Assessment of *in situ* biodegradation of chlorinated solvents in aquifers and constructed wetlands using an integrative approach

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Mots clés en français : éthènes chlorés, benzènes chlorés, dégradation, milieux humides, aquifère, isotope stable, hydrochimie, dehalococcoides, communautés microbiennes, approche holistique

Mots clés en anglais : chlorinated ethenes, chlorinated benzenes, degradation, wetlands, aquifère, stable isotopes, hydrochemistry, dehalococcoides, microbial community, integrative approach

Abstract

Knowledge about biogeochemical processes associated with natural attenuation of chlorinated solvents in the environment is currently limited. Though processes such as sorption, volatilization or dilution may contribute to contaminant natural attenuation, *in situ* biodegradation is the only process leading to destructive removal of contaminants. However, the distribution of hydrological and hydrochemical processes over both spatial and temporal scales influence degradation reactions, and thus should be taken into account when assessing *in situ* biodegradation in complex systems. This thesis aimed at gathering knowledge about *in situ* biodegradation of chlorinated solvents and associated biogeochemical processes within both contaminated wetlands and aquifers. The specific objectives of this thesis were i) to develop and apply an approach in order to demonstrate *in situ* degradation of toxic reductive dechlorination intermediates such as dichloroethenes (DCE), vinyl chloride (VC) and chlorobenzene (CB), ii) to investigate the biogeochemical development of model wetlands treating intermediate dechlorination compounds, iii) to identify the prevailing degradation pathways within the investigated systems, and iv) to investigate the relationships between the microbial community and ambient hydrochemical conditions. The different investigations carried out in the framework of this thesis were based on an integrative approach, which couples Compound Specific Isotope Analysis (CSIA) to hydrogeochemical and molecular techniques. The various resulting sets of data were explored and linked by means of multivariate statistics in order to gain additional insights into the development of biogeochemical processes.

First, recent progresses made towards understanding how mechanisms attributed to various organic chemicals removal interact to form a functioning treatment wetland were reviewed (*Chapter 1, section 2*). In particular, possible complementary techniques and integrative approaches to follow up *in situ* degradation processes were discussed.

Second, the biogeochemical processes and the *in situ* biodegradation activity were characterized in two different model constructed wetland systems for contaminated groundwater-surface water interfaces (*Chapter 2*). In a first study (*Chapter 2, section 1*), the biotransformation of CB was investigated in a constructed wetland supplied with contaminated groundwater by means of a detailed geochemical characterization, stable isotope composition analysis and *in situ* microcosm systems loaded with ^{13}C-labelled CB. Significant shift in the isotopic composition of CB over the flow path under prevailing iron reducing conditions and the detection of ^{13}C-labelled benzene indicated reductive dehalogenation of CB.
In a second study (*Chapter 2, section 2*), the spatial and temporal biogeochemical development of a model system loaded with *cis*- and *trans*-1,2-dichloroethene contaminated groundwater was characterized over 430 days by means of hydrogeochemical and CSIA. The hydrogeochemistry dramatically changed over time from oxic to strongly reducing conditions, as emphasized by increasing concentrations of ferrous iron, sulphide and methane over time. δ^{13}C values for *trans*- and *cis*-DCE substantially changed over the flow path and correlated

over time with DCE removal. The carbon enrichment factor values (ε) retrieved from the wetland became progressively larger over the investigation period, ranging from -1.7 ± 0.3 to -32.6 ± 2.2 ‰. These changes mirrored a parallel change in the dominant degradation mechanism at the system scale. Indeed, less fractionating DCE oxidation progressively succeeded to biotic reductive dechlorination, which was associated with a more pronounced isotopic effect and further confirmed by the detection of VC and ethene.

In a complementary study (*Chapter 2, section 3*), the model wetland microbial community was characterized during the transition phase from a prevailing aerobic to an anaerobic regime. Non-metric dimensional scaling analysis of microbial fingerprints revealed that a dynamic community was associated with changes of redox-sensitive processes at the system scale. Microbial analyses emphasized the possible involvement of several microbial groups in the observed biogeochemical processes potentially influencing DCE transformation. The presence of a complex guild of putative dehalorespirers (*Dehalobacter* spp., *Dehalococcoides* spp. and *Geobacter* spp.) during the anoxic phase, where reductive dechlorination prevailed, was detected. 16S rRNA gene libraries revealed substantial changes of the bacterial composition between the supplied groundwater and the model wetland pore water. *Proteobacteria* accounted for > 50 % of 16S rRNA gene clone library of the wetland, and about 17 % of the sequences could be related to sulphate reducers.

Overall, the investigation of the model system treating DCE contaminated groundwater i) demonstrated the linkage between the hydrogeochemical variability and the ongoing degradation processes, ii) highlighted the potential of CSIA to trace the temporal and spatial changes of the dominant degradation mechanism of DCE in natural or engineered systems, and iii) underscores progressive changes of both wetland microbial community structures and degrading guilds during the transition from mostly oxic to anoxic conditions that also coincided with changing degradation mechanism. The use of an integrated approach enabled monitoring *in situ* biodegradation of DCE and CB in model wetland systems, and to gather parallel information on the associated biogeochemical processes and prevailing contaminant transformation pathways.

Third, i*n situ* biodegradation of chlorinated solvents was studied in two different groundwater systems (*Chapter 3*). In a first study (*Chapter 3, section 1*), biodegradation of mostly recalcitrant chlorobenzenes was assessed at an anoxic aquifer by combining hydrogeochemical and stable isotope analyses. *In situ* microcosm analysis evidenced microbial assimilation of CB derived carbon, whereas laboratory investigations asserted mineralization of CB. An isotope balance was applied to overcome the limit of interpretation posed by the simultaneous enrichment and depletion in ^{13}C of reductive dechlorination intermediates during sequential *in situ* degradation. The enrichment obtained by cumulating the concentration and isotopic composition values of single chlorobenzene species indicated CB biodegradation at various zones of the aquifer. Additionally, the relationship between hydrogeochemical conditions and degradation activity was investigated by principal component analysis. This analysis underlines variable hydrogeochemical conditions associated with degradation activity at the plume scale.

In a second study (*Chapter 3, section 2*), the ongoing *in situ* biodegradation of chlorinated ethenes within a hydrogeologically complex groundwater system (Bitterfeld, Germany) was investigated at the plume scale. The assessment of hydrogeochemical species and chlorinated ethenes concentrations distribution by principal component analysis (PCA), in combination with carbon stable isotope composition analysis, revealed that chlorinated ethenes were subjected to substantial biodegradation. Changes in isotopic composition values up to 20.4, 13.9, 20.1 and 31.4 ‰ were observed between geological units for tetrachloroethene (PCE), trichloroethene (TCE), *cis*-dichloroethene (cDCE) and vinyl chloride (VC), respectively. The use of specific biomarkers (16S rRNA gene) indicated the presence of *Dehalococcoides* sp. DNA in 20 of the 33 evaluated samples. Analysis of bacterial community structures variation in the aquifers using canonical correspondence analysis (CCA) indicated a predominant influence of the contaminant concentrations.

To gain additional knowledge on biogeochemical variability within this groundwater system, a third study assessed the bacterial community structures, hydrogeochemical indicators and the carbon stable isotope composition of chlorinated ethenes at discrete-depth intervals along a hydrogeologically heterogeneous vertical profile (*Chapter 3, section 3*). Substantial variation of the hydrogeochemistry, bacterial community structures and the distribution of putative dehalorespirers (*Dehalobacter* spp.*, Desulfitobacterium* spp.*, Dehalococcoides* spp. or *Geobacter* spp.) were observed with depth, according to the contaminant concentration. β- and γ-*Proteobacteria* accounted for > 18 % of 16S rRNA genes clone libraries and sequences could be affiliated with at least 10 classes of the domain *Bacteria*. The compound specific isotope analysis of the single chlorinated ethenes species was interpreted globally based on an isotope balance. Hydrogeochemical and microbiological indicators as well as the isotope balance, revealing isotopic enrichment above 8 ‰ between the sources and the plume fringe area, clearly demonstrated the occurrence of reductive dechlorination of chlorinated ethenes at the investigated depths. Isotope balance values revealed enrichment from -14.4 to -4.4 ‰ over the vertical profile, which suggested that chlorinated ethenes biodegradation reaction varied spatially. Overall, a relationship could be established between bacterial community structures, hydrogeochemical variables and hydrogeological conditions.

In summary, the different investigations presented in this thesis underscored the relevance of conducting integrative studies based on several, complementary techniques to improve the assessment of relevant chlorinated solvents *in situ* biogeochemical processes in complex systems. In order to facilitate the exploration and interpretation of the relationships between subsurface biocenoses and biogeochemical contaminant removal processes, complex microbial and hydrochemical sets of data can be efficiently treated by multivariate analysis. Several concepts related to the isotopic analysis of chlorinated solvents developed and applied in the framework of this thesis (i.e. the elucidation of DCE degradation pathways, the isotope balance and the use of in situ microcosms experiments) may be adapted and employed for further investigation of the environmental fate of chlorinated solvents as well as for other organic chemicals. Furthermore, the investigation demonstrated that concepts and approaches currently being applied in groundwater systems for assessing *in situ* biodegradation and elucidating degradation pathways of chlorinated solvents can be efficiently transposed to monitor and study organic chemicals in hydrologically and hydrochemically heterogeneous wetland systems.

Abbreviations

BTEX	Benzene, Toluene, Ethylbenzen, Xylene
CB	Chlorobenzene
CCA	Canonical Correspondence analysis
CSIA	Compound-specific stable isotope analysis
DCB	Dichlorobenzene
DCE	Dichloroethene
DGGE	Denaturing Gradient Gel Electrophoresis
DIC	Dissolved inorganic carbon
DIN	Deutsches Institut für Normung
DNA	Deoxyribonucleic acid
DNAPL	Dense non aquous phase liquid
DOC	Dissolved organic carbon
ENA	Enhanced Natural Attenuation
Eq.	Equation
FA	Fatty acid
FAME	Fatty acid methyl ester
FISH	Fluorescence in situ hybridization
GC-C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GC-FID	Gas chromatography-Flame Ionisation Detector
GC-IRMS	Gas chromatography-Isotope Ratio Mass Spectrometry
GC-MS	Gas chromatography-Mass Spectroscopie
HCH	Hexachlorcyclohexane (Lindane)
IAEA	International Atomic Energy Agency
MCB	Monochlorobenzene
MFA	Multiple Factor Analysis
MNA	Monitored Natural Attenuation
MPN	Most Probable Number
MTBE	Methyl-tert-butylether
nMDS	Non-Metric Multidimentional Scaling
NA	Natural Attenuation
n.a.	Not assessed
n.d.	Not determined
PAH	Polycylic aromatic hydrocarbon
PCA	Principal Component Analysis
PC1/2	Principal Component axis 1/2
PCE	Tetrachlorethene (Perchlorethene)
PCR	Polymerase chain reaction
REACH	Registration, Evaluation, Authorisation of Chemicals
PLFA	Phospholipid-derived fatty acid
RDH	Reductive dehalogenation
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SAFIRA	Sanierungsforschung in regional kontaminierten Aquiferen
SI	Supporting Information
SIFA	Stable isotope fractionation analysis
SIP	Stable isotope probing
SSCP	Single Strand Conformation Polymorphism
TCB	Trichlorobenzene
TCE	Trichloroethene
TEA	Terminal electron acceptor
TEAPs	Terminal electron acceptor processes
TLFA	Total Lipid Fatty Acid
TMCS	Trimethylchlorosilane
TOC	Total organic carbon
UFZ	Helmholtz-Zentrum für Umweltforschung (Centre for Environmental Research)
US-EPA	United States Environmental Protection Agency
VC	Vinyl Chloride
V-PDB	Vienna PeeDee Belemnite
V-SMOW	Vienna Standard Mean Ocean Water
α	Isotopenfraktionierungsfaktor
$\delta^{13}C_{(x)}$	Carbon isotopic composition of a compound (*x*)
ε	Enrichment factor

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1

Introduction

Section 1: General Introduction

Section 2: Assessing and monitoring processes of organic contaminant removal in constructed wetlands

Section 1: General introduction

1. Chlorinated solvents in the environment

Throughout the United States of America and Europe, chlorinated solvents are among the most common contaminants observed in groundwater systems (Bouwer, 1994; McCarty & Semperini, 1994; Vogel, 1994; Alvarez *et al.*, 2004). Chlorinated ethenes are of major significance to public health, due to their widespread presence, toxic effects and persistence (Henschler, 1994). Some selected facts and properties related to chlorinated ethenes are summarized in **Table 1**. Higher chlorinated ethenes comprise tetrachlorethene (PCE) and trichloroethene (TCE). PCE and TCE have high boiling points, and are lipophilic, volatile, and non-flammable. These compounds are commonly used in extraction processes, dry cleaning and degreasing agents. Lower chlorinated ethenes include *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), 1,1-dichloroethenes (1,1-DCE) and vinyl chloride (VC). The presence of dichloroethenes in groundwater systems is most often due to *in situ* degradation of the higher chlorinated counterparts (Bradley, 2000). VC is a flammable gas used in the polyvinylchloride (PVC) manufacturing operation (Hartmans, 1995). Global production of VC amounts to more than 10,000 Kt y^{-1}, which represents around 90 % of the global chlorinated ethenes production (Sierra-Alvarez & Field, 2004). Though only vinyl chloride is a human carcinogen, all chloroethenes are suspected toxic or carcinogenic (EC Regulation N° (1907/2006, 2006)) and have maximum contaminant levels in water ranging from 2 (VC) to 100 (*trans*-DCE) $\mu g\ L^{-1}$ (Kielhorn *et al.*, 2000; EPA, 2003).

Chlorinated aromatic compounds are mainly used in the synthesis of pesticides and other chemicals but also result of microbial transformation of hexachlorocyclohexane, of which the γ-isomer (Lindane) is worldwide used as pesticide (van Agteren *et al.*, 1998; Phillips *et al.*, 2005). Large entry of these chemicals into the environment occurs primarily as a result of military actions and industrial production, transportation and application, improper waste disposal and spills as well as leakages (Häggblom & Bossert, 2003). Global industrial emissions of chlorinated ethenes to air and water were substantially reduced over the last two decades However, VC and PCE transferred in European water is estimated at 4.7 and 2.9 tons y^{-1}, respectively (Lecloux, 2003). Once released, chlorinated solvents may be subjected to non-destructive processes, including volatilization, dissolution, sorption, advection, dispersion and diffusion, and/or destructive processes, such as abiotic or biotic degradation. The main abiotic degradation reactions, to which chlorinated ethenes are subjected to, are substitution and dehydrohalogenation (Vogel *et al.*, 1987). However, these processes have been mainly reported to occur at pH-value higher than 8, and associated half-life values are one order to magnitude lower than biotic transformation, and mainly concern PCE and TCE (Butler & Hayes, 1999; Dayan *et al.*, 1999; Lee & Batchelor, 2002; Jeong & Hayes, 2007). Additionally, light-induced chemical transformations or photochemistry represents an abiotic process, which can be relevant as a contaminant sink. The respective contribution of destructive and non-destructive processes mainly depends on the characteristics of the contaminated hydro-system and on the physico-chemical properties of the contaminant species. Together with the entry point, this influences the environmental fate of chlorinated contaminants.

Moreover, laboratory and field evidence has accumulated in the last decade that microorganisms can transform chlorinated solvents to non-toxic products under a variety of environmental conditions (Holliger *et al.*, 1998; Lee *et al.*, 1998; Ellis *et al.*, 2000). The main research developments in the area of chlorinated solvents´ biodegradation are largely focusing on the use of dehalorespiring microbial cultures to dechlorinate chlorinated solvents to environmentally acceptable, non-chlorinated end-products (Holliger *et al.*, 1993; MaymoGatell *et al.*, 1997). So far, several microbial mechanisms for transformation of chlorinated solvents have been identified as potentially significant in hydro-systems (Semprini, 1995; Olaniran *et al.*, 2004).

Table 1. Consumption, production, annual global fluxes and selected physical and chemical properties of chlorinated ethene compounds.

		Estimated European consumption	**Industrial emission in water at 80 European production plants[4] (in 1997)**	**Maximum level for drinking water[5]**	**Aqueous solubility (25°C)**	**Henry´s law constant (25°C)[6]**	**Density[6]**	**Adsorption coefficient[8]**
		[K tons yr^{-1}]	[tons yr^{-1}]	[µg L^{-1}]	[mg L^{-1}]	[Atm-m^{3}/mol]	[g cm^{-3}]	[Log K_{oc}]
Chlorinated ethene species	**VC**	5700 (PVC, in 2001)[1]	4.7	2	2700[6]	0.027	0.912[6]	0.91
	***cis*-1,2-DCE**	n.a.	n.a.	70	3500[6]	0.0041	1.284[6]	1.5
	***trans*-1,2-DCE**	n.a.	n.a.	100	6300[6]	0.0094	1.257[6]	1.77
	1,1-DCE	12 (in 1998)[2]	n.a.	7	2250[6]	0.0261	1.175[7]	1.48
	TCE	30 (in 2005)[3]	n.a.	5	1100[7]	0.0103	1.462[7]	2.1
	PCE	50 (in 2005)[3]	2.9	5	162[7]	0.0184	1.625[7]	2.82

[1] Focus on Polyvinyl chloride, An International Newletter Monitoring Commercial and Technical Aspects of PVC (ISSN 1468-5736); [2] (Field & Sierra-Alvarez, 2004); [3] EuroChlor, European chlorine industry, 2005 (www.eurochlor.org); [4] (Lecloux, 2003); [5] US EPA maximum contaminant level (MCLs) for chlorinated ethenes in drinking water as of June 2003; [6] EPA Superfund Chemical Data Matrix; [7] Merk Index; [8] EPA National Primary Drinking Water Regulations Technical Fact Sheet,

.

n.a: not assessed

2. Microbial transformation of chlorinated solvents at biogeochemical interfaces

During the 1980s, there were several reports of accumulation of PCE and TCE transformation products in the environment, which was attributed to microbial reductive dechlorination (Vogel *et al.*, 1987). By the mid 1990s, microorganisms that gain energy from reductive dechlorination of chlorinated ethenes were discovered (Holliger *et al.*, 1993; MaymoGatell *et al.*, 1997). This led to a turning point from a predominantly co-metabolic view of chlorinated ethenes biodegradation to the concept of chlorinated ethenes serving as primary substrates for microbial metabolism. Recent evidence indicates that biodegradation of chlorinated solvents in ground- and surface-water can be achieved by two major mechanisms: reductive dechlorination (refer to section 2.1.) and oxidation (section 2.2.) reactions. Many chlorinated hydrocarbons also occur naturally in the environment, including TCE, PCE (Gribble, 2002) and vinyl chloride (Keppler *et al.*, 2002). This may have influenced the evolution of microorganisms specialized for degrading organochlorine compounds (Lee *et al.*, 1998). While degradation mechanisms can yield sufficient energy to sustain microbial growth (Dolfing & Janssen, 1994), the structural difference between the members of this class in terms of number and spatial arrangement of chlorine atom subsistents influence their respective reactivity and redox characteristics. This mainly determines the potential degradation pathways. Under anoxic condition, highly oxidized compounds, such as PCE and TCE, can be used as electron acceptors and readily undergo reduction reaction. However, the efficiency of reductive dechlorination decreases with decreasing chlorination degree. Conversely, the potential for microbial oxidation to CO_2 or CH_4 increases with decreasing chlorine number. **Figure 1** shows the main proposed pathways for chlorinated ethene biological transformation and illustrates the characteristics of lower chlorinated solvents to undergo in parallel reductive dechlorination and oxidation processes. Similarly, reductive dechlorination of higher chlorinated benzene is feasible either as cometabolic reaction or energy yielding halorespiration (van Agteren *et al.*, 1998; Adrian *et al.*, 2000; Jayachandran *et al.*, 2003), whereas lower chlorinated benzene, such as monochlorobenzene (CB) tends to accumulate under strongly reducing conditions (Kaschl *et al.*, 2005; Nijenhuis *et al.*, 2007).

The combined effort of microbial reductive dechlorination of higher chlorinated ethenes under anaerobic conditions to lower-chlorinated ethenes products and the microbial oxidation of the daughter products to ethene and CO_2 under both aerobic and anaerobic condition is relevant to reach significant and complete removal of chlorinated ethenes in soil and groundwater systems (Coleman *et al.*, 2002). Therefore, these two mechanisms might be efficiently coupled in environments, such as groundwater-surface water interfaces and wetland areas, where spatial and temporal variations in both hydrologic and geochemical conditions occur. Indeed, in contaminated aquifers, the reduced daughter products, such as DCEs and VC, generally increase in importance in the down gradient portion of anoxic contaminated hydro-systems. These products may reach hydrologically connected surface-water or wetland receptors and undergo in parallel reductive and oxidative degradation processes in vicineous micro-compartments. Their relative contribution is controlled by inherent steep and dynamic biogeochemical gradients occurring at ground- and surface water interfaces (e.g. McClain *et al.*, 2003).

