, new remediation options and tools can be developed to improve the contaminant removal efficiency. Moreover, further studies may focus on the effect of up-scaling such treatment system for groundwater decontamination, the increased size of such systems may influence the residence time of the groundwater contaminants and might increase their reactivity. However, up-scaling makes the microbial and hydro-geochemical monitoring a challenging task.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die Dissertation wurde bisher keiner anderen Prüfungsbehörde zur Begutachtung vorgelegt.

Leipzig, 15. April 2014 Marie Markantonis

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Listed oral and poster presentation as presenting author

Conference proceedings

Oral presentation

- **Markantonis, M. and Nijenhuis, I.** (2014) Investigation of the in situ microbial transformation pathways of dichlorobenzene (DCB) and monochlorobenzene (MCB). DehaloCon - A Conference on Anaerobic Biological Dehalogenation, 23-26 march 2014, Jena, Germany

- **Markantonis, M., Klein, B.S., Devakota, S., Richnow, H.H., Nijenhuis, I.** (2014) Anaerobic transformation of monochlorobenzene in a constructed wetland and in anaerobic sediment microcosms. Genesis conference: Integrated Management of Groundwater Resources and Dependent Ecosystems, 5-7 march 2014, Prague, Czech republic

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Appendix

Pilot-scale Constructed Wetlands

Figure A1-1: *Pilot-scale constructed wetland: unplanted (A) and planted (B) in summer 2009.*

Figure A1-2: *Pilot-scale planted constructed wetland in the greenhouse with inflow (groundwater tank [**A**]), in front view [**B**] and back view [**C**] in May 2012.*

Sampling of Pilot-scale Constructed Wetlands

Figure A2-1: *Sampling of the pilot scale constructed wetland at the field site Bitterfeld with water pumps: in gravel bed with lancets to obtain pore water samples (**A**); in the water pond (**B**) and by use of electrode to measure redox potential on site (**C**).*

Laboratory Microcosms

Figure A3: *Laboratory microcosms containing contaminated Bitterfeld groundwater with different electron donor and electron acceptor combinations. Detailed composition of each microcosm set is given in Chapter 5.*

Field site sampling

Figure A4: *Sampling at the field site in Ferrara (Italy), using (**A**) a flow through cell, which is inserted into the groundwater well (**B**) together with the metering rule (**C**). At the field site several groundwater samples were taken with a tube pump (**D**) and immediately preserved according to the protocol of the respective analytical methods (**E**).*

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Chapter 6:

Microbial diversity and activity in a monochlorobenzene removing sulphate reducing wetland

(Manuscript in preparation)

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Abstract

The diversity and activity of microbial communities in the sediment compartment of a planted model wetland was characterised, which effectively removed monochlorobenzene (MCB) from the supplied contaminated groundwater (dichlorobenzene (DCB), MCB and benzene) from a regional contaminated aquifer. TRFLP analysis of sediment samples obtained from different parts of the wetland revealed that the local distribution of distinct bacterial communities coincided with the hydro-geochemical conditions in the wetland, whereas two distinct community clusters were present, correlating with anoxic or micro to oxic zone. DCB, MCB and benzene were effectively removed in the anoxic aqueous phase of the sediment and iron (III) was thought to play a role as terminal electron acceptor in complete mineralisation of chlorobenzenes. An increase in benzene, as potential product of reductive dehalogenation, was not observed, however, benzene may have been further degraded.
The microbial activity with respect to the observed contaminant removal in the wetland was investigated in selected sets of anaerobic wetland sediment microcosms. MCB was a major product from DCB and further dehalogenation to benzene, being mineralised was elucidated. Additionally, mineralisation of MCB was observed similar to previous findings using only the groundwater instead of sediment. 16S rRNA gene libraries of the supplied contaminated groundwater revealed the presence of a diverse community which could be linked to methanogenic, sulphate or iron reducing activity, whereas 36 % of the 16S rRNA gene sequences were related to sulphate reducers. Additionally representatives (3 %) of the phylum *Chloroflexi* related to *Dehalogenimonas* which are potentially involved in the dehalogenation of chlorinated contaminants were detected. Based on a multiple-method approach, this study illustrates the linkage between microbial community composition and activity with hydro-geochemical conditions and processes of MCB biotransformation in a wetland system.

6.1 Introduction

Constructed wetlands find their application as water treatment systems since decades. They were found to improve the water quality due to different biochemical reactions and redox processes leading to the transformation of various organic compounds in different oxidation states (Truu *et al.*, 2009). This turnover of organic substances and biochemical element cycles take place due to physical, chemical and (micro) biological interaction. (Borch *et al.*, 2010). Indeed, wetlands are hot spots of metabolic activity with high carbon turnover rates and develop high specie-diversity, while habituating diverse plants, vertebrates and microbes (Kadlec *et al.*, 2012).

In this sense microbial communities are the basis for primary production and play an essential role in geochemical element cycles, generating and exploiting gradients of geochemical parameters such as oxygen, iron, nitrate and sulphur. In the rhizosphere of wetlands plant exudates, including acids, sugars and polysaccharides, arrive in the saturated sediment zone, creating different redox zones with respective microbial activities, which are found to be depth dependent (Hamonts *et al.*, 2013). Identifying the species diversity in the microbial community in such zones became easier using rapid molecular biological screening methods, such as TRFLP, DGGE, Illumina- or Pyro-sequencing. The resolution of the microbial diversity in wetland sediments in a depth profile, will allow the characterisation and separation of different zones according to their biological function in these gradients system. As wetland ecosystems are considered to be dynamic with redox processes developing over space and time (Imfeld *et al.*, 2008; Truu et al., 2009) it provokes the question where, when and which microbes are involved in the different wetland processes?

Groundwater contaminations with chlorinated hydrocarbons discharging into wetlands and surface water may impact redox conditions in wetland sediments and possibly both, the structure and composition of the microbial communities, including their metabolic activity. Among various chlorinated groundwater pollutants, the in absence of oxygen highly persistent chlorobenzenes are frequently detected in aquifers at industrial field sites due to former and illegal waste dumping, which may impact groundwater dependent ecosystems. This ongoing problem urges for investigations of the fate of these contaminants *in situ*.

Monochlorobenzene (MCB) is a recalcitrant groundwater contaminant (U.S.EPA, 2009), which was found to accumulate in particular under anoxic groundwater conditions at former chlorine industrial field sites, e.g. in Bitterfeld (Germany) (Wycisk *et al.*, 2003; Heidrich *et al.*, 2004) and seemed to be a problematic compound for anaerobic microbial biodegradation. Thus far a few field studies demonstrated a transformation of MCB under anoxic conditions, in an

aquifer (Kaschl *et al.*, 2005), in nature like constructed wetland systems (Braeckevelt *et al.*, 2007; Braeckevelt *et al.*, 2011; Schmidt *et al.*, 2014) and in *in situ* and *ex situ* microcosm studies (Nijenhuis *et al.*, 2007; Schmidt et al., 2014), using ^{13}C labelled MCB in a tracer experiment. Previously reductive dechlorination of higher chlorinated benzenes via MCB to benzene was demonstrated under methanogenic conditions (Nowak *et al.*, 1996). First indications for betaproteobacteria involved in the anaerobic biotransformation of MCB were reported (Martinez-Lavanchy *et al.*, 2011). Recently Fung *et al.* (2009) described complete reductive dechlorination of DCB and MCB to benzene in anaerobic microcosms and *Dehalobacter* spp. was identified in these microcosms (Nelson *et al.*, 2011). The aerobic microbial degradation of MCB was described, including several aerobic bacteria, like *Pseudomonas* sp. or *Rhodococcus* sp. (Haigler *et al.*, 1992; van Agteren, 1998; Vogt *et al.*, 2004), which involves a monoxygenase reaction with an aromatic ring cleavage to chlorocatechol as intermediate. However, thus far there is still knowledge lacking about the microbial community involved in the MCB degradation in anoxic zones and at biogeochemical gradients *in situ*.

Dichlorobenzene (DCB) and MCB may be directly mineralised to CO_2 and CH_4 with alternative electron acceptors like NO_3 and Fe^{3+} (Schmidt et al., 2014), or get reductively dechlorinated to benzene, which will be further mineralised with NO_3, Fe^{3+} or SO_4^{2-} or under methanogenic conditions (Burland and Edwards, 1999; Lovley, 2000; Vogt *et al.*, 2007; Liang *et al.*, 2013a). Both reaction pathways may also take place simultaneously. Recently we identified MCB transformation in anoxic conditions in a pilot scale constructed wetland, which was indicated to be linked to iron reduction (Schmidt et al., 2014). Additionally, MCB mineralisation was found under iron (III) and nitrate reducing conditions. However, reductive dechlorination of MCB to benzene under methanogenic conditions could not be detected. Also the microbial community potentially involved in the observed MCB removal *in situ* was not evaluated.

Depending on hydro-geochemical conditions, diverse microbial communities and taxa may be involved in the degradation of MCB, if metabolic or co-metabolic, if oxidative or reductive. Additionally evidences for functional genes for potential reductive MCB dehalogenases or enzymes were not reported yet. However, the detection of functional genes as biomarker for aromatic hydrocarbon-degrading micro-organisms, e.g. *bamA* (Kuntze *et al.*, 2008) could indicate the presence of a microbial community having the potential to degrade hydrocarbons such as benzene.

Additionally, the presence of known dechlorinating bacteria, e.g. *Dehalococcoides*, *Geobacter* and *Dehalobacter* in the wetland sediment would indicate potential dechlorination.

This study examined the microbial community in an anoxic, MCB removing, model wetland system (Figure 6-1). It is hypothesized that hydro-geochemical and prevalent redox conditions

affect the microbial community structure, prevailing degradation mechanism in the model wetland, which in turn may influence the system functioning over space and time with respect to organic contaminant removal. In the present study the objective was to investigate the anaerobic MCB transformation pathways in a small scale constructed wetland. The second objective was to find out whether the microbial community reflects the prevalent hydro-geochemical conditions, sulphate and iron reduction, in the wetland sediment. Finally, the DCB and MCB degradation activity of the wetland sediment indigenous microbial community under methanogenic and reducing conditions was analysed in anoxic sediment microcosm. Following this consecutive approach it was aimed at characterising the DCB and MCB microbial transformation pathways in a wetland treatment system for MCB contaminated groundwater.

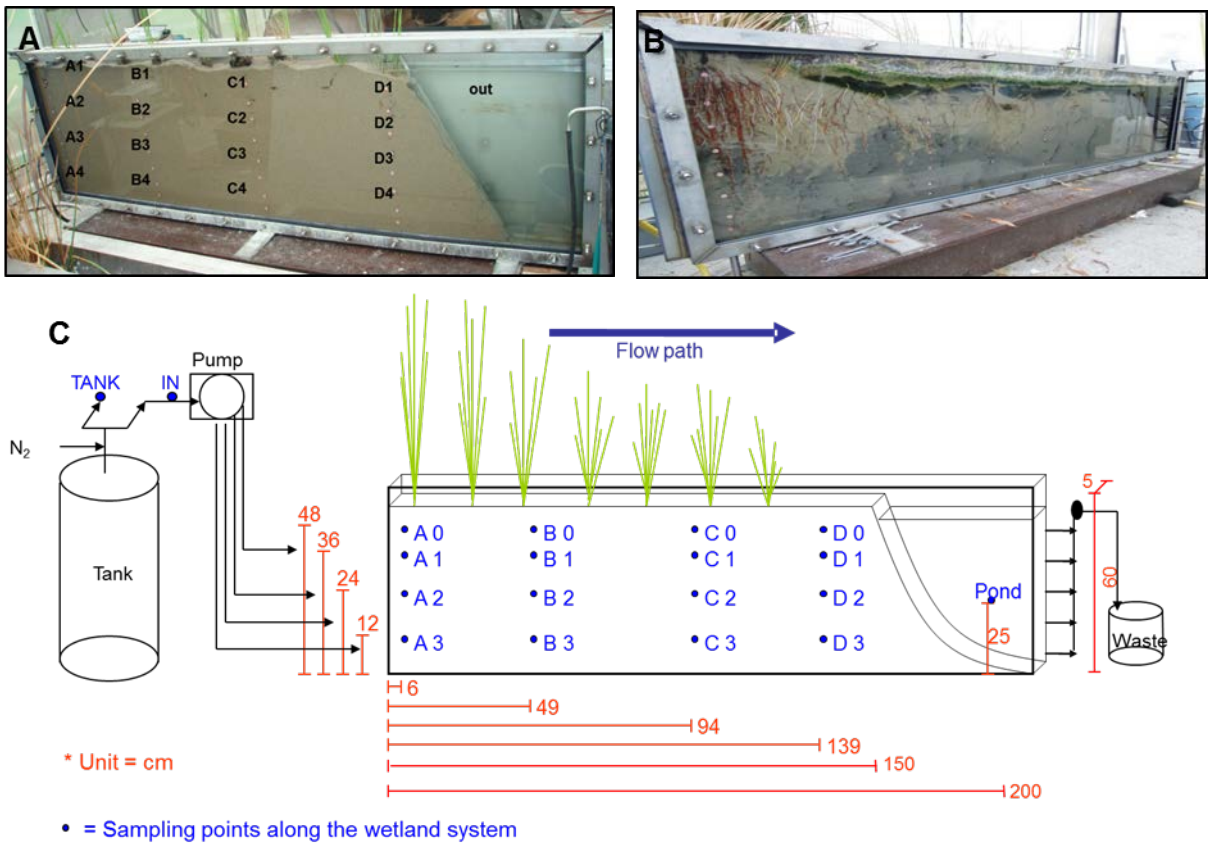

Figure 6-1: *(A) Photograph of the sub surface flow constructed wetland (CW) at the start of the experiment, filled with sediment, planted with Juncus effuses, saturated with tap water. Sampling locations are in indicated from A-D and 1-4, including the sampling point in the pond. (B) Photograph of the constructed wetland after 306 days in flow through mode with MCB contaminated groundwater. (C) Schematic view of the CW with the dimension 60 (h) x 200 (l) x 5 (w) cm, indicating the groundwater inflow with tank (T) and inflow tubes (IN) and the open water pond (P). The sampling ports are shown as blue points in the sediment compartment with the nomenclature A-D for the vertical segments and 0-3 for the horizontal depth levels with the corresponding XY position with dimensions given in cm. The nitrogen pressurised groundwater tank, the peristaltic inflow pump and the waste container are shown.*

6.2 Material and methods

6.2.1 System design, sampling procedure and analytical methods

The small-scale constructed model wetland system (CW) (Figure 6-1), in detail described in Imfeld et al. (2008) and in Rakoczy *et al.* (2011), was used to investigate the settled microbial community and to elucidate its potential role in the *in situ* biotransformation of MCB and DCB. The CW consisted of a stainless steel frame with an embedded glass pane at the front side with a dimension of 200 x 60 x 5 cm. The back site had 16 sampling ports in X-Y coordinates (Figure 6-1C), equipped with metal luer-lock valves (P339.1; Carl Roth GmbH; Karlsruhe, Germany) containing cannulas (diameter 1 mm, length. 8 mm) that extend through a butyl rubber septum into the wetland sediment. Monthly pore water sampling was done connecting the valves to plastic Luer lock syringes, to extract pore water of 44 mL per valve (resulting in an extraction radius of 2.19 cm (4.38 cm diameter). The CW was filled with quartz sand (grain size: 0.4-0.6 mm, see quartz sand composition below; Sand-Schmidt, Germany) at a length of 150 cm and was planted with common rush (*Juncus effuses*, L.). A fine mesh network was fixed at 150 cm from the top to the bottom in a 60° angle to create an artificial lake bank and a free water pond. MCB contaminated groundwater (groundwater composition, SI, Table S6-1) was pumped (multi-channel hose pump REGLO analog from ISMATEC, IDEX Health & Science GmbH; Wertheim-Mondfeld, Germany) from a stainless steel pressurized (nitrogen gas at 0.5 bar, Linde) 50 L-tank via 4 inlet tubes (di=4mm from ISOVERSINIC®; LIQUID-scan; Überlingen, Germany) into the CW. The glass front of the CW was covered with dark plastic boards, to avoid impact of light and resulting algae growth. The stainless steel backward wall of the wetland with a cooling system kept the temperature of the CW constant at 10°C. The in a water bath (10°C) cooled tank was refilled twice a month with the groundwater from the contaminated aquifer in Bitterfeld (Wycisk et al., 2003). The inflow pump rate was 2.5 L contaminated water per day, with a flow velocity of 20 cm d^{-1} in the wetland, and an estimated retention time of 26 days. The outflow consists of a movable pipe-hose construction. The chemical composition of wetland sediment(mean ± SE %; n = 5) was described previously (Imfeld *et al.*, 2010) with organic carbon 15.0 ± 0.9, SiO_2 49.6 ± 0.5, Al_2O_3 10.4 ± 1.1, MgO 2.2 ± 0.1, CaO 11.6 ± 1.1, Fe_2O_3 4.5 ± 0.5, MnO 0.1 ± 0.0, Na_2O 0.6 ± 0.1, K_2O 2.4 ± 0.2 and P_2O_5 0.4 ± 0.1. The sediment texture was (%): clay 44, fine silt 33, coarse silt 10, fine sand 5, and coarse sand 8.

6.2.2 Hydrogeochemical analysis and compound specific carbon isotope analysis

Hydrogeochemical analysis of pore water sample

Pore water, tank, inflow and outflow (pond) samples were taken monthly from the CW. Per sampling port 44 mL water were extracted and aliquots were prepared to determine the concentration of the following inorganic parameters: ammonium (NH_4^+), phosphate (PO_4^{3-}) and iron (II) (Fe(II)), nitrate (NO_3^-), nitrite (NO_2^-), chloride (Cl^-) and sulphate (SO_4^{2-}), iron total (Fe_{tot}), manganese total (Mn_{tot}) and sulphide (SH^-/S^{2-}). (Schmidt et al., 2014)Redox potential (Eh), pH value and the water temperature (T) were recorded during sampling. The methods for each parameter are described in detail in Schmidt et al. (2014). The dissolved oxygen concentration (DO) was analysed at planar oxygen mini sensors Pst3 (PreSENS; Precision Sensing GmbH, Regensburg, Germany), that were attached at the inner glass pane surface in an X-Y coordinate system (Figure 6-1). Concentrations of benzene, MCB, and DCB were analysed gas chromatographic (GC). The GC method as well as the analysis methods for the aforementioned inorganic parameter is described in Schmidt et al. (2014).

Hydro-geochemical analysis of sediment samples

After stopping the hydro-geochemical monitoring of the CW after 1.5 year runtime 30 g sediment samples (fresh weigh) were taken in triplicates per sampling location (A1-D3) (Figure S6-1), were aggraded with 100 mL ultrapure water in a 200 mL sample bottle and were shaken over head for 2 hours (rpm). Subsequent the mixture was paper filtrated. The water flow through was analysed for Cl^-, SO_4^{2-}, NH_4^+, PO_3^{4-}, Fe^{2+}, NO_3^-, NO_2^-, Fe_{tot}, Mn_{tot} and for benzene, MCB, 1,2-DCB and 1,4-DCB.

Compound specific carbon isotope analysis

Stable carbon isotope analysis was done for benzene, MCB, 1,2-DCB, 1,4-DCB, CH_4 and CO_2 was done by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). Compounds were gas chromatographically separated using the column Zebron-ZB1 (60 m × 0.32 mm × 1 µm, Phenomenex, Inc.; Torrance, USA). The carbon isotope composition is reported in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA – Vienna) (Coplen *et al.*, 2006). A more detailed description of the methods is given in Schmidt et al. (2014).

6.2.3 Anaerobic sediment microcosm

To enrich bacteria, that settled on the wetland sediment, which potentially degrade MCB and DCB under anoxic conditions, anoxic laboratory microcosms were set up. Sediment was sampled from the wetland (sampling area A3-D3), after operations at the CW were stopped after 1.5 year. Microcosms were prepared in an anaerobic glove box (98-96 % N2/ 2-4 % H2) (CoyLab, Grass Lake, MI, USA). The serum bottles (250 mL) were filled with 100 mL anoxic medium (Zinder, 1998), 10 g sediment, amended with 1 mg L^{-1} of resazurin as a redox indicator, closed with Teflon-coated butyl rubber stoppers and closed with aluminium crimps. Each of the 30 microcosm sets consisted of two autoclaved abiotic controls and three biotic microcosms. Vitamin solution (0.5 mL) and corresponding electron acceptors (SO_4^{2-} [5 mM], α-FeO(OH) [1 g/ 50 mL]) were added under sterile and anoxic conditions. Amendments of MCB, 1,2-DCB and 1,3-DCB and [$^{13}C_6$]-MCB (100 µmol L-1) were done from neat solution. Microcosms with ^{13}C-labelled/ non-labelled substrate and without amendment of additional electron acceptors were set up as biotic control. Autoclaved microcosms served as abiotic control. The potential for reductive dechlorination of MCB and DCBs, respectively was investigated by addition of lactate or hydrogen/acetate.

Microcosms were incubated stationary at 20° C in the dark and were sampled at regular time intervals (at 48, 258, 489, 558 days) to monitor the carbon isotope signature of the substrate and of possible mineralisation products (CO_2, CH_4) using GC-FID and GC-C-IRMS (see section 2.2). Concentration of chlorobenzenes was analysed at day 48 and day 489 of the experiment. Mean values and standard deviation of substrate concentration, substrate and product (CO_2) $^{13}C/^{12}C$-ratio (‰) were calculated from triplicate biotic microcosm bottles per set.

6.2.4 Molecular microbial analysis

DNA extraction

DNA extraction was carried out from filters (1 L groundwater, vacuum filtrated, Filter. 0.2 µm; Supor® 200; Pall Coporation, USA) and sediment (1 g) in triplicates using the UltraClean® Soil DNA Isolation kit (MO BIO Laboratories, Inc.) according to the manufacturer instructions and eluted in 50 µl nuclease-free water. DNA concentration and quality were assessed spectrophotometric (NanoDrop ND-1000, Thermo scientific, USA). Aliquots were stored at -20°C until analysis.

16S rRNA gene-targeted PCR

16S rRNA genes were amplified with the forward primer 27f (5′-AGAGTTTGATCMTCGTCCCTC-3′) (Edwards et al. 1989) and the reverse primer 1492r (5′-

TACGGYTACCTTGTTAGGACTT-3′) (Lane, 1990). The PCR mixtures had a total volume of 25 µl containing 19.875 µl bidest. water for molecular biological work (Fluka), 1x reaction buffer (Qiagen), 200 µM of each deoxynucleotide triphosphate , 0.2 µM of each primer and 0.625 U/ reaction of HotStar *Taq* (Qiagen). The following temperature program was used: 95°C for 15 min, 94°C for 30 sec, 52°C for 30 sec, 72°C for 75 sec and 72°C for 20 min in 25 cycles.

16S rRNA gene-targeted PCR to detect *Geobacter* was performed using the forward primer Geo73f (5′-CTT GCT CTT TCA TTT AGT GG-3′) and the reverse primer Geo485r (5′-AAG AAA ACC GGG TATTAA CC-3′) (Duhamel and Edwards, 2006) and the temperature program: 95°C for 15 min, 94°C for 1 min, 59°C for 1 min, 72°C for 1 min and 72°C for 10 min in 32 cycles.

The *Dehalobacter* 16S rRNA-gene was targeted with the forward primer Deb179f (5′-TGT ATT GTC CGA GAG GCA-3′) and the reverse primer Deb1007f (5′-ACT CCC ATA TCT CTA CGG-3′) (Schötelburg et al. 2002) with the following temperature program: 95°C for 15 min, 94°C for 1 min, 53°C for 1 min, 72°C for 1 min and 72°C for 10 min in 32 cycles.

The *bamA* gene was amplified using the primer bamA-SP9 (CAGTACAAYTCCTACACVACBG) and bamA-ASP1 (CMATGCCGATYTCCTGRC) (Kuntze et al., 2008) in the GoTaq® Green Master PCR-Mix with the following temperature program: 95°C for 3 min, 95°C for 30sec, 59°C for 30 sec, 72°C for 45 sec and 72 °C for 10 min in 34 cycles. The expected PCR product has a size of 300 bp, and is a positive biomarker for facultative aromatics degrading bacteria.

T-RFLP analysis of bacterial community structures

Samples were processed using Terminal Restriction Fragment Length Polymorphisms (T-RFLP) as described previously (Penny et al. 2010). PCR products from DNA samples isolated from the wetland sediment were generated with a combination of (6- FAM)-labeled 27f (Edwards et al. 1989) and unlabelled 1492r primers according to PCR conditions described above in the 16S rRNA gene-targeted PCR. PCRs were carried out in triplicate for each DNA sample and 1.5 kb 16S rRNA gene fragments were obtained by PCR, purified using a QIAquick PCR Purification kit (QIAGEN, Germany). DNA samples (75 ng) were digested at 37°C for 20 h with 10 U MspI (CCGG), the reaction was stopped with Na-acetate (3mM, pH 5), amplicon fragments were purified with absolute ethanol (0°C) and DNA was resuspended in 50 µl sterile ultrapure water. DNA (10 to 50 ng) in 5 µl ultrapure water was mixed with 10 µl of Hi-Di formamide (Applied Biosystems, United Kingdom) containing 1:20 (vol/vol) 6-FAM- or carboxy-

X-rhodamine (ROX)-labeled MapMarker 1000 (Bioventures, United States), denatured at 95 °C for 10 min, and cooled on ice. Denaturated restriction fragments were loaded onto an ABI Prism 3130 XL capillary sequencer (Applied Biosystems) equipped with 50 cm long capillaries and POP 7 electrophoresis matrix according to the manufacturer instructions. T-RFLP electropherograms were analysed with GeneScan V3.7 software (Applied Biosystems).

Cloning-sequencing

The extracted DNA of 1 L groundwater (CW inflow water) was used as a template for amplification of the almost entire 16S rRNA gene (~1,500 bp) using the primers 27f and1492r. The temperature program was as follows: 95°C for 5 min and then 32 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a 10-min extension time at 72°C. After amplification and gel electrophoresis verification, the PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). PCR products were ligated into pGEM-T easy vector (Promega) and then cloned into Single Step (KRX) Competent Cells (Promega) in accordance with the manufacturer's instructions. Transformants were grown on LB agar containing ampicillin (100 µg mL^{-1}), IPTG (0.5 mM) and X-Gal (80 µg mL^{-1}), and positive white clones were randomly picked to confirm the right insert using PCR with primers (T7 and SP6), complementary to the flanking regions of the PCR insertion site of the pGEM-T easy vector. The PCR program was described above. All clones containing inserts of correct size were stored in LB medium at 4°C. Amplicons of the PCR (T7 and SP6) were sequenced in 96er well plates using a sequencing service.

6.2.5 Statistical analysis

Numerical treatment and analysis of the data were carried out with R (http://www.r-project.org/index.html). A principal component analysis (PCA) was done to assess the spatial dynamics of the hydro-geochemical variables. A second PCA was run to test and evaluate a posteriori the relative importance of environmental variables on the variation of bacterial community structures. Before statistical analysis, data of bacterial abundances were transformed with the Hellinger transformation to satisfy the assumption of the statistical tests and avoid numerical hindrance associated with long gradients (Legendre and Gallagher 2001). The species matrix consisted of T-RFLP profiles from 33 different samples and an environmental matrix including 6 physicochemical parameters and contaminants (benzene, MCB and 1,2-DCB). PCA calculation was based on Hellinger transformation and Hellinger distance matrix.

6.3 Results and Discussion

6.3.1 Hydro-geochemistry, contaminants and isotopes

Hydro-geochemistry of the CW

To analyse the hydro-geochemical state and evolution of the wetland and to track the DCB and MCB removal from the contaminated groundwater all data from day 55 till 420 were exploited. Raw data are provided in the Supporting information (SI, Tables S6-2A-H, separately per each analysed parameter. The main geochemical changes in the wetland occurring, are shown for each sampling day in Figure 6-2 from 55 to 420 days for inflow, averaged data for depth 12-24 cm and 36-48 cm, and pond. In general, simultaneous indications for iron (III) and sulphate reduction were obtained from the monitoring data (Figure 6-2A/C). Hydro-geochemical conditions are characterised by a significant increase of the iron (II) concentration (Figure 6-2A) in greater depth of the sediment (0.01 to 4.18 mg L^{-1}) and a steep increase of dissolved oxygen concentrations (DO: 0-10.6 mg L^{-1}) from the saturated sediment compartment to the aqueous phase in the pond (Figure 6-2B). Over the monitoring time measured sulphide concentrations in inflow, in the water saturated sediment compartment were low and ranged from 0.03 to 0.2 mg L^{-1}, from 0.02 to 0.2 mg L^{-1} and from 0.001 to 0.3 mg L^{-1}, respectively (Figure 6-2C). However the general trends, that sulphide concentrations increased with depth and sulphate concentration increased, when closer to the aqueous pond were observed (SI, Table S6-2B to S6-2F). In contrast, occasionally spots, with lower sulphide concentration in the deeper zones (depth 36 cm) of the sediment compartment were identified. The low sulphide concentration at these specific spots might be the result of high plant root density, where exudates get transported into the pore water containing oxidised acids that enhance microbial sulphur oxidation (Armstrong, 1964; Jackson and Armstrong, 1999; Lamers *et al.*, 2012).

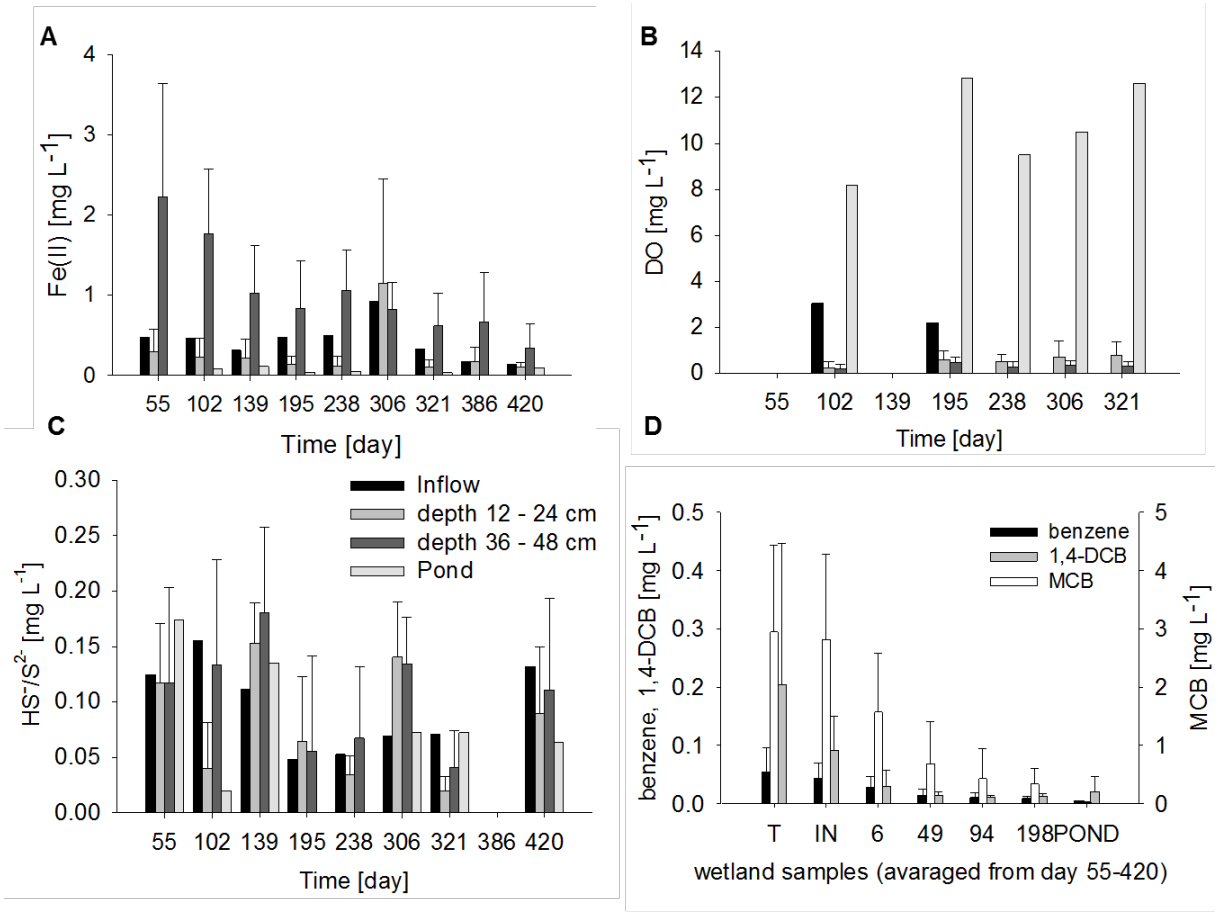

Figure 6-2: *Iron (II) (**A**), dissolved oxygen (**B**) and sulphide (**C**) averaged for inflow, sand compartment (depth 12-24 cm and 36-48 cm) and the pond are presented. The legend in C counts for A, B and C. (**D**) The (chloro)benzene mean concentrations of benzene, MCB, 1,4-DCB at the inflow, the sand compartment (vertical profiles at 6, 49 and 94, 139 cm from the inflow) and the pond in the model wetland were averaged from day 55 to 420. Error bars for sulphide, iron(II) and DO mean concentration values indicate the SDs of the 8 concentration values retrieved at the sampling ports from the sand compartment (in 12-24 cm and 36-48 cm depth, respectively). Single values for inflow and pond are presented for sulphide, iron (II) and DO. Error barsof benzene, MCB and 1,4-DCB mean concentration values indicate the SDs of the 4 values retrieved at the sampling ports from the sand compartment (at 6, 49, 94, 139 cm from the inflow, respectively).*

Contaminant removal in the CW

With respect to the contaminant removal, concentrations of benzene, MCB and 1,4-DCB decreased with increasing distance from the inflow (6, 49, 94, 139 cm) in every sampling campaign (55-420 days), whereas already after 49 cm distance a reduction of at least of 50 % of the contaminants could be determined (Fig. 6-2D). An overall decrease of 100 % was calculated for all three compounds in five out of nine sampling days, whereas initial inflow concentrations ranged from 0.015 to 0.14 mg L^{-1}, from 0.45 to 5.1 mg L^{-1} and 0.28 to 0.68 mg L^{-1} for benzene, MCB and 1,4-DCB, respectively. No increase of the benzene concentration was determined in the sediment pore water, but the opposite. Concentrations decreased rapidly along the flow

path. Thus no obvious indications for the occurrence of ongoing reductive dechlorination in the wetland, which involves a formation of benzene as intermediate or degradation product, was observed. However, benzene may have been further degraded or direct oxidation of MCB under anoxic conditions could have taken place, as suggested recently (Schmidt et al., 2014). Further, with the decrease of the contaminant concentration no strong increase in the carbon isotope ratio of the contaminants, which would coincident with a reductive dechlorination, for benzene, MCB or 1,4-DCB, was determined.