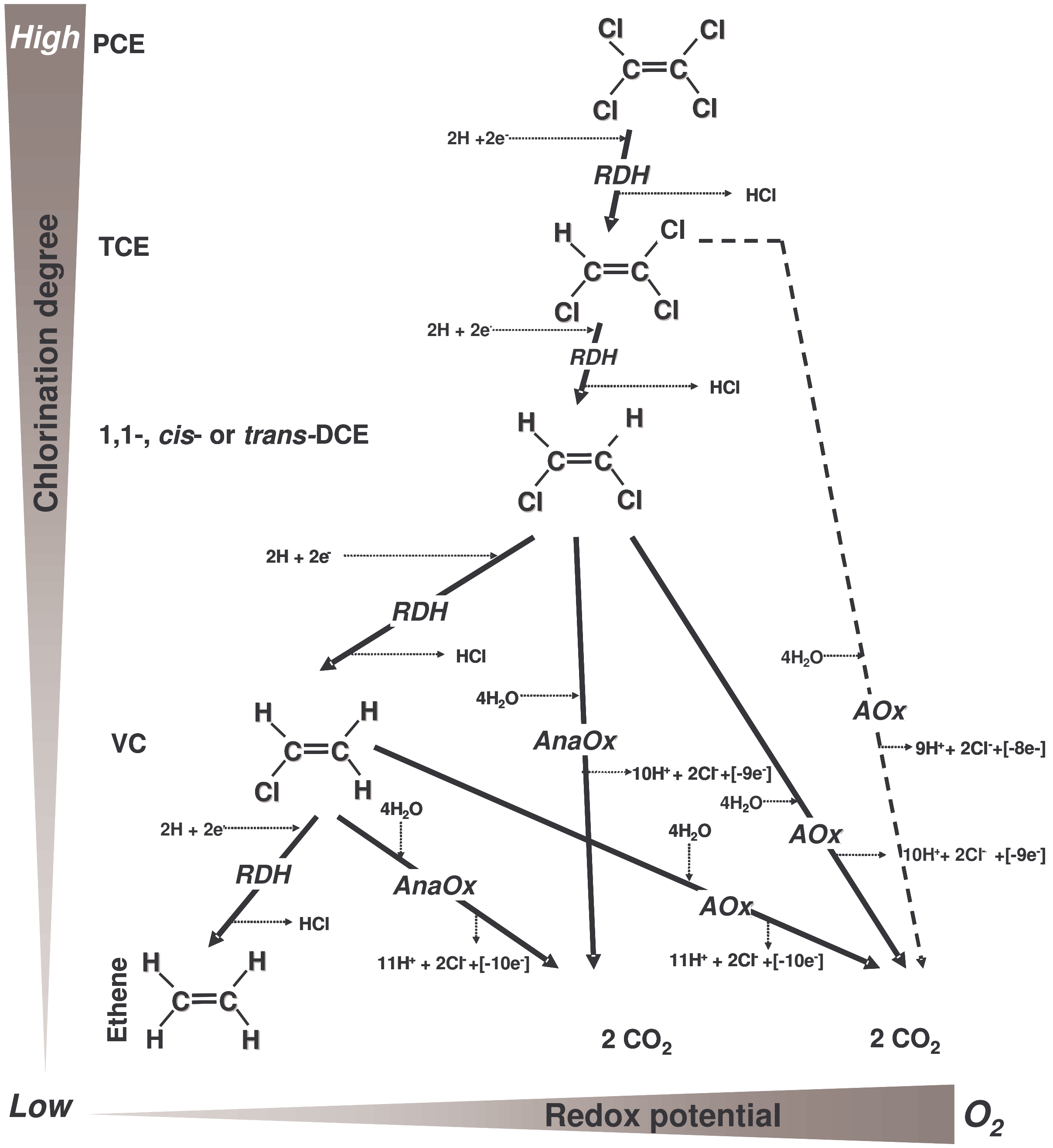

Figure 1: Proposed pathways for biological transformation of chlorinated ethenes. The different pathways are indicated as follows: RDH: reductive dechlorination; AnaOx: anaerobic oxidation; AOx: aerobic oxidation. PCE and TCE readily undergo reductive dechlorination but the efficiency of the reaction decreases with decreasing chlorination degree. Some dechlorinators sequentially dechlorinate PCE to TCE, some preferentially to *cis*-DCE, and some to VC. However, the conversion of DCE and VC as electron acceptor to non-toxic ethene is principally mediated by *Dehalococcoides* spp. affiliated bacteria. Conversely, the tendency for aerobic oxidation of chlorinated ethenes increases with decreasing number of chlorine atoms of the molecule. Both metabolic and cometabolic oxidation of lower chlorinated ethenes have been reported. However, mineralization of DCE and VC tends to increase with higher reduction potential.

2.1. Reductive dechlorination

Reductive dechlorination is a process where a chlorine atom is removed and replaced with a hydrogen atom. Typically, microbially mediated reductive dechlorination occurs under anaerobic conditions and an electron donor is required to carry out the reaction. The consensus is that microbial reductive dechlorination is essentially ubiquitous in anaerobic, chlorinated ethenes-contaminated aquifers, but that the extent of dechlorination is highly variable from site to site (Bouwer, 1994; McCarty & Semperini, 1994; Bradley & Chapelle, 1996). The proposed pathways for the sequential reductive dechlorination of PCE through the daughter products to the non-chlorinated ethene are shown in **Figure 1**. As a result of the decreasing reduction potential with decreasing number of chlorine substituents, reductive dechlorination of chlorinated compounds is often incomplete in groundwater systems, which may lead to the accumulation of *cis*-DCE and VC (Major *et al.*, 1991; Haston *et al.*, 1994; Wilson *et al.*, 1995). However, the energy available from sequential chlorinated ethenes reduction steps decrease only slightly with decreasing number of chlorine constituent (**Table 2**). For instance, the reduction potentials of all dechlorination steps from PCE to ethene remain substantially higher than those for oxidants commonly used by microbial life as respirative half-reactions, such as SO_4^{2-}. This would indicate a theoretical out-competition of sulphate reducers with respect to reducing equivalents (Dolfing & Janssen, 1994; Dolfing, 2000; Sierra-Alvarez & Field, 2004).

Cometabolic reductive dechlorination activities by sulphate reducers (Cole *et al.*, 1995), methanogens (Fathepure & Boyd, 1988) or acetogens (Terzenbach & Blaut, 1994) have been reported to mediate slow and partial PCE and TCE dechlorination to DCE. This widely held perception that this process is essentially accidental and of no benefit to the responsible organism, despite the early isolation of *Desulfomonile tiedjei* strain DCB-1 that derives energy from 3-chlorobenzoate (Deweerd *et al.*, 1990; Deweerd & Suflita, 1990). Nevertheless, current literature suggests that anaerobic reductive dechlorination of a broad spectrum of halogenated compounds is carried out by phylogenetically diverse bacterial groups (Smidt & de Vos, 2004). Isolated bacteria capable of chlorinated ethenes degradation are distributed among the Firmicutes, δ-, ε-, γ-Proteobacteria and Chloroflexi. These microorganisms able to grow using chlorinated ethenes as sole terminal electron acceptors are collectively termed halorespirers, and are capable of much higher rates of reductive dechlorination. Some dechlorinators sequentially dechlorinate PCE to TCE, some to *cis*-DCE, and some to VC (He *et al.*, 2003). Within the Firmicutes, strains from different genera have been so far isolated, e.g. *Dehalobacter* (Holliger *et al.*, 1998), *Clostridium* (Chang *et al.*, 2000) and the metabolically versatile *Desulfitobacterium* (Gerritse *et al.*, 1996; Finneran *et al.*, 2002). The δ-Proteobacteria sub-class includes *Desulfuromonas michiganensis* (Sung *et al.*, 2003) and *Geobacter lovleyi* (Sung *et al.*, 2006), which can use a large range of electron acceptors including metals (e.g. Fe^{3+}), chlorinated compounds, manganese and uranium. The sub-class ε-Proteobacteria includes isolates of the genera *Sulfurospirilum* (Scholz-muramatsu *et al.*, 1995; Luijten *et al.*, 2003), whereas *Enterobacter* MS-1 that transforms PCE to *cis*-DCE belongs to the γ-subphylum (Sharma & McCarty, 1996).

More recently, bacteria affiliated to the Chloroflexi were reported to degrade DCE and VC. *Dehalococcoides ethenogenes* strain 195 was first shown to metabolically dechlorinate PCE to VC (Maymo-Gatell *et al.*, 1997), whereas *Dehalococcoides* sp. strains BAV-1, GT, and VS able to use VC as growth-supporting electron acceptor, thereby transforming this carcinogenic compound to non-toxic ethene were isolated later (Cupples *et al.*, 2003; He *et al.*, 2003; Duhamel *et al.*, 2004; Sung *et al.*, 2006).

The occurrence of reductive dechlorination of chlorinated benzenes under methanogenic and sulfate reducing conditions has been observed in a variety of anaerobic mixed cultures (van Agteren *et al.*, 1998; Adrian *et al.*, 2000), but only one bacterial strain (*Dehalococcoides* strain CBDB1) capable to couple energy conservation with reductive dechlorination of chlorinated benzenes (≥ 3 chlorine substituents) has been isolated so far (Adrian *et al.*, 2000; Jayachandran *et al.*, 2003).

Table 2: Reductive half-reactions affecting single Cl dechlorination of aqueous chlorinated species and conventional electron acceptors (Adapted from Haas & Shock, 1999). [a]The values are shown in relation to reduction potentials of oxidants commonly used by microbial life as respirative half-reactions. Each reaction shown may be coupled to a reaction oxidizing organic matter to achieve an overall metabolic reaction. Reductive potentials (E°) in mVolts are listed for each reaction at 0 and 100°C. Values were calculated by using SUPCRT (Johnson *et al.*, 1992).

Electron Acceptor	Half-reaction		E° at 0°C [mVolts]	E° at 100°C [mVolts]
Chlorinated ethenes:				
$PCE_{(aq)}$	$C_2Cl_4 + H^+ + 2e^-$	=> $C_2HCl_3 + Cl^-$	704	720
$TCE_{(aq)}$	$C_2HCl_3 + H^+ + 2e^-$	=> *cis*-$C_2H_2Cl_2 + Cl^-$	659	681
$TCE_{(aq)}$	$C_2HCl_3 + H^+ + 2e^-$	=> *trans*-$C_2H_2Cl_2 + Cl^-$	636	655
$TCE_{(aq)}$	$C_2HCl_3 + H^+ + 2e^-$	=> 1,1-$C_2H_2Cl_2 + Cl^-$	635	656
1,1-$DCE_{(aq)}$	1,1-$C_2H_2Cl_2 + H^+ + 2e^-$	=> $C_2H_3Cl + Cl^-$	572	546
cis-1,2-$DCE_{(aq)}$	*cis*-$C_2H_2Cl_2 + H^+ + 2e^-$	=> $C_2H_3Cl + Cl^-$	549	521
trans-1,2-$DCE_{(aq)}$	*trans*-$C_2H_2Cl_2 + H^+ + 2e^-$	=> $C_2H_3Cl + Cl^-$	572	547
$VC_{(aq)}$	$C_2H_3Cl + H^+ + 2e^-$	=> $C_2H_{4(aq)} + Cl^-$	522	573
Environmentally revelant oxidants[a]*:*				
$O_{2(aq)}$	$O_2 + 4H^+ + 4e^-$	=> $2H_20$	1,270	1,277
Fe^{3+}	$Fe^{3+} + e^-$	=> Fe^{2+}	726	907
SO_4^{2-}	$SO_4^{2-} + 10H^+ + 8e^-$	=> $H_2S_{(aq)} + 4H_2O$	289	346
CO_2	$CO_2 + 12H^+ + 12e^-$	=> $CH_{4(aq)} + 4H_2O$	104	115

2.2. Oxidation

Oxidation is a process where the chlorinated solvents are oxidized to carbon dioxide or other benign compounds. Aerobic cometabolic oxidation of chlorinated ethenes requires the presence of oxygen as well as a carbon source as a primary substrate to induce the production of non-specific oxygenase by the organisms involved (McCarty & Semperini, 1994). Mineralization of TCE to CO_2 with no detectable accumulation of toxic intermediates has been reported during oxidation of substrate, such as methane, propene, methanol, ammonium, vinyl chloride or aromatic compound by a broad variety of microorganisms (Semprini, 1995; Olaniran *et al.*, 2004). Similarly, cometabolic oxidation of DCE has also been described with co-substrate, such as methane, toluene, propane or phenol (McCarty & Semperini, 1994). This mechanism has been successfully applied for engineered remediation (Semprini, 1997; Semprini *et al.*, 2001) and is considered to be of long-term significance for the transformation of chlorinated ethenes under field conditions. In the absence of co-substrates, the tendency for aerobic oxidation of chlorinated ethenes increases with decreasing number of chlorine atom of the molecule (Vogel *et al.*, 1987). For instance, *cis*-DCE and VC have been shown to be aerobically oxidized and to serve as the main carbon source for microbial growth (**Figure 1**) (Hartmans & Debont, 1992; Hartmans, 1995; Bradley & Chapelle, 1998; Bradley & Chapelle, 2000; Coleman *et al.*, 2002). However, mineralization of DCE and VC has been reported to decrease with increasing reducing condition. Moreover, the presence of humic acids has also been shown to lead to their efficient mineralization (Bradley & Chapelle, 1998; Bradley & Chapelle, 1998).

The demonstration of microorganisms capable of oxidising VC and DCE under anaerobic condition underscores a anaerobic alternative to reductive dechlorination, which is potentially relevant for *in situ* bioremediation at chlorinated ethenes contaminated field sites (Bradley & Chapelle, 1996; Bradley, 2000).

3. Assessing biodegradation during natural attenuation processes

Microbial degradation of chlorinated solvents in hydro-systems can occur naturally, supported by available electron donors, electron acceptors and nutrients, or through human intervention using enhanced or engineered bioremediation technologies (Scow & Hicks, 2005). Though previous efforts to remediate chlorinated ethenes contaminated sites were often limited to ineffective and costly methods such as pump-and-treat (Biswas *et al.*, 1992; Beeman *et al.*, 1994), the investigation of contaminant biotransformation has become of primordial importance.

Natural attenuation refers to the reduction in toxicity, mass and/or concentration of a contaminant without human intervention owing to both physico-chemical (e.g. dilution, sorption and precipitation) and biological processes (biodegradation) (Roling & van Verseveld, 2002). The concept of natural attenuation is operationalized by the Monitored Natural Attenuation (MNA), also referred to as intrinsic or passive remediation (USEPA_ORD, 1998; US_NRC, 2000; Grandel & Dahmke, 2004; Rittmann, 2004). The viability of MNA as a remediation option is based on a phased approach, which generally relies on several lines of evidence. These lines of evidence incorporate data relative to 1) the reduction in contaminant mass or concentration with time, 2) hydrogeochemical and geochemical changes providing indirect evidence of contaminant transformation (section 3.1.), and 3) *in situ* or microcosm studies providing direct evidence of biodegradation (Scow & Hicks, 2005; SRNL, 2006). The third line of evidence generally relies on various complementary investigations, such as culture-dependent and independent microbial analysis (section 3.2.) and/or compound-specific isotope analysis (section 3.3.).

3.1. Hydrogeochemical analysis

The combined evaluation of footprints of biogeochemical reactions and the variation in contaminant and metabolites concentrations in groundwater and soil systems is often a prerequisite for understanding the behaviour of a contaminant at a field site. Indeed, as hydrological and geochemical conditions in aquifers change, the dominant terminal electron-accepting processes (TEAPs) may shift, potentially resulting in different rates of degradation or the organic contaminant (NRC, 1993). Therefore, the chemical framework affecting the transformation of the targeted contaminants needs to be evaluated for interpreting system-scale processes with respect to natural attenuation potential (Reddy & DAngelo, 1997). Most likely the redox activity in the field is limited by one or more factors, such as transfer, availability of electron donors and acceptors, the thermodynamic characteristics of the processes, and microbial kinetics (Christensen *et al.*, 2000). Because groundwater systems can be isolated from the atmosphere, O_2 tends to be consumed along aquifer flow paths. Under anoxic conditions, terminal electron-accepting processes follows an order of electron acceptor utilization that is driven by the next most energetically favourable naturally available electron acceptor $–NO_3^- > Mn(IV) > Fe(III) > SO_4^{2-} > CO_2$. This conceptual ecological succession may lead to the segregation of redox processes into zones or periods where a single TEAP tends to dominate (Lovley & Goodwin, 1988). This phenomenon may then lead to the formation of hydrochemical gradients over both spatial and temporal scales. The sequential nature of electron acceptor utilization and the segregation of zones where TEAPs predominate allow delineating ambient redox processes in groundwater systems. For instance, biodegradation in aquifers is often evaluated based on redox-sensitive constituents, which are characteristic of particular ongoing processes. These include the concentration of dissolved electron acceptors, mainly O_2, NO_3^-, and SO_4^{2-}, or the reduced products of electron acceptors utilization, such as NH_4^+, HS^-, Fe^{2+}, and CH_4 (Chapelle & Lovley, 1992; McMahon & Chapelle, 2008). However, hydrogeological and biogeochemical micro- and macro-heterogeneities in contaminated aquifers often result in varying local reaction conditions. Consequently, groundwater flow segments may pass zones of different reaction rates with respect to contaminant transformation (Cozzarelli *et al.*, 1999). Redox heterogeneity possibly affecting the fate and transport of natural and anthropogenenic contaminants may also be reflected in water samples indicating mixed redox processes (McMahon & Chapelle, 2008). Consequently, interpretation of contaminant degradation processes controlling non-uniform contaminant plumes in geochemically heterogeneous aquifers may be confounded (Wilson *et al.*, 2004; Kopinke *et al.*, 2005).

While the microbial potential for various major redox processes is generally present in contaminated aquifer plumes, TEAPs also can be determined by measuring microbial activity (Cozzarelli *et al.*, 2000; Weiss & Cozzarelli, 2008).

3.2. Microbial analysis

Until the relatively recent development of rapid and cost-efficient molecular techniques for microbial identification, little was know regarding relevant, intrinsic microbiological processes involved in common chlorinated solvents biodegradation reactions. Typical research focuses on the identities and functions of the microorganisms, their physio-ecological requirements, and/or development of techniques for stimulating biodegradation reactions for remediation purposes. An overview of possible microbiological and molecular techniques that might be incorporated into subsurface environment biogeochemical studies is provided in **Figure 2**. For instance, the potential of indigenous microbial populations for degrading contaminants of interest can be investigated by the use of microcosm experiments (Aulenta *et al.*, 2005; Nijenhuis *et al.*, 2007). This approach entails advantages to qualitatively illustrate important processes controlling the fate of organic contaminants and to estimate constant rates associated with contaminant transformation. However, investigation relying solely on cultivation methods may not reflect the composition and activity of the *in situ* microbial community, as a large portion of the subsurface bacteria are viable but contain yet not culturable cells (Roszak & Colwell, 1987; Amann *et al.*, 1995; Ferrari & Hollibaugh, 1999).

Complementarily, cultivation-independent methods are applied to identify enzymes, microbial species and community structures of organic chemicals degrading microorganisms in subsurface environments (Richardson *et al.*, 2002). Such methods do not rely on the cultivability of an organism, which potentially broads the proportion of the microbial community that can be targeted. Nucleic acid techniques include the sequencing of DNA or RNA fragments to determine the identity of members of a microbial community, the detection and enumeration of specific genes sequences using e.g. the polymerase chain reaction (PCR), and the molecular fingerprinting of the microbial community to determine relative abundance and diversity (e.g. Madsen, 2000; Spiegelman *et al.*, 2005). In particular, 16S rRNA-targeting techniques may yield significant information regarding the presence and structure of *a priori* unknown degrading microbial populations and communities directly from water samples (Eyers *et al.*, 2004; Feris *et al.*, 2004; Nocker *et al.*, 2007). Studying the phylogenetic diversity, composition or/and structure of indigenous microbial communities using culture independent molecular approaches may provide key-information about the functioning of contaminated hydro-systems (Feris *et al.*, 2004; Zhuang *et al.*, 2005; Rahm *et al.*, 2006; Himmelheber *et al.*, 2007). Furthermore, different levels of the intrinsic microbial community associated with *in situ* biotransformation of chlorinated solvent can be explored. For example, some bacteria affiliated to the genera *Dehalobacter* spp., *Desulfitobacterium* spp., *Dehalococcoides* spp. or *Geobacter* spp. can be used as indicators of potential dechlorinating activity (Löffler *et al.*, 2000; Hendrickson *et al.*, 2002; Duhamel & Edwards, 2006). While the functional correlation between the presence of key-microorganisms and their activity is challenging, new techniques are being developed to address the relationship between structure and function of target communities in contaminated aquatic systems (Weiss & Cozzarelli, 2008). For instance, the use of labeled-substrate in tracer experiments or stable isotope probing techniques (DNA- or RNA-SIP) using labeled compounds would permit identifying members of the communities that are actively involved in the metabolic cycling of organic chemicals (Evershed *et al.*, 2006; Kreuzer-Martin, 2007; Neufeld *et al.*, 2007). Radioactive (^{14}C) or stable isotope (^{13}C) tracers may be used to track the partitioning, transformation and mineralization of organic contaminants. Stable isotope labelling approaches based on adding a ^{13}C-labelled substrate to a microcosm, sediment, soil or culture medium, then following its fate over space and/or time. Incorporation of ^{13}C-labelled carbon derived from the target contaminant into bacterial molecules, such as phospholipids fatty acids (PLFAs), amino- or nucleic acids substantiate *in situ* degradation, and the detection of ^{13}C-labelled degradation metabolites may be indicative for a particular degradation pathway (e.g. Stelzer *et al.*, 2006). For instance, if the target contaminant is used as an carbon source, ^{13}C-labelled microbial PLFAs may confirm the presence of an active microbial community, and labeling of a specific subset of PLFAs may highlight members of the microbial community consuming the provided substrate (Evershed *et al.*, 2006). However, the use of this approach is limited by the requirement of carbon incorporation into the bacterial biomass. Therefore, it can not be used to track microorganism involved into the reductive dechlorination of chlorinated solvents, as in this case, the contaminant is used as an electron acceptor.

Tracer experiments can be efficiently use together with measurements of the natural isotopic composition of the target contaminant by means of compound-specific isotope analysis (CSIA) to document and characterize biodegradation and pathways over space and time in complex system.

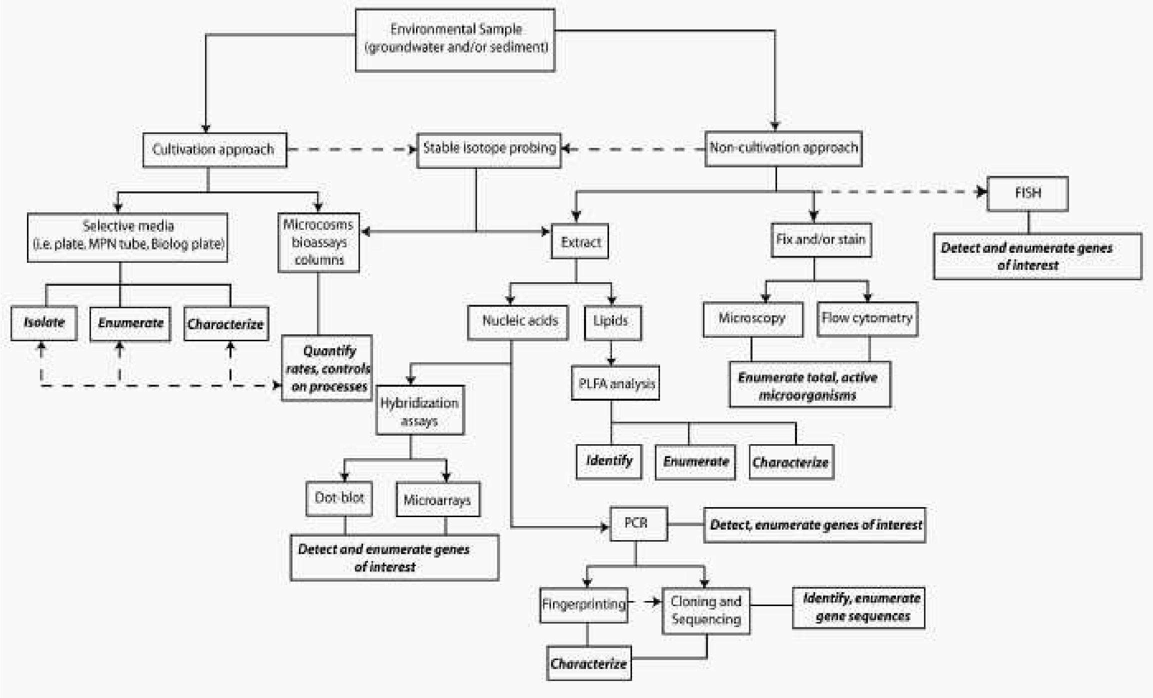

Figure 2: Flowchart of possible microbial and molecular techniques which can be incorporated in subsurface biogeochemical investigations (Weiss & Cozzarelli, 2008).

3.3. Compound Specific Isotope Analysis

Compound-specific isotope analysis (CSIA) has been shown to be an effective tool to evaluate *in situ* biodegradation processes (Meckenstock *et al.*, 2004). This method relies on the different reaction rates for molecules with light and heavy isotopes, which may generate an isotope shift in the non-degraded residual substrate fraction of contaminant and/or in the corresponding degradation products. The origin of the isotopic effect and fractionation is described in section 3.3.1. This method has been widely used to confirm, assess and/or quantify *in situ* biodegradation of various types of organic chemicals such as chlorinated ethene, BTEX or fuel oxygenates based on carbon and/or hydrogen isotopic analyses (Hunkeler & Aravena, 2002; Mancini *et al.*, 2003; Richnow *et al.*, 2003; Fischer *et al.*, 2007; Rosell *et al.*, 2007). Several laboratory studies have shown that biological degradation of chlorinated solvents can involve reproducible kinetic isotope effects, producing systematic changes in the $\delta^{13}C$ values of the residual contaminant fraction (Hunkeler *et al.*, 1999; Bloom *et al.*, 2000; Slater *et al.*, 2000; Sherwood Lollar *et al.*, 2001; Barth *et al.*, 2002; Kaschl *et al.*, 2005; Nijenhuis *et al.*, 2007). Under field conditions, isotopic enrichment is determined by comparing the isotope signals from a sample near the source to a sample further downstream. Fractionation factors determined in the laboratory may enable qualitative and quantitative assessment of *in situ* biodegradation (Meckenstock *et al.*, 2004). Isotopic investigations have also been reported in pilot field studies to estimate biodegradation rates of chlorinated ethenes (Sherwood Lollar *et al.*, 2001; Kirtland *et al.*, 2003; Vieth *et al.*, 2003).

However, the magnitude of isotope fractionation during enzymatic reactions mainly depends on the nature of the chemical bond cleavage, the reaction mechanism, and the structure of the transition state of the reaction and kinetic limitation of reaction steps previous bond cleavage (Northrop, 1981). Since the bonds and reaction mechanisms may differ according to the biochemical reaction involved in organic compound degradation, the extent of isotopic fractionation may substantially vary according to the degradation pathway (discussed in section 3.3.2.) (Cook, 1991). The isotope fractionation occurring during microbial degradation can be described by means of the Rayleigh equation following analysis of both the concentrations and isotope ratios of the residual, not yet degraded substrate fraction (section 3.3.3.).

3.3.1. The isotope effect and fractionation

The number of protons and neutrons constituting the nucleus of an atom define the element and the isotope, respectively. For example, an atom of carbon containing a seventh neutron, denoted ^{13}C and termed heavy isotope, is about 1 g per mol heavier than its predominant, lighter ^{12}C counterpart. An additional neutron in the nucleus of an atom confers slightly different physical properties to molecules bearing this atom and affects its vibrational energy. Indeed, at the chemical bond level, a change in masses of the atoms involved may influence the vibration frequency.

Because the free energy required for a reaction depends on the vibration frequency, the small discrepancy in frequency of vibration between isotopically different chemical species may generate different levels of activation energy necessary for the reaction to proceed. For instance, the presence of a heavier mass induced by a ^{13}C atom decreases the vibrational energy, which in turn increases the free energy difference between the reaction product and the transition state. Consequently, the reaction rate between heavy and light molecules, referred to as kinetic isotope effect, varies and the chemical bonds formed by isotopically lighter species theoretically react at faster rate than bonds formed by heavy isotopomers. If biotic processes are responsible for contaminant degradation, the remaining fraction would then be enriched in molecules containing the heavy isotopes and the daughter product is expected to be depleted in heavy isotopes (Meckenstock *et al.*, 2004).

3.3.2. Factors governing the isotope fractionation upon enzymatic activity

Based on the influence of isotope masses, of bonding partner(s) and of changes in bond strength, it is possible to estimate approximate *maximum* kinetic isotope effects (KIE) ("semiclassical Streitwieser limits") for the breakage of typical chemical bonds. Streitwieser Limits have, however, only semiquantitative character. Occasionally, the bond conversion is preceded by a not or only slightly fractionating process, such as transport to reactive site or formation of enzyme-substrate complexes in biotransformations. If the reverse step of this preceding process is very slow, every substrate molecule that reaches the reactive site will be converted, irrespective of its isotope composition. Hence, no or only minor isotopic discrimination will be observed in the remaining substrate. Consequently, the measured *apparent* KIE (AKIE) may differ from the *intrinsic* KIE in the actual bond conversion (Elsner et al., 2005).

The extent of the kinetic stable isotope fractionation of substrates in microbial degradation processes mainly depends on i) the rate limitation of reaction steps preceding bond cleavage, ii) the nature of the biochemical reaction mechanism and iii) the position of the heavy isotope within the molecule (Northrop, 1981; Galimov, 1985; Cook, 1991).
Rate-limiting reaction steps preceding the isotope sensitive bond cleavage may lower the extent of the "apparent" isotope effect compared to the strict biochemical cleavage reaction (Northrop, 1981). Indeed, rate limitations associated with e.g. substrate mass transfer towards the cell, uptake of substrate by the cell, transport of substrate within the cell or formation of the enzyme-substrate complex may affect the reaction step responsible of the isotope discrimination. As a consequence, the described rate-limitation in the reaction may cause significant variations of the magnitude of isotope fractionation during similar degradation pathways involving the same molecules (Nijenhuis *et al.*, 2005). Moreover, the specific structure of the enzyme that affects the catalytic properties has also been reported to affect the isotope fractionation (Nikolausz *et al.*, 2006).

Regarding the relative position of the heavy isotope in a molecule, isotope effects are referred to as “primary” if the heavy isotope is directly involved in the rate limiting step of cleavage or in formation of the chemical bond, and as “secondary” if it is located on a non-reactive position. Primary isotope effects have been reported to be significant, whereas secondary isotope effects are typically one or two order of magnitude lower (Galimov, 1985). However, various reactive positions may exist in a single molecule and the size of the molecule may consequently influence the extent of the resulting isotope fractionation (Elsner *et al.*, 2005). However, dilution of the isotopic effect with increasing molecule size may hinder the application of CSIA for large molecules.

Conversely, non-destructive processes acting on the whole chlorinated compound molecule, such as advective and dispersive transport, volatilization, sorption are likely to cause much lower changes in isotope composition than chemical and biochemical reactions. These changes are often under detection limits of commonly used analytical methods upon measurement of carbon or hydrogen isotopic composition (Harrington *et al.*, 1996; Huang *et al.*, 1999; Poulson & Drever, 1999; Slater *et al.*, 2001; Schuth *et al.*, 2003; Elsner *et al.*, 2005)

3.3.3. Notation and mathematical description of isotope fractionation

Isotope ratios of a given compound can be measured mass spectrometrically and they are reported as difference in per mil with respect to international standards and expressed in δ units (Hoefs, 1997). Carbon isotope compositions are reported relative to the Vienna Pee Dee Belemnitee standard (V-PDB, IAEA-Vienna) and can be calculated from equation 1 (Coplen *et al.*, 2006).

$$\delta^{13}\mathrm{C}\,[‰] = \frac{\left(\frac{^{13}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{sample}} - \left(\frac{^{13}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{VPDB-Standard}}}{\left(\frac{^{13}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{VPDB-Standard}}} \times 1000 \qquad 1)$$

The progressive partitioning of the isotope composition of the substrate expressed in substrate concentration changes and isotope fractionation can be described by equation 2, (Mariotti *et al.*, 1981; Clark & Fritz, 1997):

$$\frac{R}{R_0} = f^{(\alpha-1)} \qquad 2)$$

Where R and R_0 are the ratio and the initial ratio of heavy to light isotope of the substrate, respectively and f represents the remaining fraction of the substrate, and α the isotope fractionation factor, expressing the magnitude of isotope fractionation. The equation is derived from the classical Rayleigh equation, which describes fractional distillation of mixed liquids (Rayleigh, 1896) and is valid for low abundance of the heavy isotope (Hunkeler & Aravena, 2002). The fractionation factor is more conveniently expressed as enrichment factor (ε) in the equation 3:

$$\varepsilon[‰] = (\alpha - 1) \times 1000 \qquad 3)$$

3.4. Integrative approaches to assess biodegradation

Because microbial and chemical processes interact to affect the chemical evolution of groundwater, which may in turn affect the biodegradation processes, the study of aquifer biogeochemistry has recently broadened into a multidisciplinary pursuit (Haack & Bekins, 2000). Comprehensive experimental designs incorporating various geochemical and microbiological techniques are often used to enhance the understanding of contaminated aquifers biogeochemistry. Microbial transformation processes of chlorinated solvents can be documented at contaminated site by mass loss of contaminant, production of metabolites, as well as changes in redox-sensitive species. The additional use of CSIA of reactants and products to characterize contaminant transformation generally implies fair hydrological and hydrochemical knowledge of the investigated system and is generally coupled with a hydrogeochemical characterization (Grandel & Dahmke, 2004). However, few studies combine these observations with molecular techniques to investigate members of the indigenous microbial community and what factors are associated with their *in situ* distribution and activity (e.g. Roling *et al.*, 2001; Pombo *et al.*, 2005; Kleikemper *et al.*, 2005). This combination appears highly relevant in evaluating destructive contaminant removal, which is inherently dependent on the composition and function of the microbial community (Weiss & Cozzarelli, 2008). Though processes such as sorption, volatilization or dilution contribute to contaminant natural attenuation, *in situ* biodegradation is the only process leading to destructive removal of contaminants in the environment. Therefore, quantitative and qualitative evidence of efficient biodegradation processes is generally a prerequisite to use Monitored Natural Attenuation as a remediation alternative. One key objective is then to evaluate the functional linkage between the microbiology of the site and the *in situ* degradation processes. Because the structure and function of microbial communities exhibit significant spatial and temporal variability, collecting microbial data may complement geochemical data (Cozzarelli & Weiss, 2007) to substantiate the interpretation of processes.

Several recent studies have been focused on understanding the relationship between the hydrogeochemistry and the microbial community in landfill leachate (Roling *et al.*, 2000; Lin *et al.*, 2007) as wells as hydrocarbon- (Kleikemper *et al.*, 2005; Pombo *et al.*, 2005), chlorinated compounds (Davis *et al.*, 2002) or coal tar-contaminated (Bakermans *et al.*, 2002; Bakermans & Madsen, 2002) aquifers subjected to *in situ* biodegradation. In a general sense, these studies relying on a combination of methods could generally demonstrate the occurrence of contaminant degradation, while identifying the potential contribution of the microbial community to the biogeochemical processes controlling the contaminant fate and/or the microorganisms specifically involved in the degradation process. Recently, the use of *in situ* microcosm amended with ^{13}C-labelled compounds in BTEX-contaminated aquifers allowed to reliably demonstrate the active role of different bacterial populations in degrading contaminant, which could not be discerned by geochemical or culture-based methods (Geyer *et al.*, 2005; Stelzer *et al.*, 2006). The combination of molecular methods with the analysis of the contaminant isotopic composition allow to tie biodegradation processes to the responsible organisms to permit a deeper exploration of the microbial ecology of organisms associated with contaminant degradation.

Microbiological analysis can be coupled with geochemistry data and isotope signals to gain additional insight on on-going natural attenuation processes. This approach may enable a more robust evaluation of subsurface biogeochemical processes at the investigated systems. For such purpose, recent work has demonstrated the relevance of multivariate statistical methods for discerning relationships in complex data sets and for developing hypotheses regarding the relationship between community structure and measured environmental variables (Ramette, 2007). For instance, multivariate statistical analysis of DNA fingerprints has been used to relate microbial community structure to contaminant concentration and redox processes (Cozzarelli *et al.*, 2000; Roling *et al.*, 2001; McGuire *et al.*, 2003; Haack *et al.*, 2004; Mouser *et al.*, 2005). However, little is known about the relationship between indigenous microbial communities and guilds associated with contaminant degradation and ambient hydrochemical conditions in hydro-systems contaminated with chlorinated compounds. Furthermore, the distribution of hydrological and hydrochemical processes over both spatial and temporal scales influence degradation reactions, and thus should be taken into account when assessing *in situ* biodegradation in complex system.

4. Aim and approach of the thesis

This thesis aimed at gathering knowledge about *in situ* biodegradation of chlorinated solvents and associated biogeochemical processes within heterogeneous hydro-systems. The specific objective of this thesis were i) to develop and implement an integrative approach to demonstrate *in situ* degradation of toxic reductive dechlorination intermediates such as dichloroethenes (DCE), vinyl chloride (VC) or monochlorobenzene (CB), ii) to investigate the biogeochemical development of model wetlands treating dechlorination intermediate compounds, iii) to track the prevailing degradation pathways within the investigated systems, and iv) to investigate the relationships between the microbial community and ambient hydrochemical conditions.

The presented studies focused on the investigation of biogeochemical processes in both model constructed wetlands for interfaces between contaminated aquifer and surface water and anoxic aquifers contaminated with chlorinated solvents. This research has been motivated by the very limited knowledge on biogeochemical processes associated with natural attenuation of chlorinated solvents in subsurface systems as well as the potential interest of intrinsic wetland system characteristics for the elimination of chlorinated solvents in bioremediation approaches. An integrative approach combining Compound Specific Isotope Analysis (CSIA) with hydrogeochemical and microbial analyses was applied to investigate both types of systems. The approach was completed by a comprehensive analysis of these outputs by means of multivariate statistics, to gain additional insight into the development of observed biogeochemical processes over spatial and/or temporal scales.

The following section of **Chapter 1** (Section 1.2; accepted in *Chemosphere, 2008*) reviews the recent progresses made towards understanding how mechanisms attributed to various organic chemicals removal interact to form a functioning treatment wetland. Furthermore, possible complementary techniques and integrative approaches to follow up *in situ* biodegradation processes are discussed.

Chapter 2 focuses on the assessment of biogeochemical processes in model constructed wetlands treating chlorinated solvents using the described integrative approach. First, an experiment in an outdoor horizontal sub-surface flow constructed wetland treating contaminated groundwater aimed at characterizing the *in situ* biodegradation of chlorobenzene based on isotope composition analysis and *in situ* microcosm experiments was used (Section 2.1; *Environmental Pollution* 148: 428-437, 2007). Second, an experiment carried out in a model horizontal sub-surface flow wetland aimed at evaluating the spatial and temporal variability of *cis*- and *trans*-1,2-dichloroethenes degradation pathway coupling a hydrogeochemical analysis and CSIA (Section 2.2; *Environmental Science and Technology*, in Press, 2008). In addition, the microbial community structures variability and microbial composition in this last system were evaluated using molecular techniques to further substantiate the assessment of on-going processes (Section 2.3; to be submitted to *Water Research*).

Chapter 3 tackles the *in situ* biodegradation and the associated microbiology as well as hydrogeochemical conditions in anoxic aquifers contaminated with chlorinated solvents. This was achieved based on three field experiments. In the first field site, the investigation focused on the *in situ* degradation of chlorobenzene using several lines of evidence (Section 3.1; submitted to *Environmental Pollution*, 2008). In a second field site, the investigation aimed at assessing the occurrence of chlorinated ethenes biodegradation and associated microbial community at the plume scale, in a hydrogeologically complex groundwater system (Section 3.2; *Water Research* 42: 871-882, 2008). Based on the knowledge gathered in this last experiment, a third field investigation was carried out in the same field with the goal to assess at higher resolution the variability of the bacterial community structures, hydrogeochemical indicators, and the carbon stable isotope composition of chlorinated ethenes over time at a multilevel monitoring well located at the plume fringe (Section 3.3; submitted to *FEMS Microbiology Ecology*, 2008).

Chapter 4 synthesizes the main findings of this research and evaluates them with respect to current understanding on biogeochemical processes in contaminated groundwater and wetland systems and to the application of integrative approaches for the assessment of chlorinated solvents biodegradation in environmental studies. Finally, future research lines in the investigation of biogeochemical processes occurring in chlorinated solvent contaminated systems are drawn.

5. References

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Section 2: Monitoring and assessing processes of organic contaminant removal in constructed wetlands

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Abstract
Physical, chemical and biological processes interact and work in concert during attenuation of organic chemicals in wetland systems. This review summarizes the recent progress made towards understanding how the various mechanisms attributed to organic chemicals removal interact to form a functioning wetland. We also discuss the main degradation pathways for different groups of contaminants and examine some of the key characteristics of constructed wetlands that control the removal of organic chemicals. Furthermore, we address possible comprehensive approaches and recent techniques to follow up *in situ* processes within the system, especially those involved in the biodegradation processes.

1. Introduction

Constructed wetland systems may be converted natural, or constructed shallow, ecosystems designed to capitalize on intrinsic physical, chemical, and biological processes for the primary purpose of water quality improvement (Hammer, 1989). Constructed wetlands consist of four main compartments: plants, sediment and soil, microbial biomass and an aqueous phase loaded with the chemicals and typically including beds filled with poorly drained graded medium and aquatic plants. These systems are generally coupled to a drainfield or polishing pond, engineered to return the filtered water back to the environment. There are two basic designs for constructed wetlands whose primary purpose is wastewater treatment: subsurface-flow and surface-flow. In the subsurface-flow wetlands (SSF), the water may flow horizontally (parallel to the surface) or vertically (from the planted layer down) through the matrix and out of the system, whereas the water moves above the substrate surface in surface-flow wetlands (SF). The application and reliability of these systems during domestic sewage treatment has previously been reviewed (Cooper et al., 1996; Sundaravadivel and Vigneswaran, 2001; Griffin, 2003). In recent years however, the applicability of constructed wetland technology (CWT) as a cost-effective and operational alternative to conventional technologies for the elimination of various contaminants of industrial relevance has been explored (Kadlec et al., 2000; Pardue, 2002; WetPol, 2007). In particular, developments have focused on organic chemicals, defined as undesirable substances not normally present in surface or groundwater, or naturally occurring substances at an unusually high concentration and displaying harmful environmental effects. Though, this technology has the potential to become an important remediation strategy, its successful application remains challenging. Indeed, numerous environmental factors and system-inherent processes influence organic contaminant removal efficiency and may complicate the maintenance of acceptable levels of system control. Although several approaches and methods have been described in the literature, the physicochemical and biogeochemical processes associated with the transformation of organic chemicals in constructed wetlands are rarely evaluated. The results of a short literature survey are provided in Table 1. The majority of studies in constructed wetlands are orientated towards efficiency or performance (>20%), whereas studies integrating microbial, molecular or microcosm investigations account in total for about 11% of the total. The investigation of processes, such as degradation, sorption, and volatilization accounts for another 18%, whereas studies focusing on specific compartments (biofilms, sediment, rhizosphere) amount to less than 25% of the contributions. This brief survey permits depicting overall trends (**Table**

1), however, it is likely that many studies on related topics do not contain the researched keyword in their title or abstract and have therefore not been included.

Table 1: Output from a literature search performed using Thomson ISI research tool, with the following variables (Doc type: all document type; language: all languages; databases: SCI-EXPANDED, SSCI, A&HCI; Timespan: 1957-2007) on November 13, 2007. Only the titles and abstracts of the articles were searched. Each keyword (wetland, constructed wetland) was additionally combined with a designation embedded in the following categories: wetland type, type of investigation, processes and compartment. The values are provided in percent of the corresponding total number of publications enumerated for each keyword, the absolute values are given in parentheses.

	Keywords	**Natural Wetland**		**Constructed Wetland**	
		Relative proportion (%)	*Number*	*Relative proportion (%)*	*Number*
Wetland type	Free-surface	1.2	191	5	102
	Sub-surface	0.8	124	2.3	47
Type of Investigation	Performance	5.2	835	22.9	469
	Efficiency	4.9	786	19.1	391
	Microcosm	0.5	81	1.5	30
	Molecular	1.1	176	0.9	19
	Process	5.4	872	8.2	168
Processes	Degradation	3.8	613	5.3	109
	Sorption	1.7	273	5.4	110
	Volatilization	0.7	119	2	40
	Bacterial	2.3	364	5.1	105
Compartment	Microbial	4.4	714	8.1	166
	Plant	21	3’392	26	534
	Sediment	11.4	1’846	12.9	265
	Rhizosphere	1.5	249	3.2	65
	Total number		16’164		2’050

Future challenges will surely consist of optimizing CWT for more sustainable and reliable treatment of both industrial and agricultural organic contaminants. This would necessarily imply that the ability to assess design characteristics has reached an acceptable level of understanding, and reliable predictions about the mechanisms associated with organic contaminants removal could be performed. This review focuses on key processes determining the fate of organic chemicals in constructed wetlands and aims to improve their assessment in pilot studies and active treatment plants. It mainly focuses on selected categories of contaminants of worldwide relevance, namely the volatile organic compounds (VOCs), the organochlorines, the PAHs, as well as some pharmaceuticals. In the first part, main physicochemical and biological mechanisms contributing to organic chemical removal in constructed wetland are successively reviewed. Second, relevant characteristics of wetland systems that determine the feasibility and efficiency of organic chemical removal are briefly discussed. The final part addresses approaches to assess processes leading to contaminant depletion in constructed wetlands. It provides some insights into experimental designs necessary for process investigations and succinctly presents traditional and emerging methods that have been proven relevant. Overall, special emphasis is placed on degradation, as it generally represents an expected sink of organic chemicals in constructed wetlands.

2. Removal processes in constructed wetland

Several elimination pathways may occur in a complex constructed wetland system. Kadlec (1992) listed volatilization, photochemical oxidation, sedimentation, sorption and biological degradation as the major processes affecting the organic compound loads in wetlands. Additionally, processes such as plant uptake and phytovolatilization, contaminant accumulation and metabolic transformation may be relevant for some plants and organic chemicals (Susarla et al., 2002). The relative importance of a particular process can vary significantly, depending on the organic contaminant being treated, the wetland type (e.g. SSF or SF, horizontal flow (HF) or vertical flow (VF)) and operational design (e.g. retention time), the environmental conditions, the type of vegetation within the system, as well as the soil matrix. Clear treatment goals and an evaluation of the occurrence and extent of putative removal processes are preliminary requirements for defining appropriate design and operation parameters. This evaluation is particularly critical when targeting organic chemical treatments. The assessment of organic carbon removal in conventional wastewater treatment, mainly based on COD and BOD values, has been well documented since the early 1950s (Vymazal, 2005), but the treatment of organic chemicals in constructed wetlands is still at its infancy. Organic chemicals exhibit a wide range of physicochemical properties, numerous specific toxicity effects and often a degree of recalcitrance rarely encountered in common contaminants of domestic and agricultural sewage. Therefore, evaluating the physicochemical properties and biological effects of specific groups of organic chemicals with respect to their potential and observed fate in constructed wetlands may help refine artificial wetland design and operation modes. Physico-chemical properties for various organic contaminant groups of interest regarding constructed wetland treatment are listed in **Table 2**. The relationship between these physico-chemical characteristics and the fate of contaminants in constructed wetland systems is highlighted in the following sections.