Compound specific isotope analysis as indicator for contaminant transformation

Detection of isotope ratios of (chloro) benzenes in samples of pond and lower depth (12 cm) was analytically challenging, frequent not possible due to the detection limit for the compounds (developed method, Herrero-Martin et al.). The relative difference of the carbon stable isotope ratio ($\Delta(\delta^{13}C_{wetland} - \delta^{13}C_{tank})$ of benzene, MCB and 1,4-DCB for the wetland samples over time (55-386 days) is presented in the supporting material Figure S6-1. For all three compounds the majority of isotope ratios lay in an interval of 1 ‰, however single analysis of some samples (e.g. for 1,4-DCB) showed a slight isotopic enrichment at days 306 and 321. Also for benzene an increase of up to 8 ‰ for single samples at day 139 was determined. This suggests that 1,4-DCB reductive dechlorination potentially happened, as well as benzene was degraded, however MCB degradation (removal), if reductive dechlorinated or directly mineralized as recently inferred (Schmidt et al., 2014), did not result in a significant strong isotope fractionation of the parent compound. A weak fractionation for MCB was already shown in a wetland study (Braeckevelt et al., 2007).

Exemplarily the decreasing concentration and the related change in the carbon isotope ratio of MCB shown for day 418 (the last sampling day) in a sediment depth of 24 and 36 cm along the flow path. While MCB concentrations decreased along the flow path from 38 µM to ~1 µM a weak isotopic enrichment from -26.7 ‰ to -24.8 ‰ in the remaining fraction of MCB was determined. The logarithmic presentation of the changes in concentration and isotope composition for MCB (Figure 6-3A) resulted in a slope that was used to estimate the isotopic enrichment factor ε (± confidence interval of 95%) using the Rayleigh equation (Chapter 1.III.4). The isotope enrichment factors were -0.4 ‰ ± 0.7 ‰ (◊, depth 24 cm) and -0.4 ‰ ± 1 ‰ (Δ, depth 36 cm), shown in Figure 6-3B. Plotting the logarithmic concentration and isotopic change of MCB from all 16 sampling points of the wetland plus inflow and pond at day 420, resulted in the same enrichment factor with ε = -0.4 ‰ ± 0.7 ‰ (SI, Figure S6-2). This bulk enrichment factor, including the confidence interval (CI 95 %), lies in the range of to date published ε´s for aerobic MCB mineralisation with values from 0.1‰ ± 0.1 ‰ to 0.4 ‰ ± 0.1 ‰ for aerobic MCB-

degrading strains, including *Acidovorax facilis* B517, *Pseudomonas veronii* B547, *Ralstonia* sp. DSM 8910 and *Rhodococcus erythropolis* B528. (Kaschl et al., 2005). However, since MCB may be simultaneously produced during DCB dechlorination and degraded in the wetland (although the amount of production will be small), the bulk enrichment factor is not easy to interpret with respect the hypothesised degradation pathways. Also, for anaerobic *in situ* degradation of MCB in the contaminated aquifer of Bitterfeld, where MCB is potentially produced and degraded, an enrichment factor of -0.50 ‰ ± 0.04 ‰, based on seven field samples, was determined by (Kaschl et al., 2005), which is similar to the εbulk calculated from 6 wetland samples. In contrast, recently for the first time an enrichment factor of 5.0 ‰ ± 0.2 ‰, which is an order of magnitude greater, was measured during anaerobic reductive dechlorination of MCB in a methanogenic microcosm (Liang *et al.*, 2011), which demonstrates that a stronger isotope fractionation is expected for such reaction.

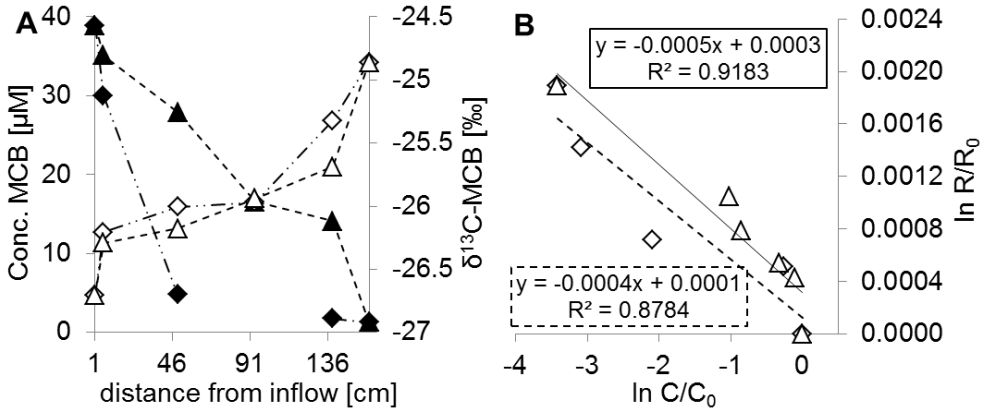

Figure 6-3: *(**A**) Concentration (filled symbols) and carbon stable isotope composition ($\delta^{13}C$) (open symbols) of MCB is shown for pore water samples in the wetland along the flow path (from 1-198 cm) in the two depths: 24 cm (◊) and 36 cm (Δ). (**B**) Rayleigh plots showing linear regression for MCB removal in 24 cm (◊, dashed line) and in 36 cm depth (Δ, solid line). Single analysis data of $\delta^{13}C$ isotope ratios of MCB are shown.*

Synthesis: Hydro-geochemistry, contaminants and isotopes

The hydro-geochemical changes in the wetland sediment, showing a progressive development of anoxic conditions in the wetland over space (depth or distance), rather than over time (55-420 days), reflected a prevailing contaminant removal. The PCA of hydro-geochemical variables (Cl^-, chloride; SO_4^{2-}, sulphate; Fe^{2+}, ferrous iron; HS^-/S^{2-}, sulphide; Mn_{tot}, manganese total; PO_4^{3+}, phosphate; B, benzene; MCB, monochlorobenzene, Fe_{tot}, iron total, NH_4^+, pH; DO, dissolved oxygen; Eh, redox potential; $\delta^{13}C\text{-}CO_2$, CO_2 carbon isotopic composition) represents

the major hydro-geochemical trends in the wetland averaged for data from day 55 to 420 (Figure S6-1) and confirmed conclusions made of the data presented in Figure 2, aforedescribed. Hydro-geochemical profiles of the tank and the inflow clustered together over time (Figure S6-3A). This underlines that the quality of the supplied water did not significantly change over the investigation period. Four additional clusters were identified, corresponding to samples of the upper layers (depth 12-24 cm), of deeper layers (depth 36-48 cm), of the first sediment segment after 6 cm and of the pond. Both axes together explain 68 % of the variance of the data set. MCB, benzene and 1,4-DCB correlated positive with ammonium, iron and manganese total and sulphide. With increasing distance from the inflow the concentration of these parameters decreases, whereas at the same time the chloride and sulphate concentration increased. Additionally, the positive correlation of iron (II), sulphide and sediment depth, represent the observed iron and assumed sulphate reduction in greater depth. The quantification of sulphide was difficult, as it was found frequent at the limit of detection, probably because of forming Fe_2S-species with ferrous iron as result of microbial activity, which adsorb to the sediment matrix (Billon *et al.*, 2001). This led to underestimations of sulphide and ferrous iron in the system. Visible back and orange-brown precipitation (SI, Figure S6-4), supposing were Fe_2S-species or Fe-oxides which appeared/disappeared depending on the night-day respiration cycle of the plants, introducing oxidised exudates into the anoxic sediment (Armstrong, 1964).

The investigation of the sand geochemistry from day 450 (SI, Table S6-3) revealed a similar spatial variability over the flow path and over depth for the parameter Cl^- and NH_4^+ compared to the hydro-geochemical parameter conducted for the pore water samples from 55-420 days. However values obtained had a different magnitude. The iron total, iron (II) and manganese total values were higher (one order of magnitude) than concentrations determined for pore water samples, demonstrating that the main part of the compounds is not dissolved but bound to the sediment. With increasing depth as well as with greater distance from the inflow the iron and manganese concentration increased, however values for the Fe (II) declined over the flow path in general. As sulphide could not be determined in the sediment samples and concentrations in pore water samples were frequent at the limit of detection, it was assumed that the dissolved Fe (II) and sulphide formed FeS_2-oxides; insoluble for detection. The sulphate concentration did not follow a typical pattern, but was surprisingly lower in the middle sediment layer (at 24-36 cm depth) along the flow path.

6.3.2 Microbial community

Groundwater microbial community

The microbial community composition in the contaminated aquifer water was described by two clone libraries, constructed for bacteria (192 clones) and archaea (132 clones). For the bacterial clone library 132 OTUs were obtained after sequencing (Table 6-1), whereas the phylogenetic affiliation is presented in Figure S6-5 for 31 OTUs (complete 16S rRNA genes + one partial 16S rRNA gene sequence of 724 bp). As sampling strategy, DNA extraction and 16S rRNA gene amplification affect the result of a clone library, detection of minor populations of the bacterial communities, potentially involved in contaminant degradation, was difficult. Therefore the determined frequency of the 16S rRNA gene sequences are no absolute numbers, but represent the relative abundance of present bacterial taxa (Table 6-1).

The microbial analysis revealed the picture of a diverse community of anaerobic bacteria, where Proteobacteria appeared to be the most abundant group. Besides, minor abundant clone sequences were determined, belonging to the groups Bacteriodetes, Chlorobi, Elusimicrobia, Firmicutes and *Chloroflexi*. Out of 132 16S rRNA gene sequences 46 % were only assigned to Bacteria phyla with a confidence level of 80 %. The Proteobacteria included three groups, one major assigned to Deltaproteobacteria (36 %) and two less frequent branching in Beta- (3 %) and Alphaproteobacteria (1 %) with a defined confidence level of 80 %. Within the order of Deltaproteobacteria most sequences were clustering in a subgroup closely related to the sulphate reducing and obligate anaerobic genera *Desulfobacterium* and to a minor extent (1 %) to *Desulfarculus*, *Desulfobacca* and *Synthrophobacter*. Other sequences within the δ-group were affiliated with *Syntrophus* sp., which were reported to degrade benzoate and fatty acids in syntrophic association with hydrogen/formate-using microorganisms (Jackson et al., 1999). Some clone sequences were closely related (2 %) with *Burkholderia* spp. belonging to the Betaproteobacteria. With respect to MCB degradation even two *Burkholderia* strains, PS12 and PS14 have been reported to grow on MCB, 1,2-DCB and 1,4-DCB as sole source of carbon (Field and Sierra-Alvarez, 2008). One partial 16S rRNA (724 bp) sequence was closely affiliated with *Geobacter* spp., known to reduce iron (III) and manganese (IV) and to dehalogenate chloroethene (Sung *et al.*, 2006). Interestingly, three 16S rRNA gene sequences were affiliated to the genus *Dehalogenimonas* that clusters in the phylum *Chloroflexi* and is reported for the ability to reductively dehalogenate halogenated alkanes but it does not grow on MCB or DCB (Moe *et al.*, 2009). Thus far, a single MCB degrading strain was not identified, however the involvement of Proteobacteria, Fibrobacteres and microbial members of the candidate division OD1 in the anaerobic MCB degradation were suggested to play a role (Martinez-Lavanchy et al.,

2011). Moreover the dechlorination of DCB and MCB to benzene by *Dehalobacter* spp. was reported (Fung et al., 2009; Liang et al., 2011). In the present study *Dehalobacter* ssp.-like 16S rRNA gene sequences were not obtained in the groundwater microbial clone library. Recently complete degradation of MCB to CH_4 and CO_2 was reported (Liang *et al.*, 2013b), whereas a MCB dechlorinating culture of *Dehalobacter* ssp. (Fung et al., 2009) was combined with the benzene degrading bacterial consortia (Nales *et al.*, 1998; Ulrich and Edwards, 2003). Similarly, the potential for complete degradation of MCB was determined in sediment microcosms (6.3.3), indicating a microbial community being capable for both, reductive dechlorination and mineralisation of aromatic compounds.

The consortium of abundant microorganism in the contaminated aquifer composed a typical anaerobic microbial community, which probably settled well in the identified geochemical wetland niches on sediment as growth substrate (e.g. oxic or anoxic parts, with iron (III) - and sulphate reducing conditions). Among the identified clones specific taxa might contribute to iron- and sulphur cycling within the lower wetland parts and thereby potentially degrade certain organic pollutants.

Table 6-1: *Relative clone frequencies in major phylogenetic groups of the clone library from the original groundwater (supplied to the wetland). The terminal restriction fragments (tRF) are shown for bacterial 16S rRNA gene sequences, obtained for the bacterial clone sequences.*

	Relative frequency (%)	tRF (bp) (MspI)
Group	Groundwater	
Proteobacteria	41	
Alphaproteobacteria	1	
Methylocystis	1	
Betaproteobacteria	3	
Burkholderia	2	
Sulfuricella	1	
Sulfuritalea	1	483
Deltaproteobacteria	36	
Desulfarculus	1	
Desulfobacterium	20	171
Geobacter	1	
Desulfobacca	1	
Synthrophus	5	490,510
Synthrophobacter	1	
Bacteriodetes	2	
Chlorobi	1	
Ignavibacteria	1	
Ignavibacterium	1	
Chloroflexi	2	
Dehalococcoidetes	2	
Dehalogenimonas	2	418
Elusimicrobia	1	
Elusimicrobia	1	299
Elusimicrobium	1	
Firmicutes	8	
Clostridia	8	
Saccharofermentans	1	
Pelotomaculum	2	
Unclassified Bacteria	46	
Number of clones	192	
Number of OTUs	132	

Microbial community pattern and detection of catabolic gene *bamA*

TRFLP analysis of PCR-amplified partial 16S rRNA genes was applied to assess changes in bacterial community structures. To analyse if the bacterial community pattern is varying in the wetland sediment in different depth and distance from the inflow and to identify the relationship between the bacterial communities pattern, a nonmetric multidimensional scaling (nMDS) analysis was performed. In the nMDS plot, the distance between the points reflects the degree of similarity of the TRFLP profiles (Figure 6-4A). Hence, samples displaying a similar community structure are found close to each other in the plot. The 16S rRNA gene-TRFLP

patterns of the wetland sediment samples originating from the upper sediment layer at 49 (B1) and 94 - 139 cm (C1-D1) distance from the inflow were found to be close together, forming one cluster and the other samples in particular from anoxic zones formed another cluster, which emphasizes the similarity of community structures in the anoxic zone of the sediment.