Table 2: Range of values for selected physicochemical properties of various organic contaminant groups of interest regarding treatment in constructed wetland. Representative organic compounds are listed in the order of increasing water solubility. Superscript numbers associated with the organic compounds refer to the literature sources of the provided values for physicochemical properties. Superscript numbers associated with the potentially relevant processes in constructed wetlands for the mentioned compound refer to the literature concerning its treatment and/or behaviour in wetlands. (+) or (-) signs associated with specific removal processes are provided if the cited literature contains explicit information or estimates on the contribution of these removal process to overall contaminant losses from constructed wetland systems.

[a]Water solubility; [b]Vapour pressure; [c]Octanol-water partition coefficient; [d]Henry's law constant; [e]calculated. n.a.: not available.

Organic compound	**Physico-chemical properties**				**Expected processes in constructed wetlands**
	S_W[a] 25°C [mg/l]	**P_V[b] 25°C [hPa]**	**log Kow[c] (25°C)**	**H[d] 25°C [Pa*m^3/mol]**	
PCBs[1]	$5*10^{-8}$-5.9	$7*10^{-9}$-$5*10^{-2}$	3.9-9.6	0.9-97	Sorption(+), microbial degradation, plant uptake, accumulation & metabolism(-)[8-13]
PCDD, PCDF[1]	$4*10^{-7}$-4.2	$1*10^{-12}$-$3*10^{-4}$	4.8-11.3	0.3-12.6	Sorption (+), microbial degradation (hypothesised)[8]
PAH (3-6 rings)[1]	$1*10^{-4}$-16.1	$1*10^{-15}$-$1*10^{-2}$	3.6-7.6	0.1-24.4	Sorption(+), microbial degradation(+), plant uptake & metabolism(-)[10,14-16]
chlorobenzenes (3-6 Cl substituents)[1]	$4*10^{-3}$-52	$2*10^{-15}$ - 0.3	4-5.7	41-375	Sorption, sedimentation, microbial reductive dechlorination, volatilisation[17-19]
Fuels: kerosene C9-16, diesel C10-19, heavy fuel oil C20-70[2]	≤5 (20°C)	<$1*10^{-4}$-35 (21°C	3.3-7.1	6-749805 (20)	Microbial degradation, sorption & sedimentation, volatilisation[20-26]
Ibuprofen[3-5]	21-49	$6*10^{-5}$-$3*10^{-4}$	3.5 (pH8: 0.5)	$2*10^{-2}$ (cal.)[e]	Microbial degradation, sorption[27-29]
Carbamazepin [3-6]	17.7	$3*10^{-7}$	2.7	$2*10^{-6}$	Sorption[28]
Naproxen[4]	15.9	n.a.	3.3 (pH8: -0.3)	n.a.	Microbial degradation, sorption (protonated form)[29]
Ketoprofen[4]	51	n.a.	3.6 (pH8: -0.4)	n.a.	Sorption (protonated form)[29]
Diclofenac[3]	2.4	$8*10^{-8}$ (cal.)[e]	4.5 (pH8: 0.7)	$5*10^{-7}$ (cal.)[e]	Sorption (protonated form)[29]
gasoline C4-12[2,7]	120	35-90 (20°C)	2.1-4.9	5-334373	Microbial degradation, volatilisation
chlorobenzenes (1-2 Cl substituents)[1]	31 - 503	1.3-15.9	2.8-3.5	159-398	Microbial degradation, volatilisation(+), sorption[18 19,30-32]
BTEX[1]	131-2167	8.8-131	1.6-3.4	272-959	Microbial degradation, volatilisation(+), sorption[30,33-36]
Chlorinated solvents (1-2 C-atoms)[1]	150-20000	4.7-5746	0.6-3.4	25-3080	Plant uptake & metabolism(+), volatilisation(+), phytovolatilisation, microbial degradation(-), sorption[36-43]
MTBE[1]	35500	327	0.9	72	Plant uptake & volatilisation(+), plant & microbial degradation(-)[44-45]
Phenol, Cresols[1]	21000-87000	0.2-0.5	1.5-2	$3*10^{-2}$-0.3	Microbial degradation(+), sorption, plant uptake (-), volatilisation(-)[46-48]
Acetone[1]	miscible	305-308	-0.2	3-4	Microbial degradation(+), phytovolatilisation(-), sorption[49]

[1]Mackay et al., 2006; [2]ATSDR 1995; Connecticut College Office of Environmental Health & Safety, 2004; NIST, 2006; Marathon Petroleum Company, 2006; [3]US National Library of Medicine, 2007;

[4]Wishart et al., 2007; [5]Mersmann et al., 2002; [6]Doll, 2004; [7]Hess Corporation, 2007; OMV 2005; Poulsen et al. 1992; [8]Campanella et al., 2002; [9]Donnelly and Fletcher, 1995; [10]Olson et al., 2003; [11]Chu et al., 2006a; [12]Chu et al., 2006b; [13]Moza et al., 1974; [14]Machate et al., 1997; [15]Giraud et al., 2001; [16]Harms et al., 2003; [17]Pardue et al., 1993; [18]Leppich, 1999; [19]Jackson, 1999; [20]Thurston, 1999; [21]Salmon et al., 1998; [22]Wright et al., 1997; [23]Kadlec, 1992; [24]Groudeva et al., 2001; [25]Boopathy, 2003; [26]Omari et al., 2003; [27]Gross et al., 2004; [28]Matamoros et al., 2006; [29]Matamoros and Bayona, 2005; [30]Keefe et al., 2004; [31]Lee et al., 2003; [32]MacLeod et al., 1999; [33]Bedessem et al., 2007; [34]Wallace, 2002; [35]Wallace and Kadlec, 2005; [36]Williams, 2002; [37]Wang et al., 2004; [38]Bankston et al., 2002; [39]Pardue et al., 2002; [40]Ma and Burken, 2003; [41]Amon et al., 2007; [42]Kassenga et al., 2003; [43]Kassenga et al., 2004; [44]Hong et al., 2001; [45]Winnike-McMillan et al., 2003; [46]Polprasert et al., 1996; [47]Abira et al., 2005; [48]Wood et al., 2000; [49]Grove and Stein, 2005.

2.1. Non-destructive processes

The mere reduction of contaminant concentration within the aqueous phase via non-destructive partitioning processes, such as sorption and volatilization, may only relocate the contamination. Therefore, the mass transfer of contaminants from the aqueous phase to other compartments (soil and atmosphere) has to be considered carefully when evaluating potential environmental hazards.

2.1.1. Volatilization and phytovolatilization

In addition to direct contaminant emission from the water phase to the atmosphere (volatilization), some wetland plants take up contaminants through the root system and transfer them to the atmosphere via their transpiration stream, in a process referred to as phytovolatilization (Hong et al, 2001; Ma and Burken, 2003). In the case of helophytes, this transfer may also occur via the aerenchymatous tissues (Pardue, 2002).

VOCs are defined as substances with a vapor pressure greater than 2.7 hPa at 25 °C (NPI, 2007). The Henry coefficient (H) is expected to be a valuable indicator for predicting volatilization behaviour of organic contaminants. It comprehensively describes the transfer of volatile contaminants from the water phase to the atmosphere. In unsaturated soil zones, additionally the diffusion transport determines effective VOCs emission. A high Henry coefficient is a characteristic of a number of organic contaminant groups frequently treated in constructed wetlands such as chlorinated solvents, BTEX (benzene, toluene, ethylbenzene, xylene) compounds and MTBE (methyl tert-butyl ether) (**Table 2**).

Direct volatilization and phytovolatilization are expected to be moderate for hydrophilic compounds such as acetone (Grove and Stein, 2005) and phenol (Polprasert et al., 1996). In contrast, volatilization may be an important removal process for volatile hydrophobic compounds such as lower chlorinated benzenes (MacLeod et al., 1999; Keefe et al., 2004), chlorinated ethenes (Bankston et al., 2002; Ma and Burken, 2003) and BTEX compounds (Wallace, 2002). In constructed wetland treatment of MTBE, which is characterized by a moderate Henry coefficient, high water solubility and additionally by strong recalcitrance under anaerobic conditions (Deeb et al., 2000), various processes may result in the release of the compound to the atmosphere. Uptake by the transpiration stream and subsequent phytovolatilization through the stems and leaves may be a major removal process and significantly contribute to contaminant mass loss; additionally, the vegetation increases the upward movement of water into the unsaturated zone, where enhanced volatilization occurs (Hong et al., 2001; Winnike-McMillan et al., 2003). If the atmospheric half-lives of VOCs are reasonably short like the one for MTBE (three days at 25°C (Winnike-McMillan et al., 2003), and the toxicological risk is assumed to be low, the water-to-atmosphere contaminant transfer occurring in wetlands may constitute a possible remediation option. However, volatilization of VOCs may also lead to air pollution and to a dispersal of the contaminant in the environment. This fact and the lack of reliable risk assessment currently discourages regulatory acceptance of phytoremediation as a strategy for VOCs removal (McCutcheon and Rock, 2001).

Phytovolatilization may be of particular relevance in SSF systems, where direct volatilization is restrained due to slow diffusion rates of contaminants through the unsaturated zone as well as laminar flow in water saturated soils zones that may result in relatively low mass transfers.

Direct contaminant volatilization is expected to be more pronounced in SF wetlands, as water remains in direct contact with the atmosphere (Kadlec and Wallace, 2008).

2.1.2. Plant uptake and phytoaccumulation

Uptake of organic chemicals into plant tissue is predominantly affected by the lipophilic nature of organic pollutants, which can be characterized by the octanol water partition coefficient (K_{ow}) (Ryan et al., 1988). Hydrophobic organics with a log $K_{ow} > 4$ are believed not to be significantly taken up through the plant cell membrane because of significant retention within the root epidermis (Trapp, 1995), but exceptions may occur. Reed and rice plants have been shown to take up highly lipophilic PCBs (Chu et al., 2006a). Only under the condition of significant contaminant uptake by the vegetation, processes like phytovolatilization, phytoaccumulation and plant metabolic transformation have to be considered as potentially significant removal processes for organic contaminants.

Phytoaccumulation occurs when the sequestered contaminants are not degraded or emitted rapidly and completely in the plant, resulting in an accumulation within the plant tissue (Susarla et al., 2002). The accumulation of chlorinated contaminants, e.g. PCDD/Fs and chlordane, has been studied in *Cucurbita pepo* species that seem to have a special uptake mechanism for these contaminants (Campanella et al., 2002; Mattina et al., 2007). Long-term storage of organic chemicals in plant biomass has only been observed for particularly persistent organic chemicals. For example, PCBs with more than two chlorine substituents and DDT have been shown to accumulate in rice plants, and could still be found in different plant compartments 60 days after incubation (Chu et al., 2006a).

2.1.3. Sorption and Sedimentation

Sorption of a chemical to soil or sediment may result from the physical or chemical adhesion of molecules to the surfaces of solid bodies, or from partitioning of dissolved molecules between the aqueous phase and soil organic matter. To evaluate the sorption behavior of organic compounds in soils and sediments, the organic carbon partition coefficient (K_{oc}) is a reasonable parameter to use, and is defined as the ratio of contaminant mass adsorbed per unit weight of organic carbon in the soil to the concentration in solution. It can be roughly estimated from the K_{ow} using empirical equations and alternatively from the water solubility of the chemical compound (Karickhoff, 1981). However, variations in K_{oc} for a given compound are affected by the sorption properties of soil organic matter. Grathwohl (1990) suggests that an empirical relationship exists between the K_{oc} and the atomic hydrogen/oxygen ratio in natural organic matter. The extent of sorption depends on the compound's hydrophobic characteristics as well as on the organic carbon content, the chemical structure and composition of soil organic matter.

During the early stages of constructed wetland operation, sorption onto soil substrate will naturally be higher due to the high adsorption capacity of previously unexposed material (e.g. Omari et al., 2003). As long as no sorption-desorption equilibrium is reached, the system acts as a sink for the contaminant. After reaching steady-state conditions, contaminants will still be retained by reversible sorption processes, but further net losses net loss of contaminants will not occur. This retention may increase contaminant residence time within the constructed wetland and support bioremediation by increasing exposure to degrading microorganisms (Pardue, 2002). However, sorptive processes may also negatively affect the bioavailability of contaminants. The bioavailability has currently been defined as the fraction of the compound in soil that can be taken up or transformed by living organisms at any time (Semple et al., 2007; Wick et al., 2007). Limited bioavailability of contaminants is one of the central attributes governing the recalcitrance of chemicals in soil-sediment systems. Biodegradation may be limited due to slow desorption kinetics especially when dealing with aged sediments (Lee et al., 2003). Aging results from chemical reactions sequestering contaminants into organic matter, diffusion into very small pores or inclusion of non-aqueous phase liquids into semi-rigid films (Bosma et al. 1997; Alexander, 2000). Different fractions of the contaminant pool can even display very low desorption rates, as observed for dichlorobenzenes by Lee et al. (2003). Deposition of contaminants sorbed to the solid phase can also lead to long-term sources of contamination in soils and sediments.

Mineral fractions in soils also affect sorptive interactions with solved organic compounds in aqueous systems. However, it is generally believed that, in saturated soils, clay mineral adsorption sites for organic compounds are effectively blocked by water molecules (Teppen et al., 1998). At least for non-polar compounds like chlorinated solvents, sorption is almost completely due to partitioning into soil organic matter (Breus and Mishchenko, 2006).
Most organic chemicals can be potentially affected by sorption at least to a certain extent. Highly hydrophobic persistent organic pollutants (POP) like PCBs, PCDDs (Campanella et al., 2002), PAHs (Cottin and Merlin, 2007) and highly chlorinated benzenes (Pardue et al., 1993) are strongly affected by sorption and therefore accumulate in sediments of constructed wetlands. It is also generally believed that pharmaceuticals such as Carbamazepin are removed from the water phase by sorptive effects due to their hydrophobicity (Matamoros et al., 2005). Significant sorptive effects have also been observed for fuel hydrocarbons in wetland soils and sediments (Thurston, 1999; Omari et al., 2003), as well as lower chlorinated benzenes (MacLeod et al., 1999; Lee et al., 2003) and chlorinated ethenes (Kassenga et al., 2003; Lorah and Olsen, 1999). Due to their high water solubility and low hydrophobicity, sorption of polar compounds such as MTBE and acetone should be of minor importance in constructed wetland systems.
In addition, sedimentation occurs when contaminant molecules are associated with particulate organic matter (POM) that settles, or is mechanically retained, within the constructed wetland. In contaminated waters containing high amounts of POM, mechanical filtration may be the most viable approach for the attenuation of organic compounds sorbed to particles, as demonstrated for petroleum hydrocarbons (Thurston, 1999) and hexachlorobenzene (Pardue et al., 1993).

2.2. Destructive Processes

2.2.1. Phytodegradation

The term phytodegradation is defined in this context as the metabolic degradation or breakdown of organic contaminants by plant enzymes or enzyme cofactors (Susarla et al., 2002). Metabolic transformations of different organic chemicals have been shown to occur in a variety of plants (Newman and Reynolds, 2004), including typical constructed wetland plants like the common reed (*Phragmites australis*), the broad-leaved cattail (*Typha latifolia*) and some poplar species (*Populus sp.*) (Bankston et al., 2002; Wang et al., 2004). The extent to which plants can degrade organic chemicals mainly depends on the specific compound of interest. For example, *Phragmites australis* has been shown to possess enzymes degrading PCB with up to three chlorine atoms, whereas higher chlorinated PCBs were not transformed (Chu et al., 2006a; 2006b). A well-known example of plant metabolic transformation of organic chemicals in constructed wetland research is the degradation of chlorinated solvents by hybrid poplar trees (*P. trichocarpa x P. deltoides*) (Newman et al., 1997; Newman et al., 1999; Wang et al., 2004) and other wetland plants (Bankston et al., 2002). Plant metabolic degradation can be very effective for this class of contaminants. For example, Wang et al. (2004) demonstrated that phytodegradation was the dominant removal process in a poplar treatment of carbon tetrachloride contaminated water.

2.2.2. Microbial degradation

The nature and extent of microbial degradation of organic chemicals within a constructed wetland is also expected to strongly depend on the physico-chemical properties of the contaminant. Indeed, the biological degradability or recalcitrance of organic compounds may often be explained by its chemical structure, for instance the presence of secondary, tertiary or quaternary carbon atoms as well as functional groups. It is designative that all compounds classified as POP in the Stockholm convention carry chlorine substituents (Ritter et al., 1995); thus cleavage of carbon chlorine bond is of particular interest for bioremediation applications in constructed wetlands.

Reddy and D'Angelo (1997) have discussed pathways and indicators for toxic organic compound removal in constructed wetlands. According to these authors, removal of toxic organics is largely a microbially mediated process, and can be subdivided into aerobic and anaerobic microbial degradation processes. Several authors have reported investigations of organic chemical removal in constructed wetlands where at least part of the contaminant elimination was assigned to microbial degradation. In the following sections, an overview of important contaminant groups will be presented. Overall, experimental evidence that allowed identifying microbial degradation pathways and quantifying organic chemical degradation potentials in constructed wetlands is scarce to date. However, indirect approaches like quantification of alternative elimination processes (sorption, volatilization) and simple gaps in mass balances without process identification are often applied to assess microbial degradation.

2.2.2.1. Highly chlorinated compounds and PAHs. An important factor limiting the degradation of highly chlorinated compounds with very low water solubility, such as PCB or PCDD/Fs, is the low bioavailability of these compounds, resulting from binding to the soil or sediment matrix (Campanella et al., 2002; Leigh et al., 2006). The compounds become more soluble after some initial reductive dechlorination steps, and thus more bioavailable. For example, Leigh et al. (2006) demonstrated that willow and pine trees are associated with enhanced rhizospheric abundances of PCB degraders at a contaminated site. However, it should be clear that efficient microbial degradation takes time, due to the slow degradation rates of dechlorination under anaerobic conditions, and the requirement for subsequent aerobic degradation steps breaking down the remaining carbon skeleton. Similar to the PCDD/Fs and PCBs, most PAHs display low bioavailabilities in soils (Manilal and Alexander, 1991). In contrast, non-chlorinated PAHs readily undergo aerobic degradation (Reddy and D´Angelo, 1997). For example, the elimination of phenanthrene in a vertical flow filter was shown to be greater than 99.9%, which corroborated with enhanced numbers of phenanthrene degraders and the formation of a known phenanthrene metabolite (Machate et al., 1997). Moreover, Giraud et al. (2001) demonstrated the degradation of fluoranthene and anthracene by several fungal isolates from a constructed wetland.

2.2.2.2. Petroleum hydrocarbons. The large and diverse group of petroleum hydrocarbons mainly consists of paraffines, naphtenes, and, to a lesser extent – aromatic, as well as polar hydrocarbons in variable portions. In constructed wetland research, they are often monitored as composite parameters like total hydrocarbons (THC), total petroleum hydrocarbons (THP) or diesel/gasoline range organics (D/GRO). In contrast to the high molecular weight compounds associated with wax and tar fractions, most petroleum hydrocarbons found in fuels are considerably more water soluble than PCDD/Fs, PCBs and PAHs. This class of contaminant displays a significant sorption potential, but is generally more easily degraded and readily mineralized under aerobic conditions. Several authors have previously reported significant petroleum hydrocarbons removal rates in constructed wetlands (Omari et al., 2003; Gessner et al., 2005; Ji et al., 2002). Groudeva et al. (2001) assessed the relationship between crude oil removal and the associated indigenous bacterial and fungal microflora in a constructed wetland. Salmon et al. (1998) demonstrated hydrocarbon removal rates of up to 90% in a constructed wetland with a porous mineral substrate matrix. For instance, 10% of the removal was assigned to sorption processes, volatilization was estimated as < 25%, and microbial degradation and eventual plant uptake were assumed to account for 60% of the observed losses.

2.2.2.3. Volatile organic compounds. Removal of chlorinated VOCs like chlorinated ethenes and monochlorobenzene (CB) from constructed wetlands has been increasingly studied in recent years (Williams, 2002; Haberl et al., 2003; Keefe et al., 2004). Some microbial degradation pathways for these compound classes are well known, and chiefly include

reductive dechlorination and aerobic oxidation. However, the most likely removal process that significantly competes with microbial degradation of these compounds is volatilization. Reductive dechlorination of tetrachloroethene (PCE) to trichloroethene (TCE), dichloroethenes (DCEs) and vinyl chloride has recently been investigated in an upward-flowing vertical flow constructed wetland (Amon et al., 2007). Bankston et al. (2002) could only assign 5% of labelled ^{14}C-TCE removal to microbial mineralization in wetland microcosms planted with broad-leaved cattail and eastern cottonwood, and point out that volatilization is the dominant removal process in their system (>50%). Kassenga et al. (2003; 2004) studied reductive dechlorination of *cis*-DCE in upflow wetland mesocosms and anaerobic microcosms derived from the former systems. After an operation time of twelve weeks, over 90% of the *cis*-DCE was degraded to vinyl chloride within the wetland mesocosms. It was concluded that reductive dechlorination can actively proceed in anaerobic zones adjacent to aerobic zones in the wetland rhizosphere (Kassenga et al., 2004). CB is preferentially degraded under aerobic conditions via a dioxygenase catalysed pathway (Reineke and Knackmuss, 1988). CB may also be degraded via reductive dechlorination (Nowak et al., 1996; Jackson, 1999) or mineralized by other anaerobic processes (Nijenhuis et al, 2007). According to MacLeod et al. (1999), mineralization of CB in a reed bed system accounted for approximately 25% of the observed losses after 47 days. Braeckevelt et al. (2007) found evidence that reductive dechlorination of CB or other anaerobic degradation pathways may simultaneously occur with aerobic degradation pathways in a constructed wetland ecosystem.
Similar to chlorinated solvents, BTEX compounds are relatively water soluble and significantly volatile. They may be microbially degraded under aerobic as well as anaerobic conditions. Removal efficiencies ranging from 88 to 100% have been reported in BTEX-treating constructed wetlands with inflow concentrations below 2 mg L^{-1} (Bedessem et al., 2007). Primary removal was assumed to be microbially mediated, whereas other authors assigned a significant role to volatilization, especially in a SF system (Machate et al., 1999) and a system with forced bed aeration, which probably enhanced emissions of BTEX compounds (Wallace, 2002).

The gasoline additive MTBE is characterized by a high water solubility and high Henry coefficient, resulting in its ubiquitous occurrence in the aqueous environment (Hong et al., 2001), and its great volatilization potential in constructed wetlands. Additionally, MTBE displays especially low microbial degradation rates under anaerobic conditions, even though degradation pathways under varying environmental conditions have been described (Somsamak et al., 2006; Haggblom et al., 2007). Hong et al. (2001) reported that in laboratory systems with poplar cuttings, the main ^{14}C MTBE removal mechanism was sequestration and volatilization by the poplar plants after approximately 10 days. In a similar experiment, Winnike-McMillan et al. (2003) found that approximately 3.5% of the MTBE label was recovered as $^{14}CO_2$, indicating a minor MTBE mineralization by microorganisms or poplar plants.

3. Metabolic potentials of constructed wetlands

Constructed wetlands may support a large spectrum of biogeochemical reactions and various environmental conditions at the wetland system scale. This function is essential for organic contaminant transformation. Indeed, the prevailing conditions generally determine both the thermodynamic feasibility of chemical reactions and the activity of indigenous microbial guilds harboring the enzymatic capacity to achieve the target biochemical reactions. In several respects, constructed wetlands are complex bioreactors characterized by considerable fluxes of material and energy governing chemical reactions over spatial and temporal gradient. Those fluxes are particularly pronounced in certain zones, such as the rhizosphere. These fluxes permit the maintenance of thermodynamic non-equilibrium conditions, and enable various reactions with exergonic free energy changes to occur (Hanselmann, 1991). In constructed wetlands, the biogeochemical reactions affecting contaminant removal mainly depend on two types of processes simultaneously occurring at different scales: (1) the various and co-existing redox processes at the wetland system scale, and (2) the processes occurring at the rhizosphere scale.

3.1. Redox processes at the constructed wetland system scale

3.1.1. Oxic-anoxic interfaces

Oxic-anoxic interfaces are dynamically established in wetlands as a result of water table fluctuations, oxygen diffusion/advection through the water column and soil, and active oxygen transport throughout the rhizosphere via plant tissues (D'Angelo, 2002) (**Figure 1**). First, the progressive constitution of sharp redox and dissolved oxygen gradients leads to the creation of sequentially adjacent anaerobic and aerobic zones (Bezbaruah and Zhang, 2004; Wiessner et al., 2005). These interfaces are then mechanically and chemically sustained and shaped via biogeochemical activities (Burken and Schnoor, 1998). At the soil-water interface of mineral soil wetlands, a thin layer of oxidized soil matrix (evident from reddish-brown iron oxide precipitates) is usually formed (Chen et al., 1980). Conversely, the deeper sediments generally remain anoxic, a state reflected by the presence of the reduced forms of redox sensitive species (Reddy and D´Angelo, 1997; Diakova, 2006). Organic chemicals supplied to a constructed wetland undergo removal process analogous to naturally occurring organic matter. D'Angelo and Reddy (1999) showed that, amongst the relevant soil factors regulating potential rates and modes of organic carbon mineralization in wetland soils, electron donor and acceptor availability appear to be essential. For instance, redox potentials were the main variables governing the observed differences in the removal patterns, and efficiently explained variation in the treatment of linear alkyl-benzene sulfonates (Huang et al., 2004) and pharmaceuticals (Matamoros and Bayona, 2006; Matamoros et al., 2005, 2007). Similarly, Meade and D'Angelo (2005) showed that redox interfaces significantly influenced pentachlorophenol mineralization rates within rice plant rhizosphere. However, degradation was significantly faster under strictly anaerobic conditions, which lack redox interfaces, when compared to treatments displaying extensive interfacing within the rhizosphere and soil matrices. While aerobic transformation is generally faster than anaerobic transformation for low-chlorinated compounds, the aerobic degradation rate is relatively slower for highly chlorinated compounds, which are more efficiently transformed via reductive dechlorination (Amon et al., 2007). This example highlights the potential interest in coupling reductive and oxidative processes in wetland systems to reach an efficient transformation for certain contaminants and their metabolic endproducts (Armenante et al., 1992; Master et al., 2002).

3.1.2. Reduction and oxidation processes

As hydrologic and geochemical conditions in a constructed wetland change over space and time, the dominant terminal electron-accepting processes (TEAPs) undergo a concomitant shift, resulting in different rates of degradation for the organic chemical. Eco-thermodynamic considerations may also be important when calculating the probability of occurrence and the direction of a biochemical transformation in constructed wetland systems (e.g. low vs. high theoretical bio-energetic capacity for a given compound, under a given condition) (Hanselmann, 1991; Dolfing and Janssen, 1994). Furthermore, the occurrence and relative contribution of a particular pathway is strongly related to oxygen and alternative electron acceptor concentrations, availability and spatiotemporal distributions. In anaerobic zones, reductive biotic and abiotic processes may directly compete for the consumption of electron equivalents with reductive degradation processes of organic contaminants. The reduction of alternative electron acceptors (NO_3^-, SO_4^{2-}, HCO_3^- and possibly FeOOH and MnO_2) in wetlands mainly depends on the soil type, and the electron donor to acceptor ratios of the influent water, but also may depend on the presence or absence of other electron accepting species (Burgoon et al., 1995; Reddy and D´Angelo, 1997). The electron acceptor and donor reactions catalysed by metal species within the iron cycle may be relevant for biogeochemical reaction in the vicinity of the capillary fringe separating water saturated and unsaturated soil zones. Fe(II) may be oxidised by oxygen penetrating from the unsaturated zone to form Fe(III), which may in turn act as electron acceptor for oxidation reactions. These processes may be important in case of water table fluctuation leading to formation of Fe(III) oxides at the capillary fringe, which may become electron donors if the water table rise again and anoxic conditions are prevailing.

However, in more aerobic zones of the water column, degradation is coupled to oxygen. Biochemically relevant oxygen transfers in wetland sediments often permit an important theoretical bio-energetic capacity ($\Delta\ G << 0$, expressing a high potential for an exergonic reaction) with respect to a given contaminant transformation. Oxygen can be transferred via three main pathways: 1) with the influent water, 2) physical transfer from the atmosphere into the water column and 3) transport via the plant tissues into the water column through root oxygen release within the rhizosphere (Tanner et al., 2002). However, the physical and phytological oxygen transport into water and sediments is generally of relatively low significance at the system scale (Rousseau, 2007), and the coupling of respiration processes leads to microaerophilic sediments in most parts of the systems. The concurrent oxygen consumption during microbial degradation of naturally-occurring organic matter outside of the contaminant pool, and the parallel oxidation of common redox-sensitive species may result in a limited supply of electron acceptors and further hinder complete oxidative contaminant transformation. Hence, the relevance of anaerobic degradation pathways is generally expected to be high at the system scale. This underlines an important point, that the significance of oxidative transformation processes may be bound to the rhizosphere and surface water zones. Some of these aspects are illustrated in **figure 1**, showing the spatial patterns of several biogeochemical processes distributed in a cross section of a model wetland treating *cis-* and *trans*-1,2-DCE contaminated groundwater (Imfeld et al, 2008).

3.2. Processes at the rhizosphere scale

Constructed wetlands are considered to be particularly valuable in cases where contaminations are initially inaccessible by the rhizosphere, and may be of special relevance when contaminated groundwater is conveyed towards the surface (Newman et al., 1998; Newman and Reynolds, 2004). Indeed, the rhizosphere represents an interface of intense soil-plant-microbe-aqueous phase interactions, enabling spatial and temporal variations of redox conditions at the root system scale. The zone is mainly characterized by concentration gradients occurring both in a radial and longitudinal axis along an individual roots and rootlets of mineral nutrients, pH, redox potential, rhizodeposition and microbial activities. In the case of several hydrophytes, the simultaneous release of oxygen and organic carbon in their rhizosphere may result in redox gradients displaying spatial and temporal variations (Liesack et al., 2000; Wei et al., 2003). These properties may in turn enhance the degradation of organic compounds in the rhizosphere as a result of the higher densities and greater activities of microorganisms when compared to the surrounding soil (Cunnigham et al., 1996; McCutcheon and Schnoor, 2003). A correlation between microbial activity at the rhizosphere level and enhanced total petroleum hydrocarbon degradation could be observed, while the microbial metabolic diversity appeared to vary between vegetated and unvegetated contaminated soils (Banks et al., 2003a; 2003b). Similarly, a conceptual-based model simulation documented the relationship between simultaneous growth of roots and associated microorganisms and increased biodegradation of crude oil contaminants (Thoma et al., 2003a, 2003b).

While some concepts tend to neglect plant mediated oxygen transfer (Wu et al., 2000; Nivala et al., 2007), spatial and temporal redox gradients of putatively enabled oxidative processes are generally expected to decrease with increased distance from the roots. Besides the plant related factors, temporal aspects of the redox variability in the rhizosphere (Wiessner et al., 2005), as well as several environmental factors such as light intensity, and water temperature (Soda et al., 2007), redox conditions and microbial oxygen demand in the sediment (Laskov et al., 2006), have to be considered when employing constructed wetlands for the treatment of organic chemicals.

Rhizodeposition products, composed of exudates, lysates, mucilage, secretions and decaying plant material, significantly contribute to the flow of organic matter across the boundaries of the rhizosphere zone. Rhizodeposition products represent an important class of diverse compounds potentially relevant to the context of contaminant removal (Rentz et al., 2005), and may differ qualitatively from the organic substrates originating in the influent water. They can be used as carbon and energy sources by the microorganisms (Jones et al., 2004), and may stimulate co-metabolic degradation of xenobiotics (Donnelly and Fletcher, 1994; Horswell et al., 1997; Moormann et al., 2002). For instance, Chung et al. (2007) recently observed a substantial organic acid release during the operation of treatment systems with bacterial biomass. This increased acid load may favor the mineralization rate of phenanthrene under waterlogged conditions. In contrast to these findings, Rentz et al. (2004; 2005) showed that the phenanthrene-degrading activity of *Pseudomonas putida* ATCC 17484 was repressed when exposed to different types of root exudates.

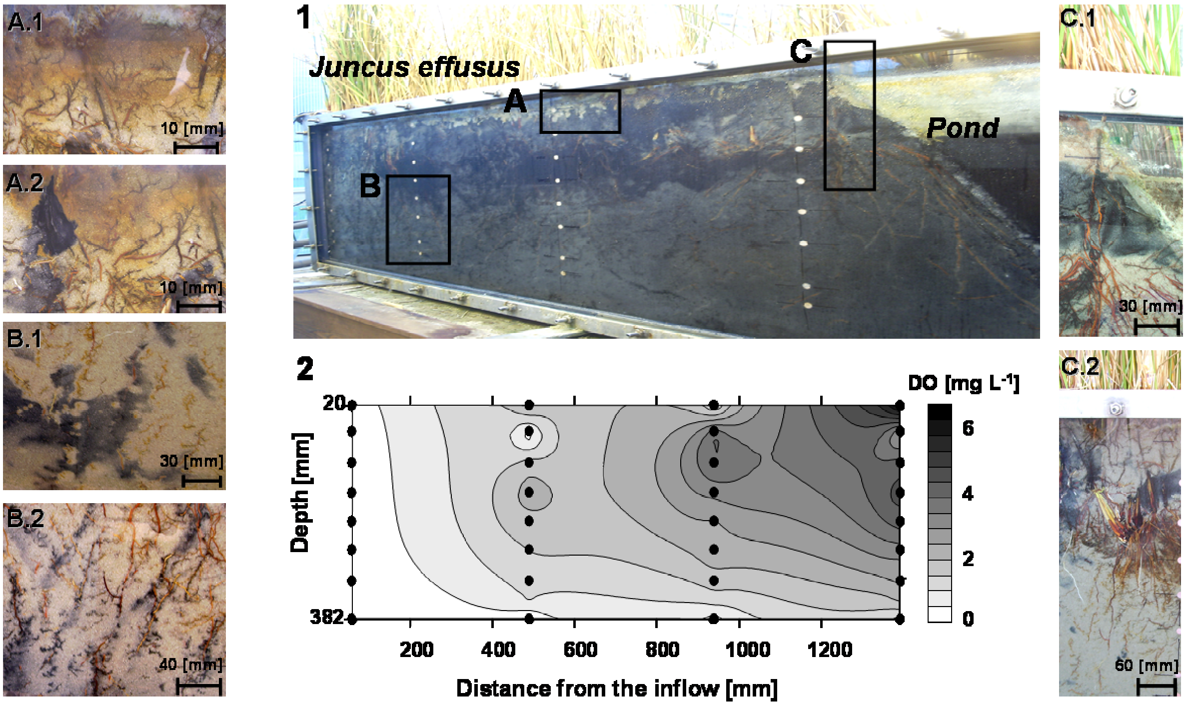

Figure 1: Spatial patterns of visible biogeochemical processes in a cross section of a model subsurface horizontal-flow constructed wetland treating *cis*- and *trans*-1,2-dichloroethene (DCE) contaminated suboxic groundwater. A complex coloration pattern of the filling sand matrix is observed at the wetland system scale through a glass board (1) after 350 days of contaminated groundwater supply, reflecting zones of iron sulphide mineral precipitation. Three zones of enlargement are depicted by rectangles and correspond to the soil-water interface in the unsaturated layer of the wetland (A), the saturated rooted zone (B) and the wetland sand matrix-polishing pond interface (C). A conceptual mapping of dissolved oxygen concentration values corresponding to 150 days of contaminated water supply (2) was obtained by planar oxygen sensor spots measurements deployed across the system (represented by the black spots), and emphasizes the occurrence of both horizontal and vertical dissolved oxygen gradients. In the unsaturated zone, the formation of reddish-brown iron oxide precipitates at the soil-water-atmosphere interface (A.1) may also occur in close vicinity to zones of black iron sulphide formation (A.2). The precipitates of iron sulphide in the saturated zone may appear as distinct and homogeneously sized black patches associated with the rootlets (B.1) or root (C.2) in the sand bed, as well as at the sand matrix-polishing pond-atmosphere interface (C.1). The precipitates may also form biogeochemically reactive meso-compartments, such as specked spots associated with the rootlets along a vertical axis (B.2). Further information on this model wetland can be found in Imfeld et al. (2008).

4. Investigation of processes in constructed wetland systems

Due to the complex fate of organic chemicals in constructed wetland systems, sole mass balance and budget calculations between the influent and the effluent water are often inefficient when characterizing relevant processes within the system. An appropriate monitoring strategy would ideally enable 1) assessing the status and contribution of the different removal processes occurring in the system, and 2) evaluating the long-term maintenance of functionality in regard to mobilization and/or transformation of organics. Integrative experimental designs based on both *in situ* hydrogeochemical and microbiological indicators may garner complementary information and create stronger basis for evaluating the *in situ* biogeochemical processes in constructed wetlands. Concepts and methods used in Monitored Natural Attenuation (MNA) approaches are currently being applied to provide quantitative and/or qualitative information about the reactive transport processes of contaminants in groundwater systems (e.g. Haack and Bekins, 2000; Grandel and Dahmke, 2004; Meckenstock et al., 2004; Rugner et al., 2006), and could be efficiently used for constructed wetland studies. Figure 2 summarizes the possible integration of several complementary experimental approaches for assessing organic contaminant removal processes in constructed wetlands.

4.1. Sampling design and techniques

Sampling designs must balance the costs associated with acquiring the necessary background information with the risks of developing an interpretation and decision based upon insufficient information. Stratified, clustered and systematic sampling strategies (Cochran, 1963; Christman, 2000) are particularly relevant, and may be used to assess *in situ* removal processes in constructed wetlands (Moustafa and Havens, 2001). For example, Amon et al. (2007) used a systematic sampling approach (66 piezometer nests regularly spaced over the investigated wetland) to assess the fate of chlorinated ethenes. Furthermore, choosing the appropriate sampling scale for the research question is crucial when investigating biogeochemical reactions and microbial communities. As the distribution of terminal electron-accepting processes in constructed wetlands may vary over small spatial and temporal scales, the chemical heterogeneity should be characterized to avoid erroneous interpretations. For instance, Hunt et al. (1997) assessed the hydrogeochemical heterogeneity in constructed wetlands using several sampling scales, and illustrated the diverging interpretations of the occurring processes. Moreover, even the choice of the sampled wetland compartment has to be considered when discerning a research question and evaluating contaminant properties. In most of the studies, pore water samples were retrieved. The collection of core soil or sediment samples from constructed wetlands allows effective sampling, but the invasive nature of the collection could bias the information regarding autochthonous microbial degraders dynamics (White et al., 2006), or specific processes such as sorption of hydrophobic contaminants (Cottin and Merlin, 2007).

However, transformations at aerobic-anaerobic interfaces are still rather difficult to measure. Current efforts are specifically focused on developing of the capability to sample integratively over spatial and temporal scales (Petty et al., 2004; Alvarez et al., 2004). In parallel to refinements of sampling devices, reliable and accurate analytical methods are under development. For example, Huang et al. (2005) used headspace solid-phase microextraction (HS-SPME) as an on-site sampling technique (Ouyang and Pawliszyn, 2006) for evaluating the behavior of volatile fatty acids and volatile alkylsulfides in a constructed wetland.

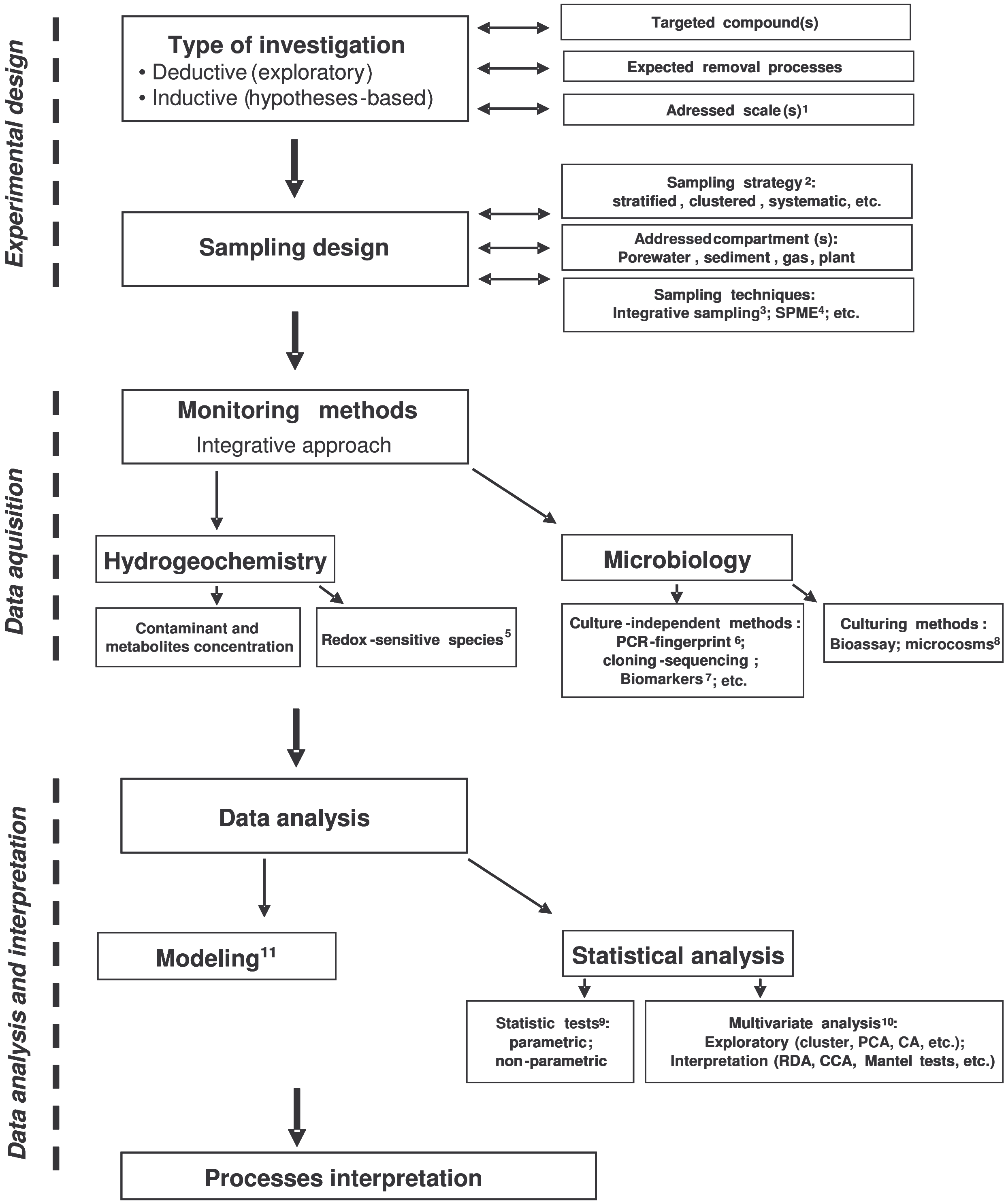

Figure 2: Flowchart of a possible integrative monitoring approach for processes investigation in constructed wetland systems.

[1]Hunt et al., 1997; [2]Cochran, 1963; Christman, 2000; Moustafa and Havens, 2001; [3]Petty et al., 2004; Alvarez et al., 2004; [4]Huang et al., 2005; [5]Reddy and DAngelo, 1997; Christensen et al., 2000; Kassenga et al., 2004; [6]Pace et al., 1986; Nocker et al., 2007; [7]Jin and Kelley, 2007; Ibekwe et al., 2007; [8]Kassenga et al., 2003; Grove and Stein, 2005; Lorah and Voytek, 2004; [9]Berryman et al., 1988; Dixon and Florian, 1993; Legendre and Legendre, 1998; [10]Legendre and Legendre, 1998; Wackernagel, 2003; Ramette, 2007; Kitanidis, 1997; [11]Wynn and Liehr, 2001; Langergraber, 2007; Keefe et al., 2004; Tomenko et al., 2007

4.2. Monitoring methods

4.2.1. Hydrogeochemistry

Understanding of contaminant behavior in a constructed wetland will require a combined evaluation of footprints of biogeochemical reactions along with variations in contaminant and metabolic-intermediates concentrations. The chemical framework potentially affecting the transformation of the targeted contaminant(s) needs to be evaluated for interpreting system observation with respect to natural attenuation potential (Reddy and D´Angelo, 1997). For example, the capacity of a system to support biological reductive dechlorination of chlorinated solvents can be assessed by monitoring concentrations of potential electron donors, typically evaluated as total organic carbon (Wiedemeier et al., 1998). Moreover, the availability of alternative electron acceptors and the distribution of microbially-mediated redox reactions can be evaluated in porewater or in reliable solid fraction analyses based on redox-sensitive constituents that are characteristic of particular processes (e.g. dissolved oxygen, iron, and manganese). A valuable review on methods and concepts for characterizing hydrogeochemistry and electron donor-acceptor interactions in ground water systems is provided by Christensen et al. (2000), which could be also applied to characterize wetland.