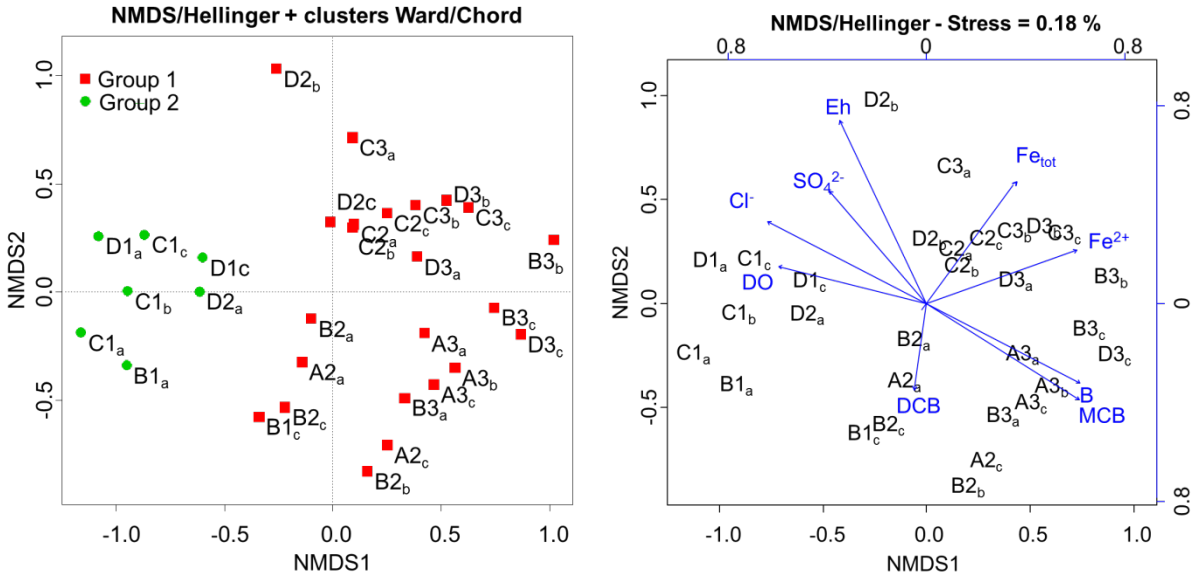

Figure 6-4: *(**A**) nMDS plot of the 16S rDNA gene TRFLP patterns of the bacterial community from wetland sediment samples, showing the community changes (stress value: 0.18 %). Objects are labelled according to location (sediment samples at distance from inflow: 6 cm (in 3 depth: A1, A2, A3), 49 cm (B1, B2, B3), 94 cm (C1, C2, C3) and 139 cm (D1, D2, D3)). (**B**) nMDS plot (two-dimensional) of the 16S rDNA gene TRFLP patterns of the bacterial community from wetland sediment samples, including the vector arrows of maximum correlation with the corresponding TRFLP patterns and correlation of nine most important characterizing components (Fe^{2+}, Fe_{tot}, SO_4^{2-}, Cl^-, 1,4-DCB, MCB, benzene, Eh, DO). The significance of fitted vectors is assessed using permutation of all assessed variables a posteriori by permutation of variables at $p<0.05$.*

The relationship between the bacterial community pattern and the observed hydro-geochemical condition (Figure 6-4B) in the wetland at day 420 was deduced statistically. The resulting characteristic variable vectors SO_4^{2-}, Cl^-, Eh and DO were positively correlated ($p<0.05$) with the microbial community structures observed in the upper sediment layer of the first 20 cm (A1, B1, C1, D1). This distribution indicated that oxic conditions forced the change in the bacterial community pattern is most probably. In contrast benzene and MCB were negative correlated to community patterns identified in the upper but positive to those in the lower and first 49 cm of wetland sediment. Further, Fe^{2+} and Fe_{tot} were positively correlated with community patterns obtained from the deeper (>32 cm) sediment compartment between 98 - 139 cm distant from the inflow. The distribution and dependencies of the community pattern and

the hydro-geochemical variables indicated the presence of comprehensive microbial network, whereas oxygen and ferrous iron seemingly influence the observed microbial pattern distribution the wetland sediment.

Some of the characteristic terminal restriction fragments identified in the overall TRFLP profile of the respective wetland sediment samples could be assigned to a specific bacterial group. The phylogenetic assignment was done by preparing a bacterial clone library of the partial 16SrRNA genes of extracted DNA from contaminated groundwater, which was pumped into the wetland. Subsequent TRFLP analysis of the clone DNA obtained was performed. Among a bigger group of unassigned tRFs, however the fragments 171 bp (*Desulfobacterium*), 299 bp (*Elusimicrobium*), 418 bp (*Dehalogenimonas*), 483 bp (*Sulfuritalea*) and 490 bp (*Synthrophus*) were identified. The in the community TRFLP profile obtained fragment of 175 bp may be assigned to *Desulfitobacterium* sp., including the uncertainty of a shift of 4 bp comparing tRFs of cloned 16S rRNA genes and to the ones of the community TRFLP profiles. Putatively, *Desulfitobacterium* sp. was found in a relative abundance ranging from 8 -22 % in the deeper sediment layers in the second part (B/D) (after 98 cm distance from the inflow) but was absent in the first part of the wetland sediment (SI, Figure S6-6). Previously isolated *Desulfitobacterium* strains were shown to reductively dechlorinate CEs (Gerritse *et al.*, 1999), suggesting that the wetland sediment settled dechlorinating bacteria. In contrast fragment 484 (bp) was identified stable over almost every sampling point in a relative abundance about 50 %, and was found to be putatively either *Sulfuritalea* sp. (483 bp) or *Synthrophus* (490 bp). Accepting a shift of 3 bp additionally the fragment 486 (bp), which was identified in a relative abundance ranging from 6-30 % in most of the samples, could account for *Sulfuritalea* sp. Recently *Sulfuritalea hydrogenivorans gen. nov., sp. nov.* was isolated from a freshwater lake, and was characterised to growth chemolithoautotrophically under anoxic conditions by the oxidation of reduced sulfur compounds and hydrogen (Kojima and Fukui, 2011). The TRFLP data indicated the presence of *Sulfuritalea* ssp.-like species in the wetland sediment, which provokes its involvement in the sulphur cycling in the wetlands. *Synthrophus* was identified in the bacterial clone library, whereas previously isolated strains were reported to degrade benzoate and fatty acids in syntrophic association with hydrogen/formate-using microorganisms (Jackson *et al.*, 1999). Moreover, in an anaerobic benzene-degrading enrichment cultures 16S rRNA gene sequences were identified being closest related to *Syntrophus* (Sakai *et al.*, 2009), indicating its involvement in the benzene degradation within a methogenic consortia. No TRFLP fingerprints were obtained for the presence of *Dehalogenimonas* ssp.-like species on the wetland sediment, although identified in the groundwater microbial community.

Geobacter and *Dehalobacter* were not detected in the wetland sediments using the 16S rRNA-gene targeting PCR approach, however the *bamA* gene was detected (Figure S6-6) in several biomass samples obtained from the sampling location A2/A3 and in C1/C2. The *bamA* gene is a positive biomarker for facultative aromatic compounds degrading bacteria (Kuntze et al., 2008) which is in accordance with the observed benzene removal in the wetland sediment.

6.3.3 Microbial pathways and MCB removal

To analyse whether the microbial community colonizing the sediment is responsible for the observed DCB and MCB removal, anaerobic sediment microcosm were prepared, amended with DCB and MCB and potential transformation pathways, e.g. reductive dechlorination or mineralisation were analysed. Routine analyses of the substrate (MCB, 1,2-DCB and 1,3-DCB) and product (MCB, benzene, CO_2 and CH_4) concentration and $^{12}C/^{13}C$-isotope ratios were performed in the different sediment microcosm set ups. These set ups were either with SO_4^{2-} or α-FeO(OH) as electron acceptor to investigate potential oxidation of the substrates (MCB, DCB), or amended with lactate and hydrogen (+acetate) as electron donor to enhance the potential for reductive dechlorination of the CBs. Nitrate was tested positive as electron acceptor for MCB mineralisation and negative for DCB transformation in previous groundwater microcosm studies (Schmidt et al., 2014), and was disclaimed in this approach as it most likely plays a minor role as alternative electron acceptor, if at all due to concentrations mainly below the limit of detection (0.05 mg L^{-1}) in groundwater as well as in wetland pore water (data not shown). In parallel, biotic controls, containing sediment and amendments of MCB or DCB without addition of alternative electron acceptors or lactate/acetate, were set up to investigate the biotransformation without potential stimulation. Abiotic controls had the same compositions as biotic controls, but microbial activity was stopped by sterilisation. In all microcosm, including biotic (with CBs; without electron acceptor/donor amendment) and abiotic controls a significant reduction in MCB (11-65 %), 1,2-DCB (13-97 %) and 1,3-DCB (41-48 %) concentration was determined after an incubation of 558 days. Despite product formation in active microcosm no product formation was found in abiotic controls. Moreover the substrate decrease in the abiotic set up was determined already in previous groundwater microcosm studies (Nijenhuis et al., 2007; Schmidt et al., 2014) and found to be not related to biotic degradation, and is likely a result of sorption to e.g. brown coal particles in the groundwater (Wycisk et al., 2003).

In Table S6-4, the substrate concentration of 1,3-DCB,1,2-DCB and MCB and product concentrations or $\delta^{13}C$ of potential mineralisation products (CO_2, CH_4) at t_0 and t_{558} in the different microcosm set ups, are presented. Remarkably, reductive dechlorination of 1,2-DCB

[~50 µM] to proportionately MCB was determined in triplicate microcosm with addition of lactate as electron donor. Detected methane (16 µM) in triplicate microcosms indicates 1,2-DCB reductive dechlorination under methanogenic conditions. Produced MCB had an isotope signature (-29.1 ± 0.3 ‰) (Figure S6-7 B2) corresponding to the 1,2-DCB signature (-28.6 ± 0.3 ‰) determined in the abiotic control (Figure S6-7 B1). No $\delta^{13}C_{1,2\text{-}DCB}$ could be determined in the active microcosm due to concentrations below the detection limit. Moreover benzene was detected using GC-IRMS in one microcosm (single value: -37.4 ‰), probably due to low concentration of ~1 µM. The methane formation was confirmed in triplicate bottles using the isotope technique ($\delta^{13}C_{CH4}$: -49.6 ± 4.2 ‰). Potentially 1,2-DCB was reductively dechlorinated in the above describe conditions in a sequence via MCB to benzene and eventually mineralized.

In contrast to the observed reaction under methanogenic conditions, selective reductive dechlorination of exclusively 1,2-DCB [49 µM] to MCB [48 µM] (Table S6-4) in one out of 3 triplicate bottles could be demonstrated containing both DCB isomers (1,3-DCB and 1,2-DCB) and goethite (α-Fe(OH)) as electron acceptor. The concentration of 1,3-DCB remained stable and its isotope signature indicated no significant shift with -25.5 ± 0.07 ‰ (Figure S6-7 A2) compared the abiotic control (-24.69 ± 0.98 ‰) (Figure S6-7 A1). No $^{12}C/^{13}C$- ratio was determined for 1,2-DCB due to concentrations below the limit of detection, however the isotope signature of its product was determined with -28.9 ± 0.04 ‰ for MCB, which is compared to the initial substrates signature 1,2-DCB (-27.61 ± 0.5 ‰), analysed in the abiotic control slightly depleted in ^{13}C (Figure S6-7 A1/2).

Interestingly, also in one out of three microcosms amended with [$^{13}C_6$]-MCB [77 µM] and goethite, proportionately 72 µM benzene was formed (Table S6-4) which was qualitatively analysed with GC-MS. The mass spectrum (Figure S6-8) of the produced benzene corresponded to a $^{13}C_6$ fully labelled benzene with the typical given mass shift of 6, resulting in the respective ion signals m/z = 55 (C_5H_3), m/z = 68 (C_5H_3) and m/z = 84 (C_5H_5). In addition CH_4 and CO_2 were detected and in ^{13}C-enriched with 97 ‰ and 201 ‰, respectively, indicating that benzene is further mineralized (Figure 6-5 A/B).

In all microcosms with [$^{13}C_6$]-MCB ^{13}C-enriched mineralisation products (CO_2 and CH_4) were traced applying stable isotope techniques. In general MCB was found to get oxidized, resulting on day 558 in an increasing $^{13}C/^{12}C$-ratio of CO_2 under addition of SO_4^{2-} (378 ± 11 ‰), Fe^{3+} (276 ± 147 ‰) and in the biotic control (338 ± 99 ‰) without supplement of additional electron acceptors (Figure 6-5), demonstrating the potential for complete degradation of MCB by the microflora which colonized the wetland sediment. In amendment of lactate and hydrogen (+acetate) as electron donor resulted in methane production, demonstrated by increasing $^{13}C/^{12}C$-enrichment in CH_4 with 173 ± 65 ‰ (day 558) and 65 ± 32 ‰ (day 498), respectively.

Surprisingly the $^{13}CH_4$ ratio decreased until day 558 down to -31 ± 6 ‰. In abiotic controls no CH_4 but CO_2 was detected, with -15 ± 2 ‰ in average and not enriched in ^{13}C. In biotic controls, however methane was determined with isotope signatures typical for (bio-) microbial methane production (~ -56 ± 11 ‰) (Chanton *et al.*, 2005), but not related to [$^{13}C_6$]-MCB mineralisation, as found with hydrogen (+ acetate).

Comparing the isotope pattern of CO_2 and CH_4 in the microcosms with MCB and lactate methane formation stops at day 498, represented by a decreased isotope ratio for CH_4 compared to the values obtained at earlier time points. In parallel, the isotope ratio of CO_2 was enriched in ^{13}C compared to the isotope ratio determined at an earlier time point of incubation. Methane formation and methane oxidation by methanogenic bacteria has been reported (Zehnder and Brock, 1979), and would explain the observed phenomenon. At the same time this case may be explained by a microbial consortia of methanogens and sulphate reducing bacteria, which have been already discovered in the contaminated groundwater used for the wetland experiment (Alfreider *et al.*, 2002).

Carbon stable isotope analysis of substrates, intermediates (MCB and benzene) products and of potential mineralisation products (CO_2, CH_4) confirmed that both processes reductive dechlorination and mineralisation were the substrate concentration reducing processes. The microbial community on the wetland sediment was shown to be cable of both, reductive dechlorination of 1,2-DCB to MCB under methanogenic condition and surprisingly with iron oxides, which is contradicting with the common sense of electron donor/acceptor understanding (Schlegel, 1992), that with the availability of Fe^{3+} as a good terminal electron acceptor (standard redox potential E_0': 770 mV) instead the bacteria used 1,2-DCB and MCB with a E_0' of 380mV and 310 mV (Dolfing and Harrison, 1992), respectively. In previous microcosm studies, using the inflow groundwater of the wetland without sediment, reductive dechlorination of the chlorobenzenes could not be demonstrated neither in presence of lactate or hydrogen, however mineralisation was shown in separate set ups with MCB and benzene, under nitrate and iron reducing conditions (Schmidt et al., 2014).

First this indicates, that bacteria performing the reductive dechlorination of DCB and MCB may need a solid growth substrate and, second, that the, for one year continuously operating, wetland was a pilot scale mesocosm to enrich chlorobenzene degraders. Further, enriching chlorobenzene dechlorinating bacteria in groundwater without a surface for biofilm formation is challenging and necessary because a complex community is involved, and was already reported other aromatic degrading microbial consortia, including benzene degraders (Vogt *et al.*, 2002).