It is likely that the relative pore water composition with respect to redox-sensitive species will reflect the dominating redox processes at the investigated zones (Christensen et al., 2000). The analysis of this information along with contaminant mass variation may allow mass and further electron budgeting to evaluate the efficacy of intrinsic bioremediation and/or exploratory statistical analyses to depict trends in the variations (see section 4.3.1.). Indeed, a mass balance for the terminal electron acceptors (TEA) required for biochemical oxidation of a particular contaminant and the total TEA available from both the pore water and wetland sediments, permits a researcher to calculate the supply of TEA needed to sustain the targeted bioattenuation rates. However, the balance is generally rendered difficult due to sampling biases, quality variability and high backgrounds levels for some redox-sensitive species. Diakova (2006) used the concentration ratio of iron oxidation states in individual zones of a constructed wetland as a sensitive indicator for redox properties. Braeckevelt et al. (2007) correlated the mobilization of ferrous iron with monochlorobenzene removal and detected biodegradation. Furthermore, the redox-process interpretation can be improved by measuring the concentrations of expected metabolites and fermentative end-products (e.g. CO_2, methane, hydrogen, acetate, ethene). This approach has been applied successfully by Kassenga et al. (2003) while assessing the potential for dechlorination by demonstrating the co-existence of methanogenic conditions and ethene production in wetland sediment core samples. In particular, H_2 measurements may represent a powerful tool for analyzing the energetics of microbial processes (Hoehler et al., 1998) and might be relevant for evaluating the stability of *in situ* conditions with respect to bioremediation by providing an indication of the redox level dynamics (Christensen et al., 2000). For instance, Kassenga et al. (2004) studied hydrogen concentration dynamics during dechlorination of *cis*-1,2-DCE and *cis*-1,2-dichloroethane in microcosms derived from a constructed wetland, and found H_2 concentration to be a key parameter for characterizing biodegradation potentials. Moreover, this study illustrated the effectiveness of coupling measurements of microbial activity with the observation of biogeochemical processes occurring in a wetland system.

4.2.2. Microbiology

The remarkable development of molecular approaches may efficiently complement both hydrogeochemical investigations and culture-based techniques. These techniques offer the potential for assessing the structure and function of microbial populations involved in the bioremediation of organic chemicals in complex wetland systems. In particular, the application of molecular tools may enable a better mechanistic understanding of the relationship between quantitative and qualitative aspects of species diversity and environmental factors, such as contaminant load, specific nutrient requirements or prevailing TEAPs. Knowledge about these interactions may in turn contribute to enhanced organic chemical treatment effectiveness in constructed wetlands by identifying factors that need to be considered during the development of more reliable and efficient systems.

4.2.2.1. Microcosms and culture-independent techniques. Microcoms based on inoculants originating from constructed wetlands have been used to assess the occurrence and kinetics of biodegradation of *cis*-1,2-DCE (Kassenga et al., 2003) or polar organic solvents (Grove and Stein, 2005). Such investigations also contributed to the identification of anaerobic biodegradation of 1,1,2,2-tetrachloroethane (TeCA) and 1,1,2-trichloroethane (TCA), as well as associated geochemical conditions and microbial consortia identifications (Lorah and Voytek, 2004). However, their use remains limited mainly due to the difficulty associated with scaling the processes to relevant field parameters (Amann et al., 1995). Additionally, several *in situ* biotic degradation pathways are possible for most of the contaminant groups, and involve different microbial guilds (Reineke and Knackmuss, 1988; Aislabie et al., 1997; Juhasz and Naidu, 2000; Williams, 2002). Therefore, studying the phylogenetic diversity, composition and/or structure of indigenous microbial communities using culture independent molecular approaches (Nocker et al., 2007) may provide key information on the functioning of wetland systems during the treatment of organic chemicals. Molecular DNA-based approaches are generally based on amplification of genetic markers by polymerase chain reaction (PCR) using universal or specific primers. Some genetic markers, such as the 16S rRNA genes (Pace et al., 1986), permit the assessment of microbial diversity, whereas functional genes are preferentially targeted for investigating relevant metabolic capabilities. In a recent contribution, Jin and Kelley (2007) combined total phospholipid fatty acids (PLFA) identification of eukaryote and prokaryotes with PCR-denaturing gradient gel electrophoresis (DGGE) to assess the microbial community diversity and composition in different types of constructed wetlands. Similarly, Ibekwe et al. (2007) used a PCR-DGGE approach followed by bacterial sequence retrieval to correlate the water quality changes with the microbial community diversity and composition of sediment, water and rhizosphere samples of a constructed wetland. Lorah and Voytek (2004) characterized the microbial community in microcosms consisting of sediment originating from wetlands treating 1,1,2,2-TeCA and 1,1,2-TCA using terminal-restriction fragment length polymorphism (T-RFLP) and focused on a particular group of dehalogenating bacteria using a taxon-specific PCR approach.

Thus, 16S rRNA-targeted techniques may yield significant information regarding the structure of *a priori* unknown degrading microbial communities directly from wetland sediment or pore water samples. However, targeted-populations in constructed wetlands could include bacteria, but also fungi, protists, nematodes or macrophytes that are capable of bioremediation processes. For instance, the development of the ability of wetland prokaryotic or eukaryotic populations capable of adapting to and transforming contaminants of concern is a critical issue that remains difficult to holistically tackle. High-throughput technologies, such as DNA chips or gene expression arrays (Freeman et al., 2000), may provide highly resolved information about the genetic diversity of complex contaminated systems subjected to recurrent, small scale variations in geochemical conditions. Moreover, these emerging techniques may enable researchers to gather knowledge about the relationship between structure and function of these various wetland communities. In turn, an increased understanding of the intrinsic contaminant transformation processes would help supporting the development of organic-contaminant stress indicators and facilitate the assessment of spatiotemporal evolution.

4.2.2.2. Emerging techniques. Emerging techniques are being developed to provide more information about the relationship between structure and function of target communities in contaminated aquatic systems and have been recently reviewed (Weiss and Cozzarelli, 2007). Radioactive (^{14}C) or stable isotope (^{13}C) tracers may be used to track the partitioning, transformation and mineralization of organic contaminants. The use of ^{14}C labelled contaminants has been effective in several wetland studies for pentachlorophenol (Meade and D'Angelo, 2005), TCE (Amon et al., 2007; Bankston et al., 2002) and CB (MacLeod et al., 1999), however tracing of radioactive compound is regulated and mostly allowed in open systems. In addition, combined compound-specific stable isotopic approaches have been used to characterize to characterize the *in situ* biodegradation of CB in a constructed wetland. *In situ* tracer experiments based on ^{13}C-labelled CB have been used to characterize the degradation by means of transformation of labeled carbon in microbial lipids upon metabolism of CB. In parallel, compound specific isotope fractionation analysis (CSIA) of CB was employed to characterize the degradation of CB along the water flow path in a wetland system (Braeckevelt et al., 2007). CSIA represents a powerful tool for assessing biodegradation of organic chemicals in field studies. As the extent of isotope fractionation mainly depends on the biochemical reaction mechanism involved (Meckenstock et al., 2004; Fischer et al., 2007; Rosell et al., 2007). This technique may also help documenting and characterizing biodegradation and pathways during longitudinal surveys in complex constructed wetland systems. Recently, Imfeld et al. (2008) traced the temporal and spatial changes of the dominant degradation mechanism of *cis*- and *trans*-dichloroethenes (DCE) in model wetland. This was rendered possible because the mechanisms of DCE oxidation and reductive dechlorination result in different characteristic isotope effect that could be measured by means of CSIA.

Furthermore, the use of stable isotope probing techniques (DNA- or RNA-SIP) would permit the identification of microbial community members that are actively involved in the metabolic cycling of organic chemicals in wetland porewater or sediments (Kreuzer-Martin, 2007; Neufeld et al., 2007) at the system scale or at discrete depths intervals. Moreover, the study of RNA transcripts would provide insights into the potential metabolic activity (e.g. mRNA of functional genes), whereas proteome and metabolome analysis hold promise for gathering knowledge about the status of protein expression and active metabolic activity in constructed wetlands treating organic chemicals.

4.3. Data treatment

Detailed hydrogeological and microbiological investigations of complex and heterogeneous systems, such as constructed wetlands, generally results in large data sets that must be further processed in order to facilitate the interpretation of on-going biogeochemical processes (Figure 2).

4.3.1. Statistical analysis

Water quality data rarely meet the statistical assumption of normality. Therefore, non-parametric statistics are generally recommended when evaluating the significance of these trends over space and/or time. Furthermore, assessment of trends over sampling points and/or treatments (e.g. soil strata; reaches over the flowpath) can be achieved with Mann-Whitney/Wilcoxon rank sum tests or non-parametric χ^2 statistics, accordingly, whereas temporal trends in water quality data can be investigated with Mann-Whitney, Spearman and Kendall temporal tests (e.g. Berryman et al., 1988; Dixon and Chiswell, 1996; Legendre and Legendre, 1998). Multivariate analyses will be of particular interest when assessing causal processes in wetlands by evaluating trends over several variables (Legendre and Legendre, 1998). The application of these tools in several disciplinary fields of relevance for constructed wetland research has been intensively reviewed: geology and geochemistry (Wackernagel, 2003), microbial ecology (Ramette, 2007), and hydrogeology (Kitanidis, 1997). Briefly, after transformation, normalization or standardization, the data sets are separately or complementarily subjected to exploratory (e.g. cluster analyses; principal component analysis, PCA; correspondence analysis, CA; principal coordinate analysis, PCoA; and nonmetric multidimensional scaling, NMDS) or interpretation analyses (e.g. indirect gradient analyses; redundancy analysis, RDA; canonical correspondence analysis, CCA; variation partitioning, Mantel test). Exploratory multivariate analyses are used to reveal patterns in large data sets (e.g. existence of clusters or groups of objects), but do not directly explain their occurrence. For example, Imfeld et al. (2008) used PCA to enhance the interpretation of the development of hydrogeochemical processes in a model wetland. In contrast, interpretative analyses allow the researcher to address the relationship between species patterns and environmental variable (Legendre and Legendre, 1998).

4.3.2. Modeling

Several groups of simple (Tanner et al., 1995; Kadlec, 2000), Monod-type (Mitchell and McNevin, 2001) and mechanistic (Wynn and Liehr, 2001) models, as well as multi-component reactive transport models focusing on the characteristics of internal processes (Langergraber, 2007) have been developed. Keefe et al. (2004) successfully used stream modeling techniques (one-dimensional transport model with inflow and storage) for the investigation of VOCs behavior in a constructed wetland. Recently, Tomenko et al. (2007) compared the predicted efficiency of constructed wetland treatments using multiple regression analysis and artificial neural networks. Though some numerical analyses may substantially contribute to the investigation of reactive transport and support quantification of physicochemical processes in constructed wetlands, their applicability for evaluating the fate of organic chemicals in constructed wetlands remains challenging. Indeed, the numerical analysis of explicit design equations is often limited describing the complexity and interconnectivity of processes in constructed wetlands. Moreover, linking wetland hydraulics to removal pathways is particularly difficult, due to non-uniform hydraulic flow, heterogeneity of retardation factor and complexity of degradation processes. Nevertheless, values retrieved from a combined hydrogeochemical and microbiological assessment can be used to generate conceptual models of both microbially mediated TEAPs and transformation reactions to better elucidate the processes associated with natural attenuation of organic chemicals in constructed wetlands.

5. Conclusion

Constructed wetland technology for the treatment of organic chemicals is an emerging field (EPA, 2000; Schröder et al., 2007). In this context, a better understanding of the processes governing the removal of organic chemicals is crucial for further system optimization. Valuable information regarding the fate of organic contaminants in constructed wetlands can be retrieved based on their physico-chemical properties and eco-thermodynamic considerations. This information may in turn support improved testing and better optimization of system designs and operational modes. Additionally, a comprehensive follow-up study investigating the longitudinal effect of hydrogeochemical and microbial indicators of degradation is required for characterizing system-intrinsic processes and reactions controlling the fate of organic compounds. Ultimately, an integrative approach will better document wetland system efficiency and reliability with respect to organic contaminant removal. In this context, monitoring bioremediation would involve correlating specific groups of organisms with their observed degradation activities and functions in constructed wetlands. Gathering knowledge about the relationship between the quantitative and qualitative development of these organisms and key environmental factors, such as characteristic patterns of redox gradients at various scales, is relevant for improving the contaminant attenuation functions of constructed wetland ecosystems. The application of approaches and techniques recently developed in related fields, such as contaminant hydrology, environmental microbiology and biotechnology, phytoremediation, statistics and environmental modelling, will tremendously enhance these investigations and open new possibilities for process characterization and interpretation in constructed wetlands.

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2

Biogeochemical processes in model wetlands treating chlorinated solvents

Section 1: Assessment of in situ biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland

Section 2: Tracking *in situ* biodegradation of 1,2-dichloroethenes in a model wetland

Section 3: Variability of bacterial community in a model wetland treating 1,2-dichloroethenes

Section 1: Assessment of in situ biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland

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Abstract

The degradation of monochlorobenzene (CB) was assessed in a constructed wetland treating MCB contaminated groundwater using a detailed geochemical characterisation, stable isotope composition analysis and *in situ* microcosm experiments. A correlation between ferrous iron mobilisation, decreasing MCB concentration and enrichment in carbon isotope composition was visible at increasing distance from the inflow point, indicating biodegradation of MCB in the wetland. Additionally, *in situ* microcosm systems loaded with ^{13}C-labelled MCB were deployed for the first time in sediments to investigate the biotransformation of MCB. Incorporation of ^{13}C-labelled carbon derived from the MCB into bacterial fatty acids substantiated *in situ* degradation of MCB. The detection of ^{13}C-labelled benzene indicated reductive dehalogenation of MCB. This integrated approach indicated the natural attenuation of the MCB in a wetland system. Further investigations are required to document and optimise the *in situ* biodegradation of MCB in constructed and natural wetland systems treating contaminated groundwater.

Capsule

An integrated approach including isotope composition analysis and *in situ* microcosm experiments provided evidences for *in situ* biodegradation of MCB in a wetland system.

1. Introduction

Monochlorobenzene (MCB) is encountered worldwide as a groundwater pollutant, and persists in the essentially anaerobic aquifer at the large-scale contaminated site in Bitterfeld, Germany (Heidrich et al., 2004b; Wycisk et al., 2004). In recent years, interest has grown in using phytoremediation processes for the elimination of recalcitrant organic substances from waste- and groundwater (Macek et al., 1998; Schnoor et al., 1995; Shimp et al., 1993; Trapp, 2000) including chloroaromatics (Gilbert and Crowley, 1997). Wetland systems represent an effective and inexpensive option to treat groundwater polluted with organic compounds by taking advantage of the geochemical and biological processes (e.g. Baker, 1998; Dunbabin and Bowmer, 1992; Gumbricht, 1993). Indeed, rapid degradation of chlorinated organics has been observed in the rhizosphere (Anderson and Walton, 1995; Jordahl et al., 1997; Lorah and Olsen, 1999; Pardue et al., 1996).

While aerobic degradation of MCB has been well studied (Van Agteren et al., 1998), only some evidence for MCB transformation under anoxic conditions has been presented yet and the degradation pathway is unknown (Kaschl et al., 2005; Liang and Gribic-Galic, 1990; Nowak et al., 1996). Moreover, only very few studies focus on the anaerobic microbial transformation of MCB under field conditions. Recently, indications of anaerobic MCB degradation taking place in the Bitterfeld contaminated aquifer were provided on the basis of isotope fractionation patterns (Kaschl et al., 2005). Kinetic isotope fractionation processes have been employed to demonstrate the biological transformation of various contaminants (Lollar et al., 2001; Richnow et al., 2003a; Richnow et al., 2003b; Song et al., 2002). A substantial enrichment of ^{13}C in the non degraded fraction in the course of a contaminant plume indicates microbial degradation, as dilution and sorption do not affect the isotope composition of contaminants significantly (Harrington et al., 1999; Schüth et al., 2003; Slater et al., 2000). Combining stable isotope composition analysis with information obtained in simple *in situ* microcosm experiments (BACTRAPs) using isotope labelled substrate may provide a suitable approach to qualitatively support *in situ* biotransformation and to monitor spatial and temporal natural attenuation processes. Previously, BACTRAPs were exclusively installed in groundwater monitoring wells (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006a) and were deployed in sediment for the first time in the framework of this study.

For the assessment of *in situ* biodegradation in constructed wetlands and wetlands treating contaminated groundwater, it may be necessary to use several methods providing more than one line of evidence. A combined approach may be of additional benefit in particular when systems are complex, possess several compartments and convincing evidence is required. Moreover, a better understanding of the controlling geochemical processes in wetland systems is necessary to reliably predict the retention and transformation of contaminant. In this study, we evaluated the natural attenuation of MCB in a constructed wetland treating MCB contaminated groundwater using a detailed geochemical characterisation, stable isotope composition analysis and *in situ* microcosm experiments. The spatial variations of geochemical parameter were studied with the help of multivariate statistics to investigate the main processes controlling the wetland system. The concentration and carbon stable isotope composition of MCB was analysed to monitor the *in situ* contaminant degradation and *in situ* microcosms were used to provide qualitative evidences of *in situ* biotransformation of MCB.

2. Materials and methods

2.1. Design and characteristics of the wetland

The pilot constructed wetland at the experimental site in Bitterfeld was set up in December 2002. The horizontal subsurface flow wetland consisted of a stainless steel tank divided into two segments. Each segment was 6 m x 1 m and was filled to an average depth of 0.5 m with autochthonous quaternary aquifer material consisting predominantly of Bitterfeld mica sand (25%) and gravel (67%), which was embedded in lignite (10%) with an effective porosity of 28% (Vogt et al., 2002). The hydrogeochemical characteristics of the study site and the filling material originating from the local aquifer are described in previous studies (e.g. Vogt et al., 2002; Weiss et al., 2001). One of the segments was planted with common reed (*Phragmites australis*, Cav.), whereas the other side was left unplanted. In both segments, a 1 m long open water pond at the outflow side allows direct contact between the atmosphere and the water surface. The water level was maintained at approximately 10 cm below the surface of the wetland. The groundwater was collected from the MCB contaminated aquifer and conveyed from 16 to 22 m depth directly to the wetland. Both segments were operated in a flow-through mode at a flow rate of 4.7 L h^{-1}, corresponding to a retention time of 6 days.

2.2. Sampling

In the period from April to September 2005, pore water samples were collected five times (d0; d53; d66; d143; d172) in order to investigate the geochemical processes and the contaminant behaviour in the wetland system. The pore water was collected in both segments along a transect from the inflow up to the outflow of the wetland, at respectively 0 (inflow valves), 0.5, 1, 2, 3 and 4 m using a stainless steel lance. At each sampling point, three depths, 30, 40 and 50 cm were systematically investigated. Water samples were also collected at the ponds (6 m).

In addition, to assess the *in situ* biodegradation using isotope composition analysis, pore water samples from both segments were collected at day 0 and day 53, at 0, 1 and 3.5 m along the wetland at 0.5 m depth, as well as in the ponds.

2.3. Physico-chemical and geochemical parameters of the pore water samples

The redox potential was measured on-line in the field using a SenTix ORP electrode (PT 1000, PreSens, Regensburg, Germany). The temperature was determined by a temperature sensor (PT 1000, PreSens, Regensburg, Germany). Samples for the pH analysis and the quantitative ions were filtered through a 5 µm syringe filter (Ministart NML, Sartorius) for particle removal. The pH value was measured with a SenTix41 electrode with pH 537 Microprocessor (WTW, Weilheim, Germany). Oxygen measurement was carried out using an optical oxygen trace sensor system (oxygen meter Fibox-3-trace and flow-through cell type sensor FTC-TOS7) with automatic temperature compensation (temperature sensor PT 1000) (PreSens, Regensburg, Germany). For the analysis of Mn(II), total iron and Fe(II), hydrochloric acid was added and samples were diluted with deionised water (1:10, v:v). Total iron and Mn(II) concentrations were analysed by atomic emission spectrometry with ICP excitation and CCD detection (Spectro Ciros Vision CCD, Spectro Analytical Instruments, Kleve, Germany). Photometric analysis of ferric iron was carried out at 562 nm after derivatisation with ferrocin using a Cadas 100 photometer (Hach Lange, Düsseldorf, Germany). Chloride and sulphate concentrations were determined by ion chromatography (DX 500) with conductivity detection (CD 20) and a IonPacAG11 (4x250 mm) column (Dionex Corporation, Sunnyvale, USA). For the analysis of sulphide concentrations samples were spiked with sulphide anti-oxidant buffer (200 ml L^{-1} 10 M NaOH, 35 g L^{-1} ascorbic acid, 67 g L^{-1} EDTA) (1:1, v:v) and measured with an ion selective Ag/S 500 electrode and reference electrode R 503 (WTW, Weilheim, Germany).

2.4. Analysis of benzene, MCB and metabolites

Pore water samples for the analysis of benzene and MCB concentrations were collected in 20 mL glass flasks (Supelco, Bellefonte, USA), and sealed with Teflon-lined septa. Sodium azide solution was added to the samples to inhibit microbial activity. Benzene and MCB concentrations were quantified by automatic headspace gas chromatography using an HP 6890 gas chromatograph with flame ionisation detector (Agilent technologies, Palo Alto, USA). For headspace analysis a volume of 1000 µl was injected at an injection temperature of 250°C with split 1:5 (measurements in duplicates). The chromatographic separation was achieved on a HP-1 capillary column (Agilent technologies, Palo Alto, USA) (30 m x 0.32 mm x 5 µm) with the following oven temperature program: 45°C (1 min), 20°C min^{-1} to 200°C (2.5 min), 65°C min^{-1} to 250°C (1 min) and a detector temperature of 280ºC.

For the determination of the carbon isotope composition of MCB, 1 L glass bottles (Schott, Mainz, Germany) containing NaOH pellets to prevent microbial growth were filled completely with groundwater, stored at 4°C and extracted within 24 h using 2 mL n-pentane as described previously (Richnow et al., 2003b). The analysis of volatile metabolites obtained in the *in situ* microcosm experiments was carried out using a HP 6890 gas chromatograph with HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, USA). Aliquots of 1 µl liquid samples were injected at a temperature of 280°C with split 1:40 and separated on a Zebron BPX-5 column (30 m x 0,32 mm x 0,25 µm) (Phenomenex, Torrance, USA). The following oven temperature program was applied: 40°C (2.5 min), 10°C min^{-1} to 70°C (0 min), 60°C min^{-1} to 280°C (4 min).

2.5. In situ microcosms (BACTRAPs)

Preparation of microcosms and derivatisation of fatty acids

The *in situ* microcosms were prepared as described previously (Stelzer et al., 2006a). Different sets of *in situ* microcosm experiments were prepared. One set was loaded with [$^{13}C_6$]-labelled MCB (Cambridge Isotope Laboratories, Andover, USA) and another one with natural abundance MCB. A third set was kept unloaded to observe the background effects. The loading was done via gas phase under reduced pressure with approximately 40 mg MCB per g Bio-Sep®. The microcosms were deployed at 1.5, 2.5 and 4.5 m from the inflow in both planted and unplanted segments at 50 cm depth. The microcosms were collected after 6 weeks and fatty acid extraction was carried out according to Bligh and Dyer (1959). The derivatisation to obtain fatty acid methyl esters (FAME) was done according to Thiel et al. (2001). After evaporation to complete dryness and addition of heneicosanoic acid methyl ester (C21:0) as an internal standard the FAME fraction was dissolved in *n*-hexane for further identification, structural characterisation and carbon isotope composition analysis.