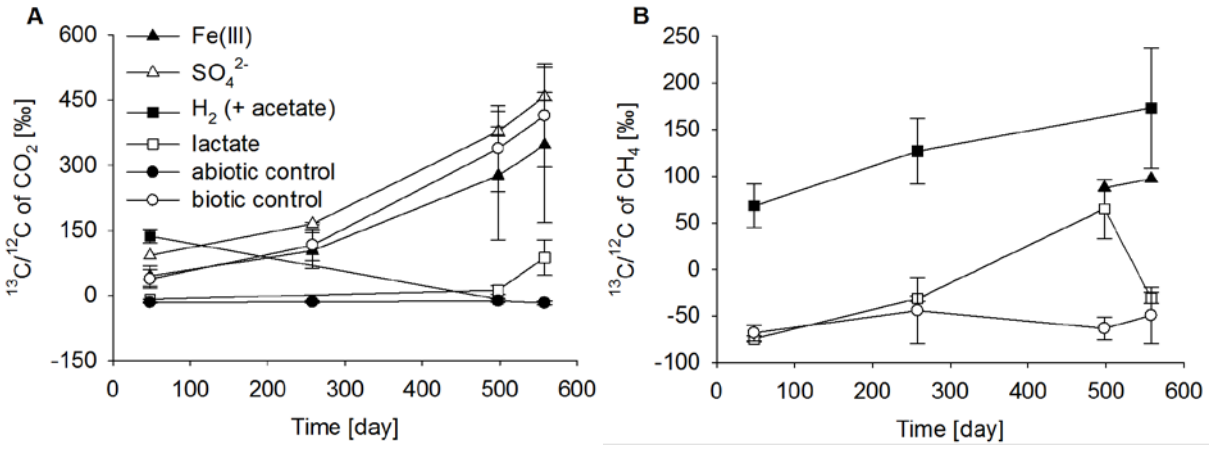

Figure 6-5. *Carbon stable isotope ratio of CO_2 (**A**) and CH_4 (**B**) produced over time (558 days of incubation), during [$^{13}C_6$]-MCB mineralisation in microcosms with wetland sediment and anoxic mineral medium, amended with α-FeO(OH) (▲), SO_4^{2-} (Δ), hydrogen in overpressure (+ acetate as carbon source, ■) and lactate (□).The abiotic (autoclaved) controls with respective amendments (see above) and biotic controls (○)without amendment of additional electron acceptors, except those present/ bound to the wetland sediment, contained [$^{13}C_6$]-MCB (●). Mean values and standard deviation of $^{13}C/^{12}C$-ratio (‰) of CO_2 were calculated from triplicate biotic microcosm bottles per set.*

6.3.4 Pathways involved in MCB removal

In this study, we assessed the biogeochemical development of a wetland treating MCB-contaminated groundwater over time, by in particular emphasizing the characterisation and distribution of the microbial community. Microbial and hydro-geochemical investigations were combined to characterize the wetland microbial community and ambient hydro-geochemistry in the wetland sediment.

In the constructed wetland system, MCB removal up to 100 % along the flow path was observed. Whereas the concentration decrease in lower depth (12 cm) of the wetland sediment was most likely linked to aerobic oxidation, MCB transformation in greater depth (>24 cm) was indicated to be linked to a decreasing iron total concentration. In parallel, DCB and benzene, present as co-contaminants as well as potential degradation substrates and products, decreased. A low but not significant isotope fractionation of the MCB was estimated over the flow path, whereas general trend of an increasing $^{13}C/^{12}C$-ratio was detected for all three contaminants. Results of the sediment microcosm confirmed the observed removal of DCB, MCB and benzene, whereas reductive dechlorination of 1,2-DCB to MCB as well as dechlorination of MCB to benzene with further mineralisation to CH_4 and CO_2 was determined. Changes of the hydrochemistry in the wetland could be identified and corresponded to a different distribution of the microbial community patterns, whereas the dissolved oxygen concentration was most likely responsible for the two different cluster of the wetland microbial

community. The community pattern of samples from the lower wetland sediment at 12 cm depth correlated with the environmental vectors DO, Eh, Cl^- and SO_4^{2-}, whereas samples from greater depth represented community pattern correlated with the contaminants and Fe^{2+} and Fe_{tot}. This distribution indicated that bacteria inhabit different niches, related to different redox conditions, with rather micro-oxic to oxic and more reduced conditions. A clear proof was found that the wetland microbial community was linked to the observed DCB and MCB removal revealed in the sediment microcosm, which demonstrated the capability of the microorganism to dechlorinate the contaminants under methanogenic and iron reducing conditions.

Moreover, the groundwater microbial community composition, composing mainly Deltaproteobacteria with representatives of sulphate and iron reducing bacteria, reflected the observed hydro-geochemistry of the pore water in the wetland sediment. Additionally the presence of *Dehalogenimonas* spp.-like communities, belonging to the group of dechlorinating bacteria of the *Chloroflexi*, in the groundwater was demonstrated, which could be involved in the observed dechlorination of MCB in the sediment microcosm.

Dehalobacter spp.-like species, which were recently reported as the first isolates to reductively dechlorinate DCB to MCB and even further to benzene (Fung et al., 2009; Nelson et al., 2011) were not detected in the microbial community. This indicates that other bacteria, potentially *Dehalogenimonas* spp. may be involved in the reductive dechlorination. This needs to be revealed by further enrichment and potential isolation of the responsible organism demonstrated to be active in the DCB and MCB dechlorination. Thus far *Dehalobacter* spp. was shown to dechlorinate up to benzene, which however accumulated in the cultivation (Nelson et al., 2011). In contrast in the sediment microcosm set up with ^{13}C-MCB and iron (III) reductive dechlorination benzene with further mineralisation was indicated, which could be previously only achieved by mixing the DCB/MCB dechlorinating *Dehalobacter* culture with a benzene degrading consortium (Liang et al., 2013b). In this study the first time enrichment cultures with strong indications for DCB/MCB-dechlorination to benzene with subsequent mineralisation were obtained. That aromatic compound degrading microorganisms are present in the wetland was confirmed by detecting *bam*A gene amplificates in some sediment samples.

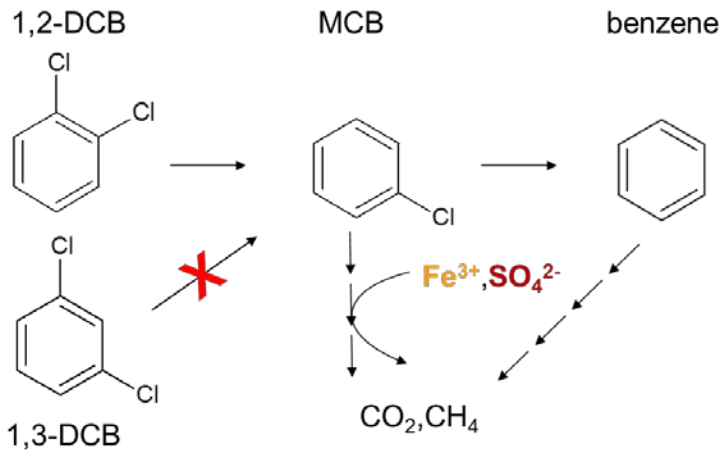

Figure 6-6: *Proposed production and degradation pathways for MCB in situ removal in constructed mode wetland. Preferential reductive dechlorination of 1,2-DCB over 1,3-DCB to MCB. MCB reductive dechlorination to benzene with further mineralisation or direct mineralisation of MCB with Fe^{3+} or SO_4^{2} as electron acceptor, which was for Fe^{3+} the first time shown in (Schmidt et al., 2014).*

Taking the results of this study altogether, we propose the following degradation pathway for MCB in the constructed wetland. Reduction and oxidation processes in wetland systems are probably inseparable linked, whereas 1,2-DCB is dechlorinated to MCB and MCB to benzene with further mineralisation or direct mineralisation of MCB with Fe^{3+} or SO_4^{2} as electron acceptor. Microbial data supported this hypothesis, whereas dechlorinating species are present (sediment microcosm), however *Geobacter*, *Dehalobacter*, *Dehalococcoides* were not detected. If the presence of other dechlorinating bacteria, *Desulfitobacterium* ssp.- and *Dehalogenimonas* ssp.-like species, identified with DNA fingerprinting methods on the wetland sediment or in the groundwater, is related to the observed biotransformation processes of DCB and MCB, still needs to be revealed.

An efficient transformation of MCB and their transformation products was achieved in the model wetland and promotes the application of wetland for treatment of MCB contaminated groundwater at this field site. Furthermore the wetland system was shown to be a stable habitat for the anaerobic groundwater microbial community and to be applicable as mesocosm system to enrich anaerobic bacteria involved in MCB transformation.

Further microbial studies in the sediment microcosms will eventually lead to isolation of new bacterial strains that are potentially capable to couple reductive dechlorination of MCB to growth. This study demonstrated the importance of integrating hydro-geochemical and microbial approaches to understand how biodegradation mechanisms and processes influence the transformation of lower chlorinated hydrocarbons in wetland systems.

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Supporting Information for Chapter 6

Microbial diversity and activity in a monochlorobenzene removing sulfate reducing wetland

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Table S6-1. *The composition of the groundwater in the tank, including inorganic, organic and physico chemical parameter, is presented (in the experimental period of 55-420 days).*

	Wetland	
Parameter	**Avarage***	**Stdev**
[mg L^{-1}]		
Cl^-	180.33	33.41
SO_4^{2-}	903.11	42.78
NH_4^+	1.89	0.34
PO_4^{3-}	3.95	0.38
Fe^{2+}	0.48	0.21
Fe tot	0.59	0.25
Mn tot	0.13	0.03
HS^-/S^{2-}	0.1	0.05
Benzene	0.05	0.04
MCB	2.94	1.49
1,4-DCB	0.20	0.242
O_2	2.43	0.36
Eh [mV]	57	57.08
pH	6.99	0.17
CO_2 [‰]	-17.49	0.83

***** avarage of 9 sampling campaign

Table S6-2. *The hydro-geochemical composition of the groundwater (tank, T), inflow (IN) and wetland pore water and pond (POND) samples, presented for* Cl^-, SO_4^{2}, Fe^{2+}, NH_4^+, PO_4^{3-}, HS^-/S^{2-}, *DO, benzene, MCB and DCB in mg* L^{-1}, *given for each sampling month (in the experimental period of 55-420 days) and indicated for the sampling location (in XY orientation, represented by column depth and distance form inflow) in the wetland (See Figure 6-1, wetland scheme, accordingly.*

Table 6-2A. *Concentration of chlorine.*

Sampling points		**Day**	**55**	**102**	**139**	**195**	**238**	**306**	**321**	**386**	**420**
Cl^- [mg L^{-1}]	**Depth [cm]**	**Distance from inflow [cm]**	**Sep**	**Oct**	**Dec**	**Jan**	**Mar**	**Ma**	**Jul**	**Aug**	**Sep**
T	0	0	246	205	187	206	169	154	154	157	145
IN	10	0	245	206	190	200	172	153	151	153	145
A0	12	6	251	212	189	215	184	162	175	185	169
A1	24	6	243	213	189	197	173	157	153	160	150
A2	36	6	238	208	195	199	169	153	155	156	146
A3	48	6	242	204	191	202	174	153	156	152	149
B0	12	49	270	225	201	198	203	178	169	178	177
B1	24	49	284	221	191	211	195	n.s.	171	173	171
B2	36	49	274	204	189	217	179	177	172	161	156
B3	48	49	291	207	189	213	174	166	158	157	153
C0	12	94	249	235	204	228	205	189	181	176	190
C1	24	94	265	220	191	221	189	185	172	166	177
C2	36	94	244	209	188	209	181	174	165	159	171
C3	48	94	247	210	190	n.s.	175	169	154	163	n.s
D0	12	139	274	217	197	218	217	171	172	187	177
D1	24	139	274	225	188	237	n.s.	n.s	171	n.s.	172
D2	36	139	264	216	193	217	194	167	159	161	168
D3	48	139	252	204	187	212	177	159	155	175	152
POND	30	200	253	220	202	227	204	174	165	174	171

n.s. – no sample obtained from sampling port

Table 6-2B. *Concentration of sulphate.*

Sampling points		**Day**	**55**	**102**	**139**	**195**	**238**	**306**	**321**	**386**	**420**
SO_4^{2-} [mg L^{-1}]	**Depth [cm]**	**Distance from inflow [cm]**	**Sep**	**Oct**	**Dec**	**Jan**	**Mar**	**Ma**	**Jul**	**Aug**	**Sep**
T	0	0	998	871	852	900	893	912	911	924	867
IN	10	0	1000	873	865	887	909	920	899	901	872
A0	12	6	1013	895	857	935	975	960	1028	1031	992
A1	24	6	987	905	856	876	914	932	916	923	895
A2	36	6	967	883	885	878	891	918	928	902	872
A3	48	6	984	860	866	902	924	915	942	893	891
B0	12	49	1053	946	928	859	1052	1026	1006	1029	1021
B1	24	49	1121	942	870	923	1021	n.s.	1039	1023	1013
B2	36	49	1082	868	860	924	959	1053	1022	925	939
B3	48	49	1175	877	864	913	916	967	952	919	911
C0	12	94	1003	954	931	973	1045	1092	1066	972	1081
C1	24	94	1071	924	870	943	981	1068	1019	940	1018
C2	36	94	992	882	854	888	987	1018	1022	905	1014
C3	48	94	1000	888	863	n.s.	928	1000	927	927	n.s.
D0	12	139	1062	897	869	922	1052	999	1004	1024	1009
D1	24	139	1075	947	837	1006	n.s.	n.s.	1012	1058	998
D2	36	139	1053	908	871	933	981	993	973	932	990
D3	48	139	1027	853	848	918	920	1000	942	968	908
POND	30	200	1012	906	899	954	990	1012	977	987	1000

n.s. – no sample obtained from sampling port

Table 6-2C. *Concentration of ammonium.*

Sampling points		Day	55	102	139	195	238	306	321	386	420
NH_4^+ [mg L^{-1}]	Depth [cm]	Distance from inflow [cm]	Sep	Oct	Dec	Jan	Mar	Ma	Jul	Aug	Sep
T	0	0	1.78	1.52	1.71	2.06	1.64	2.6	1.89	2.16	1.65
IN	10	0	1.77	1.77	1.69	1.99	1.62	2.51	1.86	2.13	1.65
A0	12	6	0.03	0.03	0.00	0.05	0.05	0.03	0.02	0.06	0.05
A1	24	6	0.07	0.02	0.00	0.05	0.04	0.04	0.02	0.02	0.02
A2	36	6	0.62	0.07	0.02	0.07	0.06	0.09	0.03	0.2	0.02
A3	48	6	1.74	0.97	0.08	0.11	0.05	0.06	0.11	0.23	0.02
B0	12	49	0.1	0.07	BLD	0.05	0.05	0.11	0.04	0.02	0.01
B1	24	49	0.07	0.03	BLD	0.04	0.04	0.03	0.04	0.02	0.03
B2	36	49	0.07	0.04	BLD	0.02	BLD	0.04	0.04	0.02	0.02
B3	48	49	0.21	0.19	BLD	0.03	BLD	BLD	0.06	0.02	0.01
C0	12	94	0.05	BLD	BLD	0.02	0.02	0.03	0.03	0.02	0.02
C1	24	94	0.07	BLD	BLD	0.02	BLD	BLD	0.05	0.02	0.02
C2	36	94	0.06	0.03	BLD	0.04	BLD	0.03	0.02	0.02	0.01
C3	48	94	0.32	0.07	BLD	BLD	BLD	0.04	0.02	0.02	BLD
D0	12	139	0.05	0.03	BLD	BLD	BLD	0.05	0.02	0.02	0.01
D1	24	139	0.04	0.03	0.03	BLD	BLD	BLD	0.03	0.15	0.01
D2	36	139	0.11	0.05	0.02	BLD	0.03	BLD	0.04	0.03	0.02
D3	48	139	0.68	0.11	0.02	0.02	0.03	BLD	0.02	0.02	0.01
POND	30	200	0.05	0.07	BLD	BLD	0.03	BLD	0.02	0.02	0.01

BLD-Below limit of detection (<0.01 mg L^{-1})

Table 6-2D. *Concentration of phosphate.*

Sampling points		Day	55	102	139	195	238	306	321	386	420
PO_4^{3-} [mg L^{-1}]	Depth [cm]	Distance from inflow [cm]	Sep	Oct	Dec	Jan	Mar	Ma	Jul	Aug	Sep
T	0	0	4.5	4.24	3.35	3.95	3.95	3.55	-	4.25	3.84
IN	10	0	4.34	4.06	3.54	3.72	3.75	3.34	-	4.66	3.71
A0	12	6	1.47	2.35	3.09	2.44	2.4	2.04	-	2.3	2.18
A1	24	6	2.96	2.47	2.73	4.39	2.81	2.6	-	3.39	2.45
A2	36	6	3.75	3.65	1.6	3.09	3.76	3.51	-	3.29	3.01
A3	48	6	3.64	4.03	3.14	3.38	3.76	3.26	-	3.66	3.33
B0	12	49	1.78	0.95	1.13	0.69	1.57	1.29	-	1.8	1.69
B1	24	49	1.67	1.01	1.3	0.99	0.69	1.62	-	1.95	1.36
B2	36	49	1.81	2.26	1.74	2.11	2.52	1.64	-	2.17	2.02
B3	48	49	1.85	2.58	2.13	2.41	2.93	BLD	-	2.89	2.27
C0	12	94	BLD	BLD	0.46	0.45	0.71	0.88	-	1.07	0.94
C1	24	94	BLD	0.18	0.16	0.4	0.78	0.93	-	1.12	1.08
C2	36	94	0.64	0.86	0.32	2.11	1.26	0.89	-	2.17	1.12
C3	48	94	1.28	1.59	1.07	BLD	2.09	1.13	-	2.76	BLD
D0	12	139	BLD	BLD	BLD	BLD	BLD	0.46	-	0.96	0.45
D1	24	139	BLD	BLD	BLD	BLD	BLD	BLD	-	1.58	0.59
D2	36	139	BLD	0.3	BLD	0.3	0.75	0.49	-	1.41	0.72
D3	48	139	0.29	0.81	0.76	1.5	1.73	0.55	-	2.44	1.11
POND	30	200	0.1	BLD	BLD	0.16	BLD	0.1	-	0.21	BLD