GC-MS analysis

For identification and structural characterisation of FAME a HP 6890 gas chromatograph coupled with a HP 5973 quadrupol mass spectrometer (Agilent Technologies, Palo Alto, USA) was used. The FAMEs were separated on a Zebron BPX-5 column (30 m x 0.32 mm x 0.25 µm) (Phenomenex, Torrance, USA) with the following temperature program: 70°C (1 min), 20°C min^{-1} to 130°C, 2°C min^{-1} to 150°C (5 min), 2°C min^{-1} to 165°C (5 min), 2°C min^{-1} to 230°C, 20°C min^{-1} to 300°C (5 min). FAME were identified by comparing with the retention time and mass spectra of an authentic standard mix (bacterial acid methyl esters mix, Sigma-Aldrich, Germany) and quantified relatively to the internal standard.

Isotopic composition analysis

The carbon isotope composition of MCB and the FAMEs was measured with a gas chromatography-combustion-isotope ratio mass spectrometry system (GC-C-IRMS) consisting of a GC unit (HP 6890, Agilent technologies, Palo Alto, USA), a combustion device (Finnigan MAT GC III, ThermoFinnigan Bremen Germany) with water-removal assembly (Nafion® membrane, 50 cm long, T = 0°C) and a mass spectrometer (Finnigan MAT 252; ThermoFinnigan, Bremen, Germany), as previously described (Richnow et al., 2003a). Helium was used as carrier gas at a flow rate of 1.5 ml min^{-1}.

Stable isotope samples were measured in triplicates and the analyses were carried out immediately after each sampling. Aliquots of 1 µl liquid samples were injected at 250°C with split 1:10 to the GC-C-IRMS and separated on a capillary column (Zebron ZB-1, 60 m x 0.32 m x 1 µm; Phenomenex, Torrance, USA). The following chromatographic conditions were applied: injector temperature 250°C, oven temperature program: 40°C (1 min), 4°C min^{-1} to 150°C, 20°C min^{-1} to 250°C (2 min). The carbon isotope composition is reported in the delta notation as $\delta^{13}C$ values [‰] relative to Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna) (Eq. 7-1) (Hoefs, 1997).

(Equation 1) $$\delta^{13}C[‰] = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{\text{Sample}}}{\left(\frac{^{13}C}{^{12}C}\right)_{\text{Standard}}} - 1 \right) * 1000 = \left(\frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right) * 1000$$

Eq.(7-2) is applied to calculate the remaining substrate fraction (f_t) using the isotope fractionation factor (αC).

(Equation 2) $$f_t = \left(\frac{R_t}{R_0} \right)^{\left(\frac{1}{\frac{1}{\alpha C} - 1} \right)}$$

The R_t and R_0 give the isotope composition of MCB at time t and zero. Eq. (7-3) is applied to calculate the percentage of biodegradation of the residual substrate fraction (B_t).

(Equation 3) $$B[\%] = (1 - f_t) * 100$$

For the separation of the FAME fractions, a Restek RTX 5 column (60 m x 0.32 mm x 0.1 µm; Restek, Bellefonte, USA) was used with the same temperature program applied for the GC-MS-analysis of FAME. Aliquots of 1 µl were injected with split 1:5. The methylation of fatty acids for gas chromatographic analysis introduces an additional carbon atom into the structure of the fatty acid molecules which affects its isotopic composition. Therefore the isotope signature of fatty acids ($\delta^{13}C_{FA}$) was corrected for the isotope effect upon derivatisation to FAME with methanol as described previously (Abraham et al., 1998; Abrajano et al., 1994; Goodmann and Brenna, 1992). The methanol used for the derivatisation had an isotope composition of -38.2‰.

2.6. Statistical analysis

Statistical analyses were carried out using the R software (R, Version 2.1.1, 2005). Statistical significance of the difference in geochemical parameters as well as concentrations and isotopic compositions of MCB between the planted and unplanted segment was determined with the unpaired Wilcoxon or the Kruskall-Wallis rank sum tests. Correlation analyses were carried out using the Spearman rank sum coefficient. Principal Component Analysis (PCA) was used to analyse the relationship between the different samples with reference to their respective pore water parameters. The sampling location corresponds to the object and the chemical parameters to the descriptors (represented by the vectors) of the multivariate analysis. The PCA were scaled as correlation biplots.

3. Results

3.1. Characterisation of pore water chemistry

Distribution of MCB and benzene

The MCB concentration was measured as a function of the distance from the inflow point in both the planted and unplanted segment (Table 1). The average amount of MCB ranged from 14.4 mg L^{-1} to 17.7 mg L^{-1} at the inflow down to 2.0 mg L^{-1} to 2.2 mg L^{-1} in the ponds for the planted and the unplanted segment, respectively. No significant difference in MCB concentration between the three depths over the study period were generally observed ($p<0.05$). Benzene was found in low concentration in both segments (<26 µg L^{-1}), with generally higher concentration values in the unplanted segment (Table 1).

Table 1: Geochemical characterisation, MCB and benzene concentrations of samples collected from (a) unplanted plot and (b) planted plot. Values represent the depth profiles average in the soil compartments (0.5 - 4 m) and average (0 m and pond) over the study period (13.04.05-29.09.05). Standard deviation is indicated in parentheses.

Sampling point [m from inflow]	pH	Temp. [ºC]	O_2 [mg L^{-1}]	Eh [mV]	Fe(II) [mg L^{-1}]	Sulphate [mg L^{-1}]	Cl^- [mg L^{-1}]	Benzene [µg L^{-1}]	MCB [mg L^{-1}]
(a)									
0	7.1 (3.6)[d]	14.8 (8.5)[c]	0.04 (0.02)[e]	38 (37)[d]	0.18 (0.23)[c]	969 (402)[c]	280 (116)[c]	25.6 (3.6)[c]	17.7 (8.0)[c]
0.5	7.0 (2.2)[b]	16.3 (5.4)[a]	0.05 (0.04)[b]	61 (26)[b]	2.43 (1.83)[a]	969 (253)[a]	273 (69)[a]	21.2 (6.1)[a]	14.6 (5.0)[a]
1	6.7 (2.1)[a]	17.1 (5.4)[a]	0.04 (0.02)[a]	84 (38)[a]	9.58 (5.80)[a]	967 (253)[a]	269 (68)[a]	23.3 (3.9)[a]	14.5 (5.1)[a]
2	6.9 (2.0)[a]	17.8 (5.5)[a]	0.04 (0.04)[a]	70 (41)[a]	12.40 (6.74)[a]	973 (254)[a]	267 (67)[a]	22.2 (5.4)[a]	14.5 (5.2)[a]
3	6.8 (1.9)[a]	18.4 (5.5)[a]	0.04 (0.03)[a]	57 (34)[a]	21.12 (7.45)[a]	974 (254)[a]	261 (71)[a]	22.5 (5.5)[a]	14.9 (5.6)[a]
4	6.8 (1.9)[a]	18.9 (5.6)[a]	0.04 (0.02)[a]	57 (37)[a]	30.23 (7.08)[a]	973 (256)[a]	267 (72)[a]	21.1 (6.3)[a]	14.4 (5.5)[a]
Pond	6.8 (3.0)[d]	20.9 (8.4)[c]	n.a.	250 (44)[d]	3.06 (8.81)[c]	986 (403)[c]	287 (118)[c]	1.8 (3.0)[c]	2.0 (7.9)[c]
(b)									
0	6.8 (3.1)[d]	14.8 (8.5)[c]	0.04 (0.02)[e]	35 (40)[d]	0.20 (0.21)[c]	977 (400)[c]	262 (109)[c]	24.0 (6.9)[c]	14.4 (6.4)[c]
0.5	6.8 (2.2)[b]	15.6 (5.5)[a]	0.13 (0.05)[b]	47 (42)[b]	5.73 (3.62)[a]	994 (251)[a]	257 (68)[a]	11.8 (5.5)[a]	8.7 (7.7)[a]
1	6.8 (2.1)[a]	16.6 (5.4)[a]	0.12 (0.09)[a]	54 (40)[a]	13.34 (7.48)[a]	980 (248)[a]	256 (68)[a]	15.7 (6.6)[a]	9.8 (7.2)[a]
2	6.7 (2.0)[a]	17.6 (5.4)[a]	0.05 (0.02)[a]	46 (36)[a]	25.48 (7.60)[a]	1006 (248)[a]	266 (68)[a]	11.9 (8.8)[a]	8.2 (6.6)[a]
3	6.7 (1.9)[a]	18.0 (5.4)[a]	0.05 (0.03)[a]	51 (34)[a]	36.57 (7.33)[a]	1000 (248)[a]	271 (69)[a]	11.3 (8.9)[a]	7.9 (6.1)[a]
4	6.5 (1.9)[a]	18.8 (5.5)[a]	0.03 (0.02)[a]	57 (34)[a]	37.40 (8.94)[a]	998 (259)[a]	266 (74)[a]	10.2 (8.0)[a]	7.7 (5.9)[a]
Pond	6.8 (3.1)[d]	20.5 (8.1)[c]	4.85 (1.89)[e]	204 (43)[d]	2.42 (9.28)[c]	1068 (397)[c]	275 (111)[c]	0.2 (0.4)[c]	2.2 (7.8)[c]

[a] Profile average of five sampling dates (n=15)
[b] Profile average of four sampling dates (n=12)
[c] Average of five sampling dates (n=5)
[d] Average of four sampling dates (n=4)
[e] Average of three sampling dates (n=3)
n.a. not assessed

Pore water geochemistry

The evolution of oxygen, redox potential, manganese, sulphide, sulphate, ferric iron and total iron were monitored along the flow path at three depths in order to characterise the geochemical conditions prevailing in the wetland system. The average values of the three depths investigated at each sampling point (0, 0.5, 1, 2, 3, 4 m from the inflow) were computed (Table 1). To investigate the differences between the sampling locations and to explore existing gradients, the data sets were analysed by principle component analysis, separately for the unplanted and the planted segments (Fig. 1). In both cases, the vectors representing the temperature, Fe(II), and total Fe parameters were orientated in the same direction (positive correlation), in the opposite direction with regards to vectors representing manganese and ammonium (negative correlation), and are perpendicular to the MCB vector (absence of correlation). The orthogonal projection of an object on a descriptor allows approximating the correlation between that object and the descriptor. For both segments, samples from the inflow part of the wetland (0.5 to 1 m) were associated with MCB, manganese and ammonium, whereas samples from the outflow (3 and 4 m) were generally associated with total Fe and Fe(II). Indeed, the Fe(II) concentration at the inflow were below 0.5 mg L^{-1} and systematically increased along the flow path in both planted and unplanted segment, indicating ferrous iron mobilisation. A clear shift in the parameters characterising predominantly the trends of variation of the samples along the flow path is operating on the first principal component. Indeed, the axis I corresponds to a spatial gradient from the inflow to the outflow of the system, and separates sampling sites accordingly (from the right to the left). The variations of geochemistry along the flow path contributed more in characterising the samples than the variations occurring along the vertical profile.

The maximum concentration of Fe(II) reached an average value of 37.4 mg L^{-1} at 4 m from the inflow of the planted segment. The levels of total dissolved iron detected throughout the experimental period ranged from 0.8 to 50.6 mg L^{-1}. Interestingly, Fe(II) mobilisation was more important in the planted than in the unplanted segment. Correlation analysis revealed, however, very similar Fe(II) mobilisation patterns in both segments over the study period ($\rho>0.9$; $p<0.01$). Iron reduction was apparently a relevant process at the three depths. Due to the high background concentration of sulphate in the supplied groundwater (700-1100 mg L^{-1}), a sensitive analysis of the microbial sulphate reduction on the basis of sulphate concentration was not possible. However, trace concentrations of sulphide (<3 µg L^{-1}) were detected along the horizontal transect, indicating sulphate reduction. Mn(II) concentrations systematically ranged below 0.2 mg L^{-1}, suggesting a low relevance of Mn as electron acceptor in the wetland (data not shown). The redox potential displayed averages of values ranging from 35 mV at the inflow to 250 mV in the ponds, and maximal concentration of oxygen in the soil compartments was 0.12 mg L^{-1}, indicating the prevalence of anoxic conditions in the soil compartments and oxic conditions in the ponds, respectively. The dissolved organic and total carbon concentrations ranged from 0.24 to 0.45 mM L^{-1} and from 2.27 to 2.41 mM L^{-1} in the unplanted and planted segments, respectively (data not shown). Nitrate concentrations were assessed in previous studies and ranged systematically under the detection threshold in the groundwater supplied to the wetland (Vogt et al., 2002), and nitrate may therefore not represent a relevant electron acceptor. The chloride concentrations did not show any significant variation along the transect.

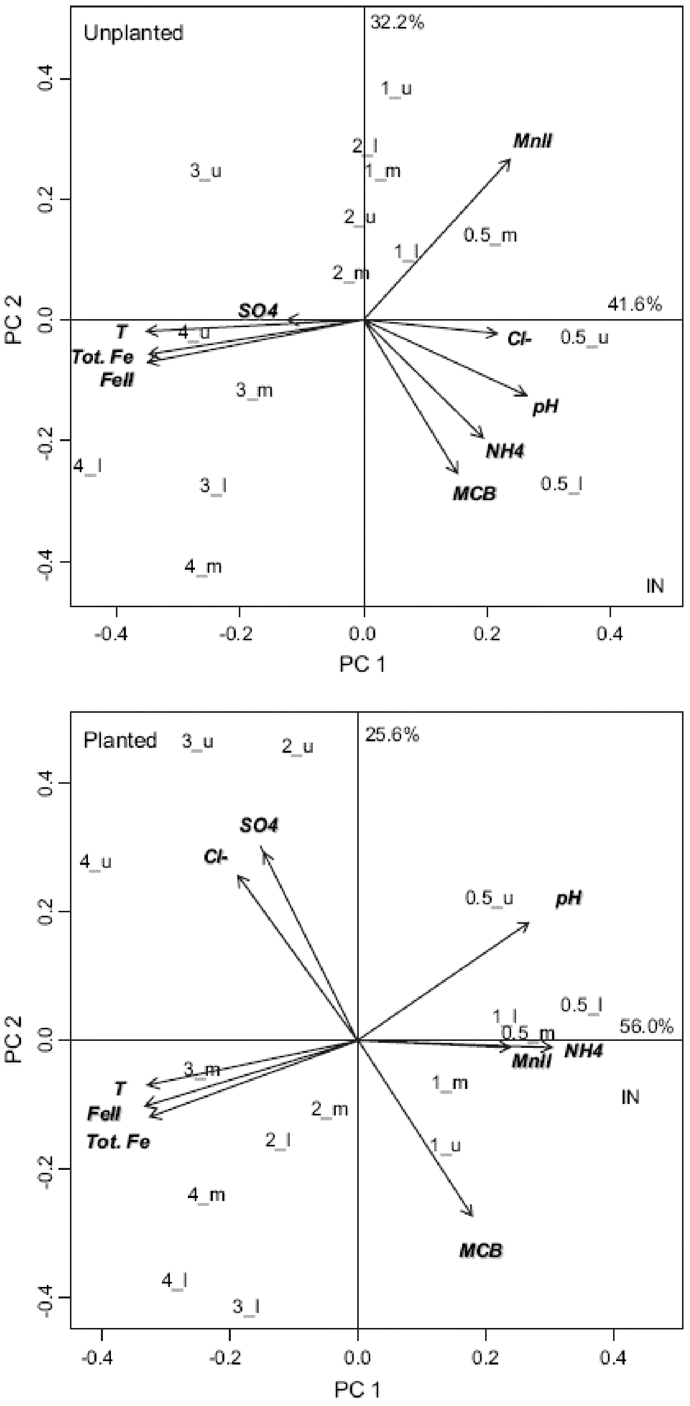

Fig. 1: Ordination plot generated by principal component analysis representing the relationship between the sampling locations and the average geochemical parameters measured in both the planted (a) and the unplanted (b) experimental wetlands over the study period. Description vectors correspond to: FeII = ferric iron; Tot.Fe = Total iron (Fe(II)+Fe(III)); MCB = Monochlorobenzene; MnII = Manganese(II); NH4. = Ammonium; T = Temperature. Objects correspond to: 0.5 to 4 = distance [m] from the inflow point; u = upper depth (30cm); m = medium depth (40cm); l = lower depth (50cm). Values on the axes indicate % of total variation explained by the axes. PC 1 = principal component axis 1; PC 2 = principal component axis 2.

3.2. Carbon isotopic composition of MCB

The concentration and isotopic composition ($\delta^{13}C$) of MCB were plotted as a function of the distance from the inflow (Fig. 2). A decrease in MCB concentrations over the flow path was systematically associated in both segments with a significant enrichment in $\delta^{13}C$. In the planted segment, MCB showed a maximal isotope shift of 0.6‰, whereas it reached 0.9‰ in the unplanted segment. This indicates that MCB is subjected to *in situ* biodegradation in both segments. Assuming an isotopically homogenous source of MCB, these values are slightly higher than the typically defined analytical error of 0.5‰ associated with compound specific isotope analysis (Dempster et al., 1997; Mancini et al., 2002).

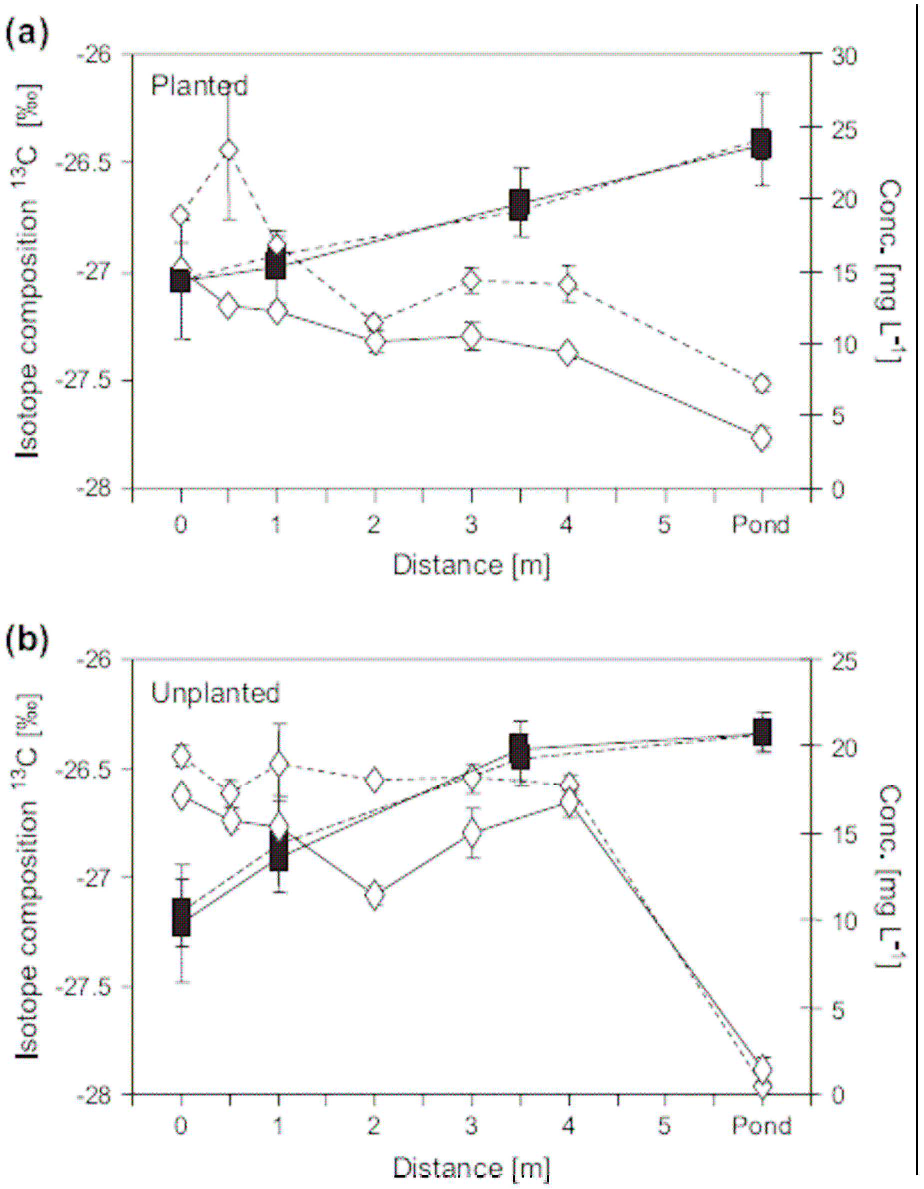

Fig. 1: Concentration and isotopic composition of MCB (diamond = concentration; squares = isotopic composition) in both planted (a) and unplanted (b) segments and for both sampling dates (line = 14.04.2005, dashed = 09.05.2005). Error bars show the standard deviation.

A quantitative assessment of *in situ* MCB biodegradation requires a fractionation factor representing the *in situ* conditions. Fractionation factors for aerobic MCB degradation are available (Kaschl et al., 2005), whereas factors retrieved under laboratory conditions for anaerobic MCB degradation are missing. Although MCB degradation under anaerobic conditions is expected in the wetland, Eq. (7-3) allows estimating the significance of degradation along the flow path throughout the wetland applying the highest isotope fractionation factors (αC) retrieved for aerobic MCB degradation by a dioxygenase reaction pathway (αC = 1.0004) (Kaschl et al., 2005). The quantification was carried out based on the isotope signature of MCB measured at the planted side at day 0. The lowest $\delta^{13}C$ value measured (inflow point, 0 m: MCB $\delta^{13}C$= -27.0 ‰) was used as the initial isotope composition of the source (R_0). The estimated percentage of biodegradation was 60% of the inflowing MCB mass at 3.5 m, whereas the observed contaminant mass decrease reached only 38% at 4 m from the inflow. Processing of isotope and concentration data of the second sampling campaign gave almost identical results.

The use of too low fractionation factors for the quantification of MCB degradation would lead to an overestimation of the effective MCB mass depletion at the planted segment. The observed absence of significant MCB mass depletions along with a higher isotopic shift at the unplanted segment obviously direct to the same conclusion. Therefore, higher fractionation factors are expected, which would be more in concordance with the observed MCB concentration values. The use of higher isotope fractionation factors would point to a dominating anaerobic fractionation process, which is compatible with the observation of anoxic conditions in the wetland. The fractionation factors would then be comparable to the ones retrieved by Kaschl et al. 2005 (Kaschl et al., 2005) (αC = 1.0005) in the local anaerobic aquifer with MCB contamination or by Griebler et al. (2004a) and Mancini et al. (2003), obtaining significantly higher fractionation factors for the anaerobic degradation of benzene or trichlorobenzenes.