BLD- Below limit of detection (<0.07 mg L^{-1})

Table 6-2E. *Concentration of iron (II).*

sampling points		Day	55	102	139	195	238	306	321	386	420
Fe^{2+} [mg L^{-1}]	Depth [cm]	Distance from inflow [cm]	Sep	Oct	Dec	Jan	Mar	Ma	Jul	Aug	Sep
T	0	0	0.75	0.57	0.35	0.37	0.75	0.65	0.2	0.42	0.25
IN	10	0	0.49	0.6	0.41	1.21	0.76	0.6	0.48	0.43	0.29
A0	12	6	0.19	0.16	0.19	0.11	0.14	2.99	0.09	0.09	0.15
A1	24	6	0.69	0.34	0.1	0.07	0.37	3.07	0.32	0.27	0.18
A2	36	6	0.86	0.64	0.2	0.22	0.67	0.96	0.14	0.17	0.12
A3	48	6	0.66	0.8	0.52	0.63	0.9	0.98	0.63	0.24	0.21
B0	12	49	0.29	0.13	0.11	0.33	0.09	0.86	0.04	0.1	0.08
B1	24	49	0.1	0.76	0.4	0.19	0.11	0.24	0.03	0.08	0.11
B2	36	49	2.93	1.64	0.85	0.48	0.68	0.6	0.36	0.34	0.24
B3	48	49	4.75	2.06	0.54	0.62	1.71	1.28	0.55	0.31	0.29
C0	12	94	0.14	0.06	0.11	0.11	0.02	0.42	0.07	0.03	0.18
C1	24	94	0.09	0.2	0.72	0.03	0.03	0.21	0.1	0.04	0.07
C2	36	94	1.75	1.23	1.3	1.35	0.7	0.23	0.28	0.51	0.1
C3	48	94	2.7	2.37	1.39	BLD	1.77	0.75	0.76	0.57	BLD
D0	12	139	0.78	0.08	0.09	0.09	0.06	0.27	0.06	0.26	0.04
D1	24	139	0.08	0.11	0.03	0.21	BLD	BLD	0.12	0.54	0.01
D2	36	139	3.15	2.71	1.43	0.64	0.6	0.62	0.78	1.83	0.46
D3	48	139	1.02	2.65	1.99	1.92	1.49	1.15	1.45	1.4	0.98
POND	30	200	0.11	0.11	0.1	BLD	BLD	0.23	0.16	0.09	BLD

BLD- Below limit of detection (<0.05 mg L^{-1})

Table 6-2F. *Concentration of sulphide.*

Sampling points		Day	55	102	139	195	238	306	321	386	420
HS^-/S^{2-} [mg L^{-1}]	Depth [cm]	Distance from inflow [cm]	Sep	Oct	Dec	Jan	Mar	Ma	Jul	Aug	Sep
T	0	0	0.06	0.17	0.15	0.03	0.06	0.06	0.12	-	0.13
IN	10	0	0.19	0.14	0.08	0.06	0.04	0.08	0.02	-	BLD
A0	12	6	BLD	0.08	0.12	0.04	0.05	BLD	0.04	-	0.10
A1	24	6	BLD	0.12	0.20	BLD	0.03	0.18	0.01	-	0.20
A2	36	6	0.22	0.15	0.19	0.03	0.07	0.18	0.02	-	0.21
A3	48	6	0.09	0.18	0.33	0.23	0.17	0.13	0.09	-	0.24
B0	12	49	0.09	0.02	0.17	0.01	0.01	BLD	BLD	-	0.12
B1	24	49	BLD	0.02	0.14	0.15	BLD	BLD	0.01	-	0.02
B2	36	49	0.18	0.13	0.13	BLD	0.02	0.20	0.01	-	0.05
B3	48	49	0.01	0.26	0.21	0.03	0.09	0.15	0.04	-	0.08
C0	12	94	0.08	0.01	0.13	0.05	0.02	BLD	0.03	-	0.10
C1	24	94	BLD	0.01	0.18	BLD	0.05	0.11	0.03	-	0.05
C2	36	94	0.03	0.02	0.05	BLD	BLD	0.09	BLD	-	0.08
C3	48	94	0.17	0.25	0.17	BLD	0.01	0.15	0.05	-	BLD
D0	12	139	0.18	0.02	0.19	BLD	BLD	BLD	0.01	-	0.02
D1	24	139	BLD	BLD	0.10	BLD	BLD	BLD	BLD	-	0.09
D2	36	139	0.02	0.02	0.18	0.04	BLD	0.09	0.01	-	0.02
D3	48	139	0.20	0.05	0.17	BLD	BLD	0.08	0.08	-	0.08
POND	30	200	0.17	0.02	0.13	BLD	BLD	0.07	0.07	-	0.06

BLD- Below limit of detection (<0.009 mg L^{-1})

Table 6-2G. *Concentration of dissolved oxygen.*

Sampling points		**Day**	**55**	**102**	**139**	**195**	**238**	**306**	**321**	**386**	**420**
DO [mg L^{-1}]	**Depth [cm]**	**Distance from inflow [cm]**	**Sep**	**Oct**	**Dec**	**Jan**	**Mar**	**Ma**	**Jul**	**Aug**	**Sep**
T	0	0	2.68	-	2.17	n.s.	n.s.	n.s.	-	-	2.68
IN	10	0	3.42	-	2.21	n.s.	n.s.	n.s.	-	-	3.42
A0	12	6	0.51	-	1.33	1.10	1.62	1.73	-	-	0.51
A1	24	6	0.01	-	0.45	0.62	0.14	0.75	-	-	0.01
A2	36	6	0.02	-	0.20	0.12	0.20	0.21	-	-	0.02
A3	48	6	0.41	-	0.53	0.39	0.63	0.54	-	-	0.41
B0	12	49	n.s.	-	n.s.	n.s.	n.s.	n.s.	-	-	n.s.
B1	24	49	0.40	-	0.52	0.67	0.69	0.53	-	-	0.40
B2	36	49	0.38	-	0.49	0.44	0.52	0.49	-	-	0.38
B3	48	49	0.05	-	0.19	0.10	0.19	0.18	-	-	0.05
C0	12	94	0.10	-	0.59	0.33	1.71	1.44	-	-	0.10
C1	24	94	0.01	-	0.23	0.08	0.10	0.11	-	-	0.01
C2	36	94	0.49	-	0.93	0.40	0.57	0.53	-	-	0.49
C3	48	94	0.11	-	0.58	0.14	0.22	0.13	-	-	0.11
D0	12	139	0.68	-	0.89	0.55	0.73	0.74	-	-	0.68
D1	24	139	0.10	-	0.24	0.27	0.14	0.16	-	-	0.10
D2	36	139	0.32	-	0.44	0.67	0.48	0.51	-	-	0.32
D3	48	139	0.01	-	0.34	0.12	0.14	0.12	-	-	0.01
POND	30	200	8.20	-	12.82	10.82	n.s.	n.s.	-	-	8.20

n.s.- no sample

***Table 6-2H**. Concentrations of (Chloro)benzenes.*

Sampling points	Day		55			102			139			195		
	Month		September			October			December			January		
Conc. in [mg L-1]	Depth [cm]	Distance from inflow [cm]	B	MCB	1,4-DCB	B	MCB	1,4-DCB	B	MCB	1,4-DCB	B	MCB	1,4-DCB
T	0.0	0.0	0.033	5.113	0.073	0.027	3.244	0.044	0.035	3.558	0.058	0.075	3.739	0.088
IN	10.0	0.0	0.033	4.92	0.099	0.025	3.078	0.033	0.033	3.428	0.075	0.077	3.777	0.103
A0	12.0	6.0	0.011	0.749	0.011	0.021	1.838	BLD	0.013	0.557	BLD	0.027	0.479	BLD
A1	24.0	6.0	0.025	2.969	0.024	0.02	2.078	BLD	0.022	1.435	0.029	0.053	1.704	0.087
A2	36.0	6.0	0.031	4.308	0.106	0.024	2.615	BLD	0.032	2.738	0.047	0.072	3.189	0.162
A3	48.0	6.0	0.029	4.022	0.071	0.023	2.81	BLD	0.028	2.551	BLD	0.063	2.927	0.095
B0	12.0	49.0	BLD	BLD	BLD	0.006	0.239	BLD	BLD	BLD	BLD	BLD	0.036	BLD
B1	24.0	49.0	BLD	BLD	BLD	0.011	0.986	BLD	0.021	1.168	BLD	BLD	0.028	BLD
B2	36.0	49.0	BLD	0.405	BLD	0.025	3.000	BLD	0.031	2.493	0.02	BLD	0.009	BLD
B3	48.0	49.0	BLD	0.518	BLD	0.025	3.045	BLD	0.027	2.519	0.033	BLD	0.007	BLD
C0	12.0	94.0	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
C1	24.0	94.0	BLD	BLD	BLD	BLD	BLD	BLD	0.009	0.496	BLD	BLD	BLD	BLD
C2	36.0	94.0	BLD	0.024	BLD	BLD	0.158	BLD	0.028	2.455	BLD	BLD	0.144	BLD
C3	48.0	94.0	0.013	1.397	0.02	0.012	0.716	BLD	0.032	3.106	0.014	BLD	0.000	BLD
D0	12.0	139.0	BLD	BLD	BLD	BLD	BLD	BLD	BLD	0.008	BLD	BLD	0.227	BLD
D1	24.0	139.0	BLD	BLD	BLD	BLD	BLD	BLD	BLD	0.025	BLD	BLD	0.013	BLD
D2	36.0	139.0	0.011	0.949	BLD	0.012	0.759	BLD	0.007	0.176	BLD	BLD	0.081	BLD
D3	48.0	139.0	0.017	1.945	0.034	0.013	0.885	BLD	0.03	2.101	0.019	0.011	0.572	BLD
POND	30.0	200.0	BLD	BLD	BLD	BLD	BLD	BLD	BLD	0.014	BLD	BLD	BLD	BLD

BLD- Below limit of detection for benzene (0.005 mg L^{-1}), MCB (0.01 mg L^{-1}), DCB (0.011 mg L^{-1})

sampling points	Day		238			306			321			386		
	Month		March			May			July			August		
Conc. in [mg L-1]	Depth [cm]	Distance from inflow [cm]	B	MCB	1,4-DCB	B	MCB	1,4-DCB	B	MCB	1,4-DCB	B	MCB	1,4-DCB
T	0.0	0.0	0.063	2.345	0.559	0.087	3.553	0.675	0.015	0.447	0.222	0.016	0.828	0.028
IN	10.0	0.0	0.055	2.160	0.130	0.073	3.246	0.222	0.011	0.400	0.053	0.016	0.785	0.028
A0	12.0	6.0	0.020	0.499	BLD	0.005	BLD	0.035	BLD	BLD	BLD	BLD	BLD	BLD
A1	24.0	6.0	0.021	0.452	BLD	0.036	0.787	0.040	BLD	BLD	BLD	BLD	0.149	BLD
A2	36.0	6.0	0.049	1.603	BLD	0.065	2.413	BLD	BLD	BLD	BLD	BLD	0.211	BLD
A3	48.0	6.0	0.040	1.341	BLD	0.060	2.249	0.014	BLD	0.104	BLD	0.009	0.507	BLD
B0	12.0	49.0	BLD	0.009	BLD	BLD	0.031	0.018	BLD	BLD	BLD	BLD	BLD	BLD
B1	24.0	49.0	BLD	0.067	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
B2	36.0	49.0	0.040	1.227	BLD	BLD	0.059	0.048	BLD	BLD	BLD	0.008	0.271	BLD
B3	48.0	49.0	0.048	1.746	0.036	0.019	0.343	BLD	BLD	0.101	BLD	0.017	0.630	BLD
C0	12.0	94.0	BLD	0.009	BLD	BLD	0.011	BLD	BLD	BLD	BLD	BLD	BLD	BLD
C1	24.0	94.0	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
C2	36.0	94.0	0.014	0.400	BLD	BLD	0.139	BLD	BLD	BLD	BLD	BLD	0.092	BLD
C3	48.0	94.0	0.044	1.697	BLD	BLD	0.053	BLD	BLD	0.041	BLD	BLD	0.342	BLD
D0	12.0	139.0	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
D1	24.0	139.0	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
D2	36.0	139.0	0.010	0.376	BLD	BLD	BLD	0.012	BLD	BLD	BLD	0.008	0.208	BLD
D3	48.0	139.0	0.043	1.628	BLD	BLD	0.192	0.034	BLD	BLD	BLD	BLD	BLD	BLD
POND	30.0	200.0	BLD	0.003	BLD	BLD	0.076	0.090	BLD	BLD	BLD	BLD	BLD	BLD

BLD- Below limit of detection for benzene (0.005 mg L^{-1}), MCB (0.01 mg L^{-1}), DCB (0.011 mg L^{-1})

Sampling points	Day		420		
	Month		September		
Conc. in [mg L-1]	Depth [cm]	Distance from inflow [cm]	B	MCB	1,4-DCB
T	0.0	0.0	0.140	3.651	0.084
IN	10.0	0.0	0.074	3.558	0.079
A0	12.0	6.0	BLD	BLD	BLD
A1	24.0	6.0	0.057	2.754	BLD
A2	36.0	6.0	0.072	3.234	BLD
A3	48.0	6.0	0.067	3.209	0.017
B0	12.0	49.0	BLD	0.095	BLD
B1	24.0	49.0	BLD	0.358	0.050
B2	36.0	49.0	0.063	2.556	BLD
B3	48.0	49.0	0.065	2.715	BLD
C0	12.0	94.0	BLD	BLD	0.022
C1	24.0	94.0	BLD	BLD	0.014
C2	36.0	94.0	0.037	1.474	0.019
C3	48.0	94.0	0.060	2.509	0.016
D0	12.0	139.0	BLD	0.027	0.022
D1	24.0	139.0	BLD	0.072	0.027
D2	36.0	139.0	0.034	1.241	0.018
D3	48.0	139.0	0.019	0.637	0.024
POND	30.0	200.0	BLD	0.025	0.022

BLD- Below limit of detection for benzene (0.005 mg L^{-1}), MCB (0.01 mg L^{-1}), DCB (0.011 mg L^{-1})

Table S6-3. *The geochemical composition of the wetland sediment, including inorganic, parameter, is presented as average (n=3) with standard deviation (±) of 11 sampling locations in X-Y coordinates (wetland scheme, Figure 6-1) (at day 450).*

[mg L^{-1}]	Cl^-	SO_4^{2-}	Fe^{2+}	Fe_{tot}	Mn_{tot}
A2	22.8 ± 3.0	99.5 ± 14.2	2.1 ± 0.8	11.5 ± 9.9	12.9 ± 14.1
A3	85.8 ±10.3	547.7 ± 33.1	2.2 ± 0.8	10.1 ± 2.2	48.1 ± 53.4
B1	17.5 ±1.6	255.9 ± 88.7	1.1 ± 0.1	18.9 ± 1.3	9.6 ± 11.2
B2	17.1 ± 2.0	61.7 ± 5.9	1.2 ± 0.3	23.8 ± 8.2	9.6 ± 10.7
B3	67.0 ± 0.6	440.9 ± 29.0	1.0 ± 0.7	9.2 ± 0.5	33.8 ± 46.9
C1	16.7 ± 1.0	264.3 ± 212.8	1.1 ± 0.3	16.2 ± 2.6	8.9 ± 11.1
C2	15.2 ± 1.1	36.2 ± 5.5	0.6 ± 0.3	18.6 ± 0.3	8.2 ± 10.0
C3	74.0 ± 5.3	477.5 ± 95.7	0.3 ± 0.0	8.7 ± 1.0	39.6 ± 48.6
D1	16.7 ± 2.5	45.9 ± 16.8	1.0 ± 0.3	18.5 ± 0.8	9.6 ± 10.0
D2	13.2 ± 0.6	21.4 ± 1.4	0.5 ± 0.2	16.5 ± 1.0	6.9 ± 8.9
D3	75.2 ± 6.1	479.3 ± 53.6	0.3 ± 0.1	8.0 ± 0.4	40.7 ± 48.8

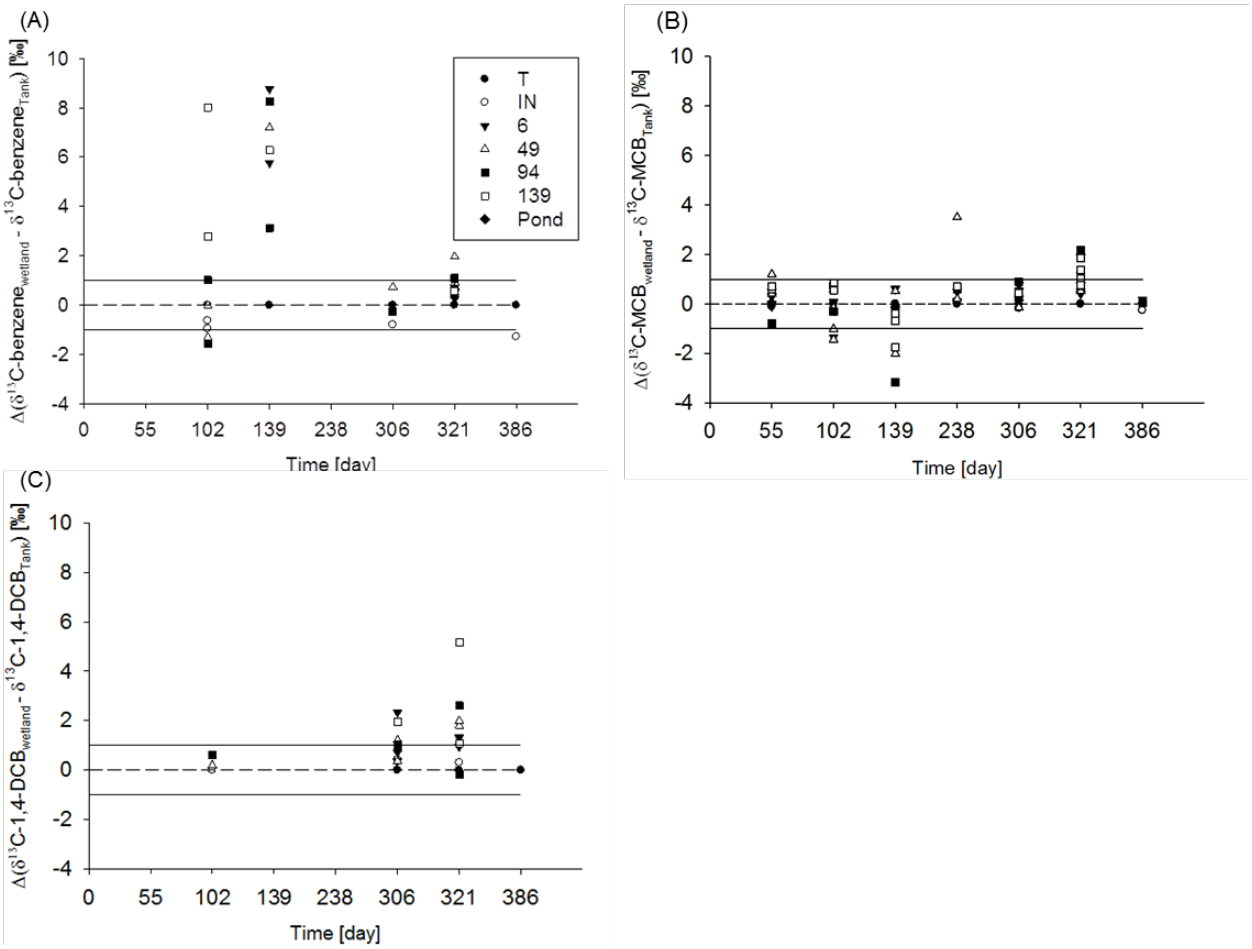

Figure S6-1. *The relative difference of the carbon stable isotope ratio ($\Delta(\delta^{13}C_{wetland} - \delta^{13}C_{tank})$ of benzene (**A**), MCB (**B**) and 1,4-DCB (**C**) for the wetland samples: tank (T), inflow (IN), sand compartment (after 6, 49, 94 and 139 cm distance from inflow) and the pond over time (55-386 days) to carbon stable isotope ratio in the tank is shown. The dashed line represents all the normalised tank samples with a deviation of zero. And the upper and lower solid lines indicate the minimum delta ($\delta^{13}C$-‰) – shift to be determined to have a significant $\delta^{13}C$ fractionation. All obtained data are depicted as single values.*

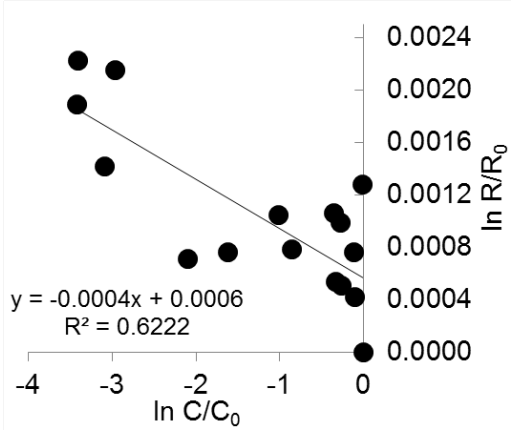

Figure S6-2.. *Rayleigh plot showing linear regression for MCB removal in the wetland at day 418. Single data analysis of $\delta^{13}C$ isotope ratios of MCB are shown at distinct MCB concentrations.*

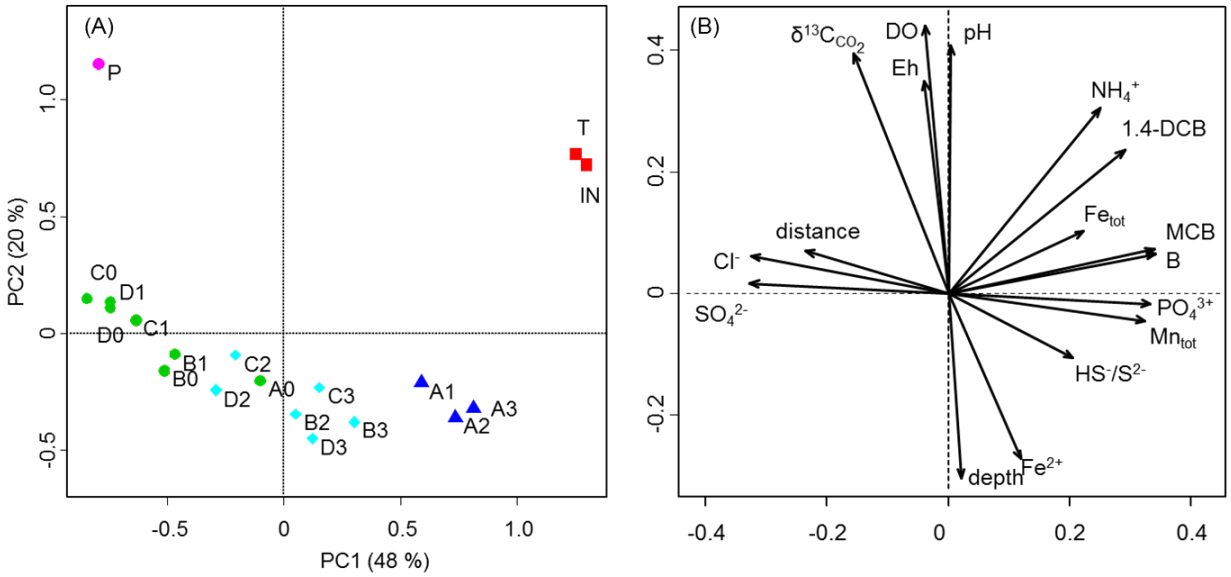

Figure 6-3. *PCA ordination plot of (**A**) hydrogeochemical characteristics of water samples and of (**B**) hydrogeochemical variables collected in the model wetland between days 55 till 420 (average values are depicted). (**A**) Values on the axes indicate % of the total variation explanation by the corresponding axis (PC 1, principal component axis 1; PC 2, principal component axis 2). The first and second principal components accounted for 68 % of the variance in the data set. Objects are labeled and were collected according to the wetland scheme (Figure S1) from (T, tank and IN, inflow (□); sand compartment at 6 (A), 49 (B), 94 (C) and 139 (D) cm from the inflow in 12 (0), 24 (1), 36 (2), 48 (3) cm depth, respectively; P, pond (○). (**B**) Description vectors correspond to: Cl-, chloride; SO_4^{2-}, sulphate; Fe^{2+}, ferrous iron; HS^-/S^{2-}, sulphide; Mn_{tot}, manganese total; PO_4^+, phosphate; B, benzene; MCB, monochlorobenzene, Fe_{tot}, iron total, NH_4^+, pH; DO, dissolved oxygen; Eh, redox potential; $\delta^{13}C_{CO2}$, CO_2 carbon isotopic composition.*

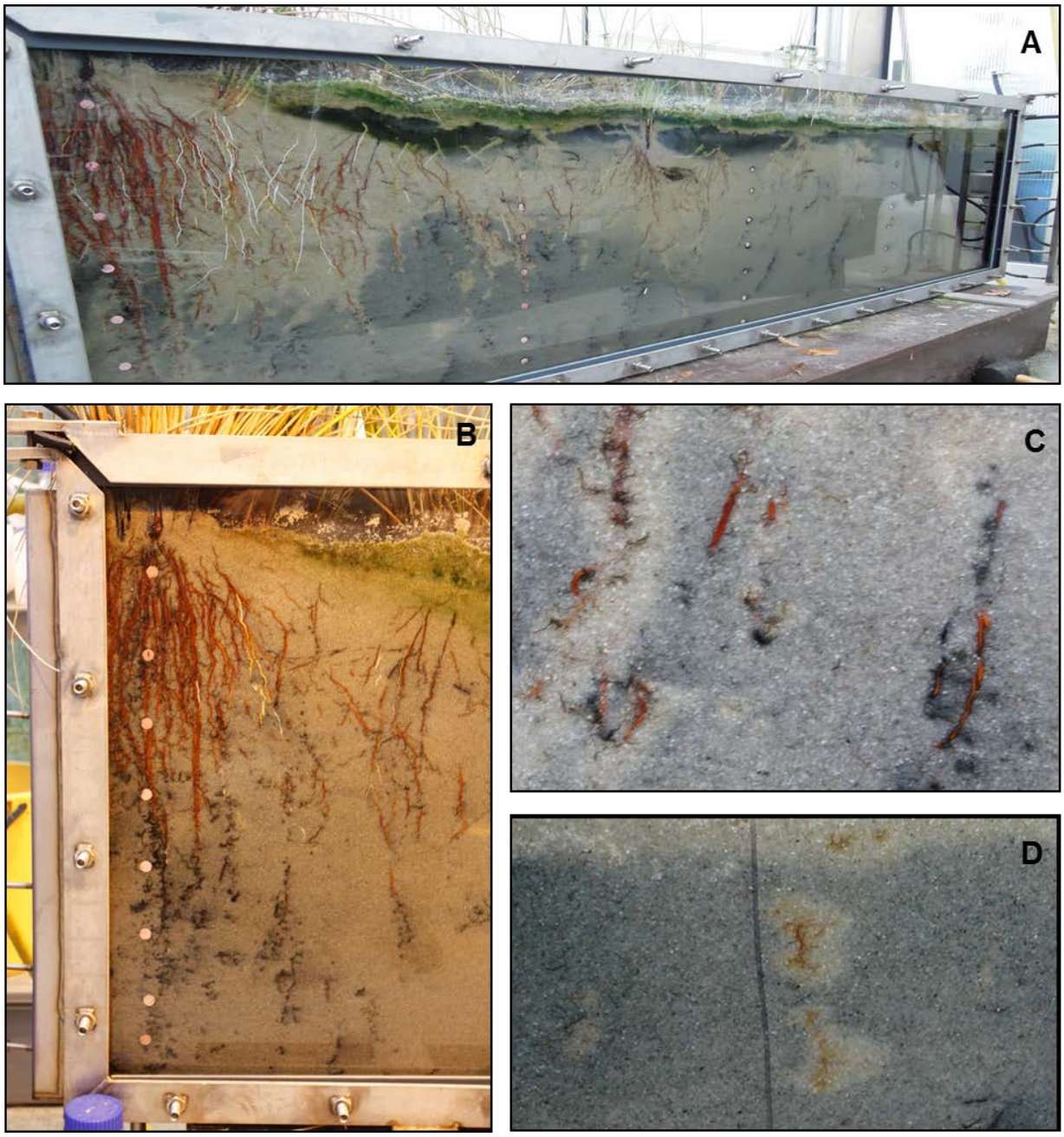

Figure S6-4. *Photographs show the front view at the constructed model wetland with blackish precipitates on the sediment in deeper zones (**A**) and the dense root system of the first Juncus effusus (L.) plant with black precipitates at fine roots (**B**). The blackish coloration of the sediment and fine roots (**C**) and the brownish coloration at the fine root (**D**) is clearly visible (likely FeS_2 species), indicating anoxic conditions in the sediment compartment.*

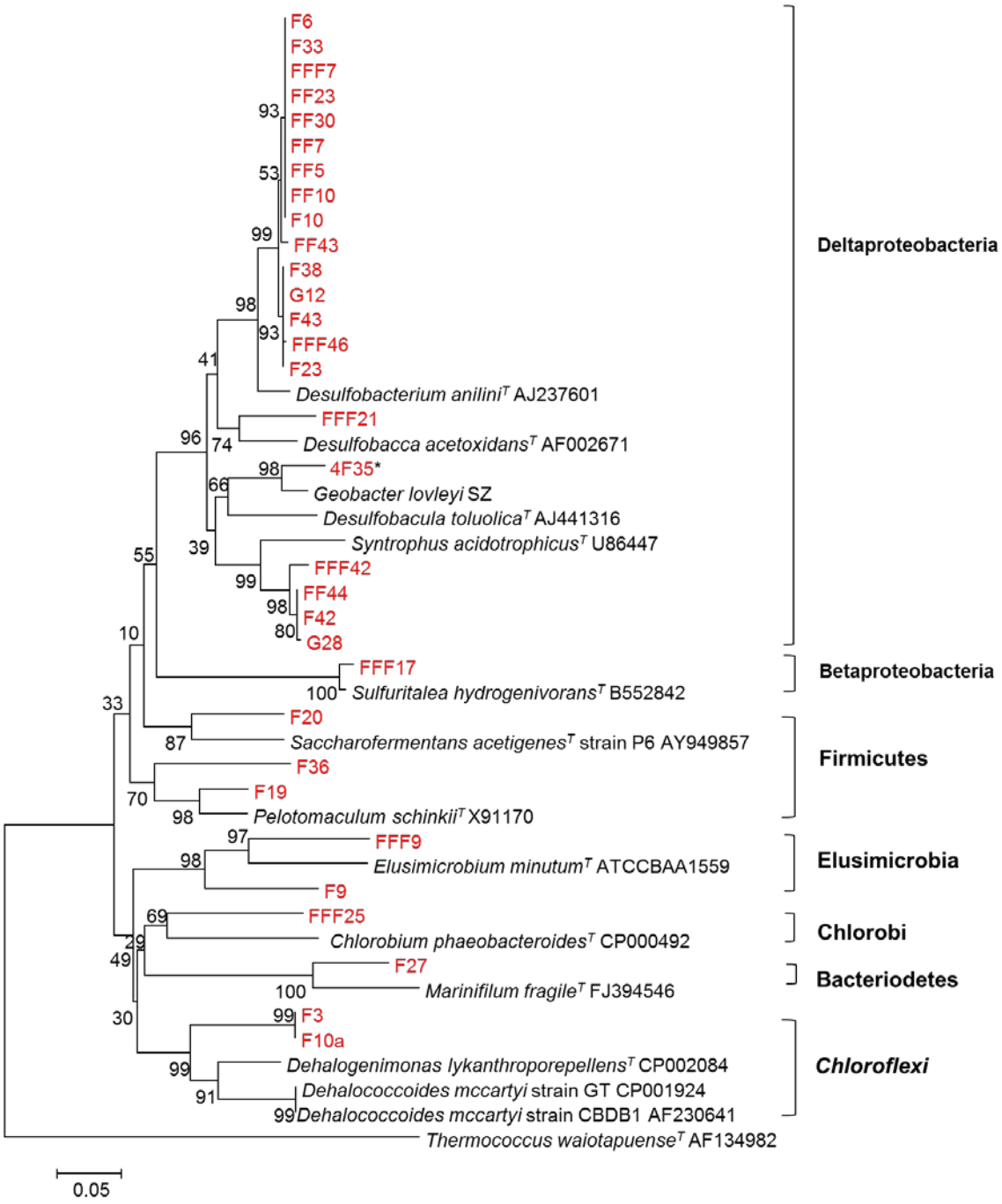

Figure S6-5. *Neighbour-joining tree of 16S rRNA gene sequences depicting the relationships among community members of the groundwater as revealed by comparative analysis of cloned 16S rRNA gene sequences (red) and those stored in the RDP database and GenBank. 16S rRNA gene bacterial sequences determined in this study are depicted and will be deposited in GenBank. Bootstrap values of <50 % are indicated at the node of the branch. Scale bar represents a 5 % estimated change. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011)*

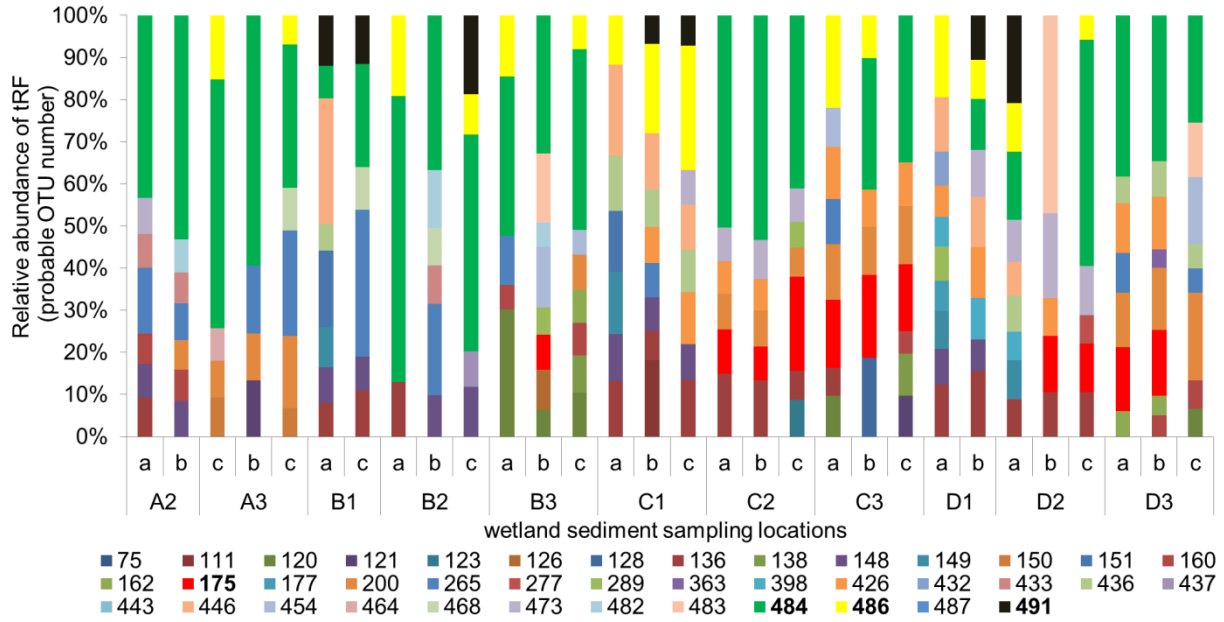

Figure S6-6: *Relative abundance of the terminal restriction fragments (tRF) = probable OTU number, determined in the TRFLP profile (enzymatic restriction with MspI) for bacterial community of wetland sediment samples of day 420, obtained from the sampling location with given sampling point nomenclature given as X axis according to Figure 6-1, wetland scheme. The tRFs (bp: 175, 484, 486, 491) highlighted in bold numbers were identified in the groundwater bacterial clone library, accordingly.*

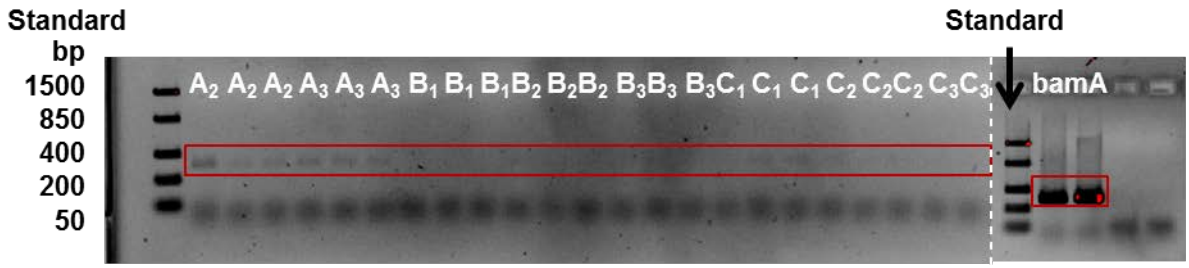

Figure S6-7. *Image of agarose gel, showing* bamA *gene ampificates with a size of 300 bp, obtained by PCR amplification of DNA extracts from different wetland sediment sampling locations A-C. Molecular standard indicates size of the amplified DNA fragments in bp (Fastruler low range DNA Marker, 1500, 850, 400, 200, 50). Positive (*bamA *gene amplificate of Geobacter metallireducens) and negative controls (water) are indicated.*

Table S6-4. *Substrate and product initial (t=0) and final concentration (t=558 days), under methanogenic and sulphate and iron (III) reducing conditions. Isotope ratio $^{13}C/^{12}C$ [‰] for the mineralisation products (CH_4, CO_2).*

Substrate	Amendement	Product	t_0	t_{558}					
			Substrate [µM]	Substrate [µM]	Product concentration [µM]			Product $^{13}C/^{12}C$ [‰]	
					Benzene	MCB	CH_4	$^{13}CO_2$	$^{13}CH_4$
MCB	**lactate**	CH_4, CO_2	107 ± 5	78 ± 26	9 (n=1)	-	18 ± 1	-2.75 ± 4.65	-56.18 ± 0.04
^{13}C-MCB	**lactate**	CH_4, CO_2	86 ± 5	56 ± 6	-	-	17 (n=1)	84.43 ± 40.98	-30.84 ± 6.03
MCB	**H_2**	CH_4	93 ± 14	79 ± 26	-	-	12 ± 9		-11.91 ± 7.47
^{13}C-MCB	**H_2**	CH_4	82 ± 8	67 ± 9	-	-	2 ± 1	-	-
MCB	**none**	CO_2	88 ± 8	67 ± 15	-	-	-	-8.34 ± 0.25	-76.97 ± 0.65
^{13}C-MCB	**none**	CO_2	98 ± 12	66 ± 21	-	-	-	414.49 ± 118.92	-49.38 ± 29.94
MCB	**Fe(III)**	CO_2	101 ± 12	90 ± 8	-	-	-	15.26 ± 0.59	-
^{13}C-MCB	**Fe(III)**	benzene, CO_2, CH4	77 ± 6	26 ± 21	72 (n=1)*	-	-	346.56 ± 179.07	97.18 (n=1)
MCB	**SO_4^{2-}**	CO_2	78 ± 28	78 ± 10	-	-	-	-9.87 ± 1.64	-
^{13}C-MCB	**SO_4^{2-}**	CO_2	88 ± 19	55 ± 6	-	-	-	458.24 ± 9.8	-
1.2-DCB	**lactate**	MCB, CH_4, CO_2	40 ± 2	1 ± 2	-	56 ± 6	-	-8.61 ± 5.49	-46.62 ± 4.24
1.3-DCB	**lactate**	CH_4	44 ± 4	23 ± 8	-	-	18 ± 5	-1.57 ± 1.64	-58.92 ± 1.97
1.2-DCB	**H_2 (+ acetate)**	CH_4	38 ± 5	42 ± 13	-	-	13 ± 11	-8.52 ± 1.09	135.88 (n=1)
1.3-DCB	**H_2 (+ acetate)**	CH_4	41 ± 4	21 ± 3	-	-	27 ± 16	-22.22 ± 1.01	20.221 (n=1)
1.2-DCB/1.3-DCB (N=2)	**Fe(III)**	MCB	36, 43/ 38, 39	38, 34/ 25, 21	-	-	-	-17.6 ± 4.09	-
1.2-DCB/1.3-DCB (N=1)	**Fe(III)**	MCB	51/ 48	2/ 29	-	48 (n=1)	-		

* ^{13}C-benzene detected using Gas chromatography mass spectrometry (See Figure S6-9 for GC-MS chromatogram)

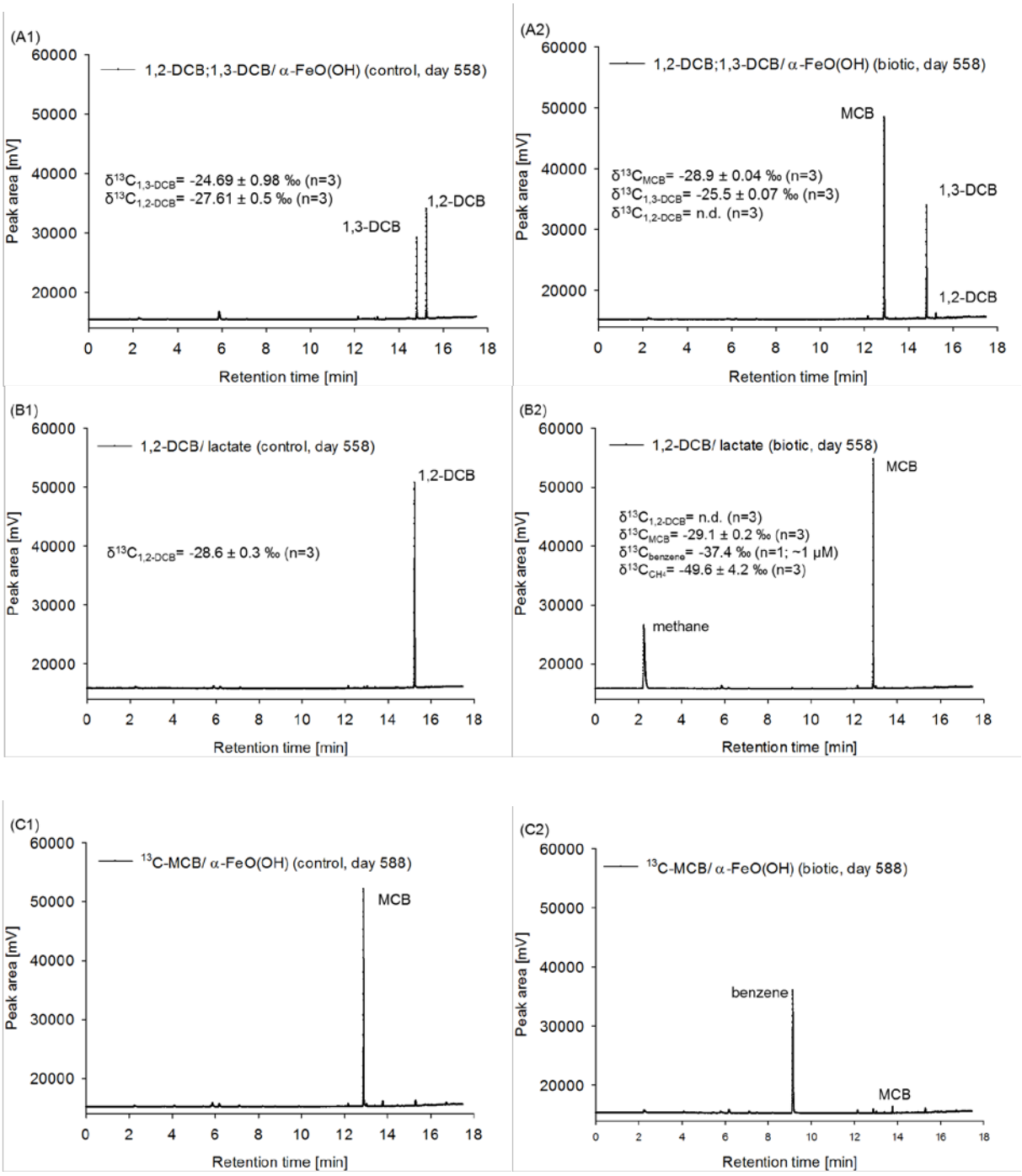

Figure 6-8. *The GC-FID chromatograms of selected sediment microcosms with 1,2-DCB;1,3-DCB/ α-FeO(OH) (biotic, A1; abiotic control, A2), with 1,2-DCB/ lactate (B1; B2) and with [$^{13}C_6$]-MCB/ α-FeO(OH) (C1, C2) are depicted after an incubation period of 558 days to present the product formation. 1,2-DCB was dechlorinated to MCB with α-FeO(OH) (A2) and degraded to MCB and methane with lactate (B2). Dechlorination of [$^{13}C_6$]-MCB to benzene with α-FeO(OH) was determined (C2). Additionally the δ^{13}C [‰] carbon isotope ratio of substrates and products is given for the non ^{13}C-labelled substrate (1,2-DCB; 1,3-DCB) and their formed products (MCB, benzene, CO_2 and CH_4) (A,B).*

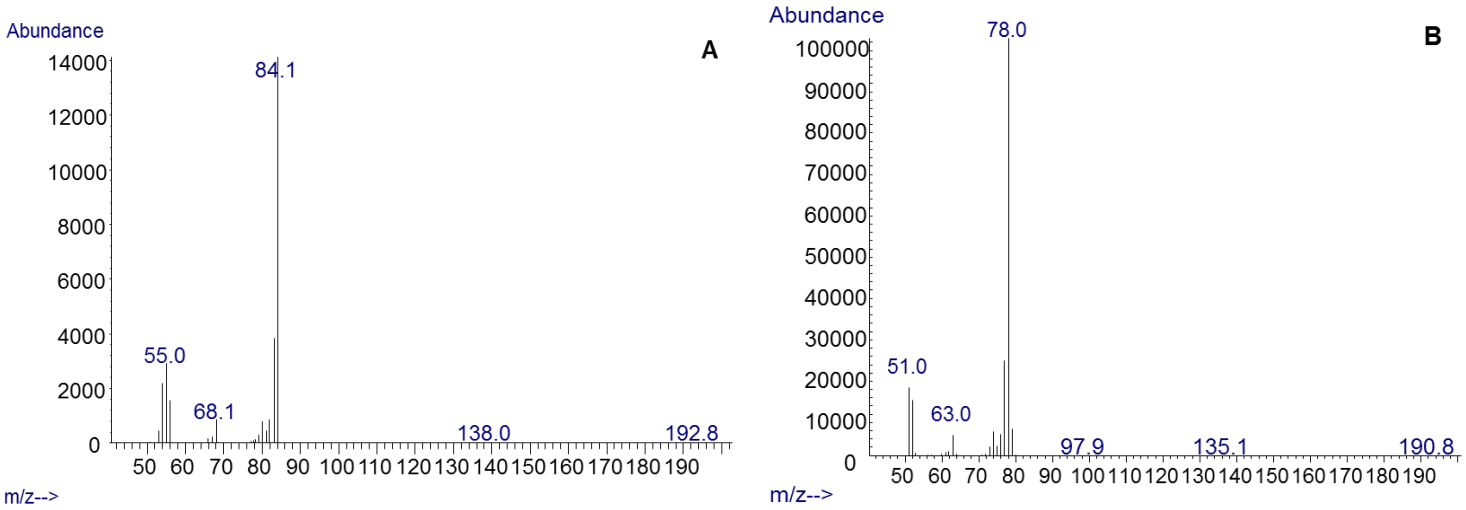

Figure S6-9. *(**A**) Biotic microcosm (set up with [$^{13}C_6$]-MCB and Fe(OH): mass spectrum of [$^{13}C_6$]-benzene and (**B**) spectra Benzene standard, the ion signals at a given m/z ratio were obtained by integration of the ion counts over the entire mass peak, from which the baseline contribution was subtracted. The ion signals at m/z = 55 (C_5H_3), m/z = 68 (C_5H_3) and m/z = 84 (C_5H_5) were measured with the typical mass shift of 6 for the main mass 78 → 84.*