3.3. In situ microcosm experiment

[$^{13}C_6$] labelled MCB was used as stable isotope tracer in the *in situ* microcosm experiment in the wetland. The BACTRAPs were incubated directly in both soil compartments, at several distances from the inflow, as well as in the ponds. The incorporation of labelled carbon derived from the labelled substrate into bacterial fatty acids provided evidence for MCB degradation in the wetland system. In addition, [$^{13}C_6$] benzene, a possible intermediate, found on the BACTRAPs from both segments demonstrated the occurrence of reductive dehalogenation of MCB (Fig. 3). Even though the employed [$^{13}C_6$] MCB contained 0.02 % of [$^{13}C_6$] benzene as impurity, significantly higher amounts of [$^{13}C_6$] benzene were detected by GC-MS.

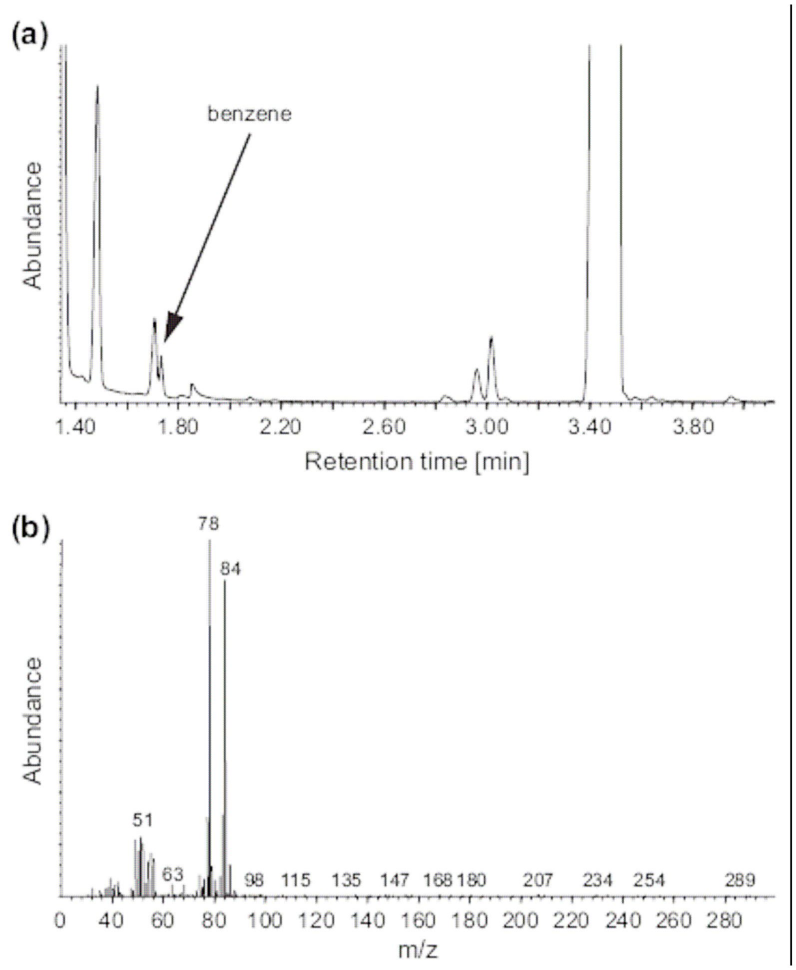

Fig. 3: GC-MS chromatogram (total ion current) of the *in situ* microcosm extract showing the ^{13}C-labelled benzene metabolite at retention time of 1.73 min and corresponding mass spectrum (a) with the molecule mass of m/z = 84 for [$^{13}C_6$] benzene and m/z 78 for [^{12}C] benzene (b).

Fatty acid composition

The composition of total fatty acid fractions extracted from *in situ* microcosms was compared to investigate variation in the microbial community. No systematic differences were observed between the oxic ponds and the anoxic segments of the wetland. However, the lowest quantity of fatty acids was retrieved from the microcosms exposed at 4.5 m in the soil compartments, and the highest accumulated biomass was retrieved from the microcosms deployed in the pond of the unplanted side of the wetland (Fig. 4).

The fatty acid patterns were dominated by high amounts of the saturated hexa- (C16:0) and octadecanoic (18:0) and the monounsaturated hexa- (C16:1) and octadecenoic (C18:1) acids which are common fatty acids in bacteria. The tetra- (C14:0), pentadecanoic (C15:0) and eicosanoic (C20:0) acids, the *iso* and *anteiso* isomers of C15:0 as well as the unsaturated C18:2 were present in lower abundance and could not be detected in all samples (Fig. 4).

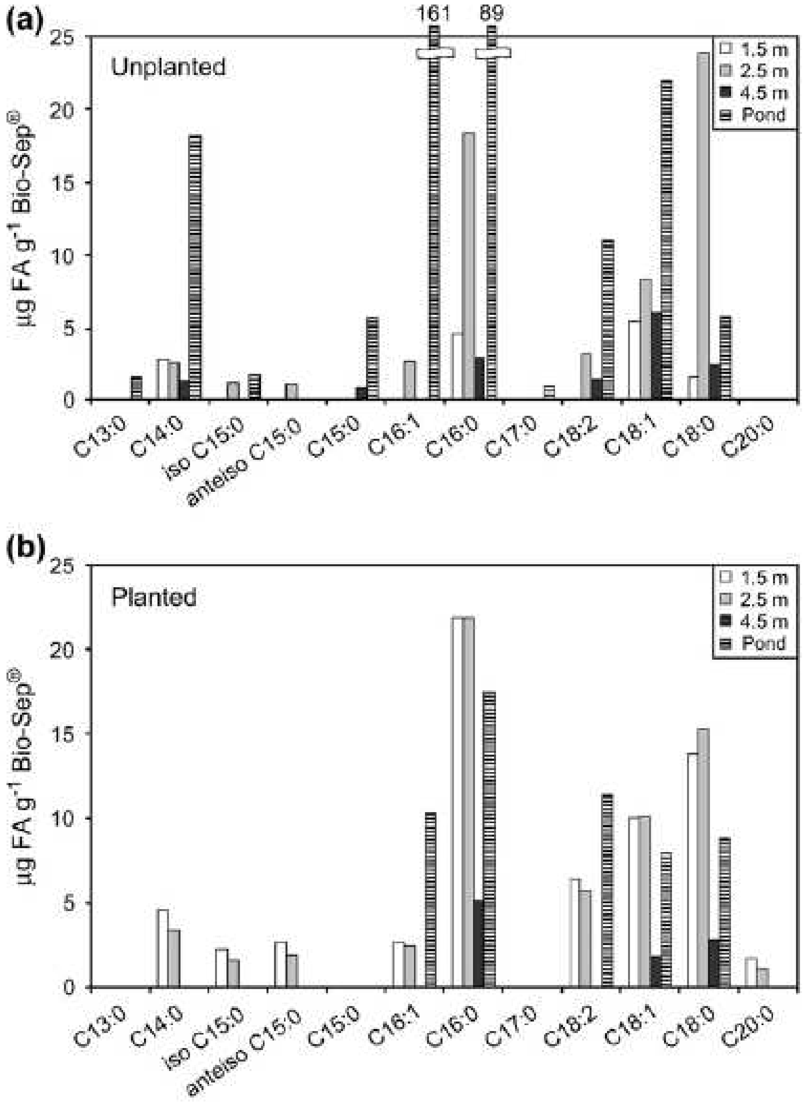

Fig. 4: Absolute abundance of extracted fatty acids [µg FA per gram Bio-Sep® beads] from *in situ* microcosms exposed in the soil compartment at different distance from the inflow and ponds at the unplanted (a) and planted (b) segments of the constructed wetland.

The variation of the geochemical parameters down gradient of the inflow were not related to a distinct change in fatty acid composition in both segments. No systematic differences in the fatty acid patterns were found between the planted and unplanted segments. Globally, no clear indications of microbial community changes were obtained on the basis of the fatty acid composition. In this experiment, fatty acid patterns were probably not sensitive enough to reflect changes in the microbial communities as a function of variation in geochemical conditions within the constructed wetland.

Isotope signatures of fatty acids

The total lipid fatty acids extracted from the *in situ* microcosms displayed some differences in the incorporation of ^{13}C into fatty acids. This was particularly obvious when comparing the samples from the soil compartments and the ponds. The isotope composition of fatty acids ($\delta^{13}C_{FA}$) extracted from the BACTRAPs amended with [$^{13}C_6$] MCB ranged between -39‰ and 7244‰ (Table 2). An enriched ^{13}C signature of fatty acids (> 0 ‰) can only stem from the microbial utilisation of [$^{13}C_6$] MCB as a carbon source. In contrast, fatty acids with an isotope signature lower than -20 ‰ showed the typical natural abundance of $\delta^{13}C_{FA}$ found in soil and aquifer material (Pelz et al., 2001b; Pombo et al., 2002). Fatty acids derived from parallel microcosms with non labelled MCB or from non amended *in situ* microcosms displayed an isotope signature ranging from -24‰ to -54‰ (data not shown). This represents a typical isotope composition of lipids derived from microorganisms feeding on organic substrates with natural isotope composition.

Table 2: Carbon isotope composition of fatty acids extracted from *in situ* microcosms incubated for 6 weeks with $^{13}C_6$ labelled monochlorobenzene at 1.5, 2.5 and 4.5 m from the inflow point as well as at the pond.

	Planted				**Unplanted**			
	$\delta^{13}C_{FA}$ [‰]				**$\delta^{13}C_{FA}$ [‰]**			
[m] from inflow	**1.5**	**2.5**	**4.5**	**Pond**	**1.5**	**2.5**	**4.5**	**Pond**
C13:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	118
C14:0	-6	40	n.d.	n.d.	138	204	116	238
iC15:0	121	89	n.d.	n.d.	n.d.	742	n.d.	1597
aC15:0	23	68	n.d.	n.d.	n.d.	3	n.d.	n.d.
C15:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-37	252
C16:1	411	453	n.d.	7244	n.d.	1832	n.d.	806
C16:0	2	9	93	1711	260	95	192	639
C17:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	242
C18:2	-31	-30	n.d.	65	n.d.	-27	-30	216
C18:1	-30	-29	-28	276	-23	-23	-24	175
C18:0	-27	-28	-24	449	-22	-27	-26	266
C20:0	-37	-39	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. – not detected

Generally, all fatty acids extracted from the *in situ* microcosms exposed in the ponds showed enrichment in ^{13}C (65‰ to 7244‰), which was generally of higher intensity than in the soil samples (-39‰ to 1832‰). In contrast, only fatty acids with up to 16 carbon atoms showed ^{13}C incorporation in the soil samples with highest enrichment in C16 species. Fatty acids with longer carbon chains such as C18:0 or C18:1 displayed no enrichment in ^{13}C (-22‰ to -39‰), indicating that the microbial community was not exclusively growing on the [$^{13}C_6$] MCB. Comparing the samples from the planted and unplanted segments no significant differences in $\delta^{13}C_{FA}$ were observed. Therefore, the putative impact of plants on the microbial community involved in MCB degradation could not be assessed using our test system.

Some fatty acids can be used as biomarker to identify specific groups of microorganisms (Kaur et al., 2005; Zelles, 1999). In some of the samples labelled *iso* and *anteiso* branched fatty acids with 15 carbon atoms could be identified, indicating that Gram-positive bacteria were involved in the biodegradation of MCB. Labelled C18:2 was only found in samples of the ponds. Linoleic acids (C18:2) can serve as a biomarker for fungi or other eukaryotic organisms (Lösel, 1988) and their presence may lead to the hypothesis that grazing organisms, not involved in the biodegradation of MCB, may feed on the microbial biofilm. However, the applied GC-MS procedure did not allow conclusive identification of the position of the double bond.

4. Discussion

The geochemical parameters indicated the overall prevalence of anoxic conditions associated with iron mobilisation in the soil parts of the wetland, whereas an aerobic milieu characterised the ponds. In the *in situ* microcosm experiments, the level of incorporation of labelled carbon into bacterial biomass was used as direct indicator of *in situ* MCB degradation. Interestingly, the analysis of the BACTRAPs incubated in the ponds revealed fatty acids patterns and ^{13}C incorporation levels differing from the ones retrieved from the soil compartments. The higher ^{13}C incorporation level observed in the ponds of both segments is indicative of a more effective microbial transformation of the [$^{13}C_6$] MCB under the prevailing aerobic conditions. Along with a significant accumulation of biomass on the microcosms retrieved from the ponds, these results suggest that some change in the microbial community dynamics may operate between the anoxic soil compartments and the more aerobic ponds. Moreover, the fact that all the extracted fatty acids showed incorporation in ^{13}C suggests that the microbial community established on the microcosms were mostly involved in contaminant degradation. However, it should be considered that cross feeding by metabolites or recycling of dead biomass within the biofilm may also channel labelled carbon into individual members of the microbial community, explaining the variation in the labelling of specific fatty acids. In general, fatty acids displaying a higher incorporation of ^{13}C were very likely synthesised by organisms feeding on [$^{13}C_6$] MCB, whereas organisms synthesising non-labelled fatty acids were likely not involved in the degradation of the labelled MCB and use different carbon sources. The analysis of the composition of total fatty acid fractions showed that this method might not be sensitive enough for investigating in detail changes in microbial communities between soil and water compartments as well as down gradient the flow path.

Additionally, the MCB degradation processes in the wetland were investigated by carbon stable isotope composition analysis. A correlation between decreasing MCB concentration and a shift in the carbon isotope signature towards the heavier isotope was visible along the flow path, suggesting the degradation of MCB. Toxic effects of MCB on the MCB-degrading population can be reasonably excluded at the observed range of concentration values (Fritz et al., 1992; Vogt et al., 2002; Vogt et al., 2004).

In the transition from the soil compartments to the ponds, a substantial contaminant mass depletion without concomitant isotope enrichment was observed. In this open system, it is likely that part of the contaminant may partition into the atmosphere, affecting the MCB concentration values without generating a significant isotope shift. Moreover, under oxic-prevailing conditions, several bacteria have the ability to use MCB as sole carbon and energy source, and may putatively adopt the well-known and described aerobic degradation pathways (Van Agteren et al., 1998). These bacteria may degrade MCB at faster rate than the degrading bacteria associated with anoxic conditions, contributing to the observed contaminant mass decrease. These oxygen-driven degradation reactions would lead to MCB mass decrease without a significant associated isotope effect (Kaschl et al., 2005). In parallel, biogeochemical processes such as oxidation of ferrous iron or mineral surfaces may compete for oxygen (Ehrlich, 1998; Søgaard et al., 2001; Warren and Haack, 2001), leading to transient conditions and oxygen gradients, which may affect the composition of the existing microbial community and rate of degradation reactions. However, the anaerobic degradation pathway of MCB is not elucidated yet.

In the anoxic soil compartments, two major hypothetical degradation pathways would come into consideration: 1) reductive dechlorination of MCB, and 2) degradation of MCB as an electron donor molecule. First, an initial dechlorination of MCB, followed by the degradation of benzene, is likely to occur in the soil compartments. Indeed, the presence of ^{13}C-labelled benzene detected in all the microcosms along with the detection of low benzene concentrations suggested that MCB is degraded reductively to benzene under anoxic conditions prior mineralisation. This pathway would suggest a similar pattern as observed previously by Nowak et al. (1996). The pH values were, however, constant, and the relatively high background level of chloride in the groundwater hindered the direct verification of MCB degradation by an increasing chloride concentration along the flow path. Bacterial breakdown of benzene could lead to the formation of benzoate or phenol as intermediates (Chakraborty and Coates, 2005; Edwards and Grbic-Galic, 1992; Lovley, 2000; Phelps et al., 2001; Ulrich et al., 2005), which were not detected in this study. Soluble organic carbon species, which could serve as electron donors for dechlorination processes, were found in low concentration in the unplanted segment, which might effectively limit the extent of degradation. Conversely,

dechlorination activity in the planted segment may be partly related to the abundance of hydrogen and reduced organic acids such as acetate and propionate (Holliger et al., 1992; Middeldorp et al., 1997).
Reductive dechlorination reaction of MCB to benzene is expected to be associated with a pronounced primary isotope effect (Griebler et al., 2004a). In contrast, the isotope composition presented in this study displayed a slight but significant enrichment ranging from 0.4 to 0.7 δ units, in the soil compartments of the planted and the unplanted segments, respectively. However, the isotope effect at zones of preferential *in situ* degradation reaction can be substantially higher. If other non fractionating processes such as sorption and volatilisation also contribute to a decrease in MCB concentrations, the isotope effect upon *in situ* degradation is expected to be relatively high. The observed isotope effect points to dominating anaerobic processes, as inferred by the estimation of biodegradation levels over the flow path. In the planted, the oxygen supplied by the plant at the rizosphere level may favour the establishment of aerobic zones. Although this process is not relevant for the electron budget, oxygen may contribute to the MCB degradation reactions, leading to MCB decrease without concomitant isotope effect. A fractionating anaerobic process and a less fractionating aerobic process may both contribute to *in situ* degradation, resulting in a mixed overall fractionation at the planted segment. Conversely, the isotopic composition shift observed at the unplanted segment suggests the occurrence of a more fractionating process.

Alternatively, MCB may be degraded as an electron donor molecule under ferric iron- or sulphate-reducing conditions. Anaerobic oxidation of benzene under these conditions has been previously observed (Anderson and Lovley, 2000; Anderson et al., 1998). For instance, geochemical footprints of iron reduction processes were found, and the ferrous iron mobilisation was increasing as a function of the flow path. Dissolved Fe in pore waters can be a result of different processes such as Fe(III) reduction (Lovley, 1991; Lovley, 1997), pyrite oxidation (Lord and Church, 1983), or Fe complexation (Luther et al., 1996). Fe(II) may be precipitated with sulphide originating from sulphate reduction activity or form complexes. Therefore the concentrations may not reflect the true extent of iron reduction in the presence of sulphate reduction. A low extent of sulphate reduction process and the availability of reactive iron may prevent accumulation of H_2S in the near-neutral conditions of the wetland. A MCB mineralisation by sulphate reduction contributing to the contaminant mass decrease is feasible and may be possible. However, due to the high concentrations of sulphate in the supplied water, a reliable estimation of the extent of sulphate reduction could not be carried out. However, a reduction of ferric iron directly linked stoichiometrically to MCB oxidation in the wetland may theoretically account for a MCB mass decrease of about 18% between the inflow and the outflow of the system at both the planted and unplanted segment.
Additionally, other unknown degradation pathways can not be excluded. These hypotheses will require further detailed investigations, along with further isolation and identification of microorganisms involved in the anaerobic MCB degradation in wetlands treating MCB contaminated water.

5. Conclusion

The integrated approach provided evidence for *in situ* MCB biodegradation in both, soil compartments and ponds of the planted and unplanted segments of a horizontal subsurface flow constructed wetland. This was supported by isotopic fractionation analysis, combined with *in situ* microcosm experiments, which can be utilized to document further the *in situ* degradation of MCB and other contaminants in wetland systems. Further investigations to elucidate the microbial degradation of MCB, facilitated by an integrated approach and combined with a high resolution sampling, are required to evaluate zones of enhanced *in situ* biodegradation of MCB and to optimise wetland systems treating contaminated groundwater.

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Section 2: Tracking in situ biodegradation of 1,2- dichloroethenes in a model wetland

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Abstract

The spatial and temporal biogeochemical development of a model wetland loaded with *cis*- and *trans*-1,2-dichloroethene contaminated groundwater was characterized over 430 days by hydrogeochemical and compound-specific isotope analyses (CSIA). The hydrogeochemistry dramatically changed over time from oxic to strongly reducing conditions as emphasized by increasing concentrations of ferrous iron, sulphide and methane since day 225. $\delta^{13}C$ values for *trans*- and *cis*-DCE substantially changed over the flow path and correlated over time with DCE removal. The carbon enrichment factor values (ε) retrieved from the wetland became progressively larger over the investigation period, ranging from -1.7 ± 0.3 ‰ to -32.6 ± 2.2 ‰. This indicated that less fractionating DCE oxidation was progressively replaced by reductive dechlorination, associated with a more pronounced isotopic effect and further confirmed by the detection of vinyl chloride and ethene since day 250. This study demonstrates the linkage between hydrogeochemical variability and intrinsic degradation processes and highlights the potential of CSIA to trace the temporal and spatial changes of the dominant degradation mechanism of DCE in natural or engineered systems.

Synopsis

The spatial and temporal variability of dominant DCE degradation pathways was evaluated in a model wetland by coupling hydrogeochemical analysis and CSIA

1. Introduction

Wetlands are widespread ecotones covering interfaces between terrestrial and aquatic ecosystems. These biogeochemically heterogeneous systems can be hydrologically connected to an underlying contaminated aquifer. Therefore, they may play a crucial role in the control of water quality of surface water systems (1). However, knowledge about biogeochemical processes associated with transformation of contaminants in wetlands remains limited. Simulating natural wetlands, constructed wetlands (CW) may capitalize intrinsic physical, chemical and biological processes for the purpose of water remediation. Recently, several attempts have been made to extend the application of CW for treating chlorinated solvents (2-5).

For instance, the *cis*- and *trans*-1,2-dichloroethene isomers (DCE) often accumulate in anaerobic aquifers and are considered as priority pollutants due to their widespread presence and toxicity (6). During microbial degradation, DCE can serve either as electron donor or as electron acceptor mainly depending on the geochemical conditions and the intrinsic microbial ecology. In aerobic systems, oxidation of DCE has been observed to occur metabolically (7,8) or cometabolically (9). Alternatively, anaerobic oxidation of DCE has been demonstrated in laboratory experiments under Mn(IV)-, Fe(III)- or SO_4-reducing conditions (10,11). Under strongly reducing conditions, biotic reductive dechlorination may result in DCE transformation to non-toxic ethene *via* carcinogenic vinyl chloride as intermediate compound (12). Thus, DCE transformation is feasible by several pathways mirroring the prevailing biogeochemical characteristics of an environmental system. DCE can then be used as model compounds to follow-up the spatio-temporal variability of environmental conditions with respect to the dominant biodegradation mechanism in complex environmental systems.

Physico-chemical and biological processes associated with natural attenuation of organic contaminants are often monitored in contaminated groundwater systems but more rarely in wetlands. The hydrogeochemical conditions in the subsurface can generally be delineated and statistically analysed over time along with variations in contaminant and metabolites concentrations (13,14). Furthermore, compound specific isotope analysis (CSIA) allows a qualitative and/or quantitative integrative assessment of biological transformation of organic contaminants over a flow path in environmental system (15). The mechanism of a chemical reaction results in a characteristic isotope effect and isotope fractionation analysis can then be used to identify biodegradation pathways. The characterization of biodegradation processes in environmental systems generally relies on laboratory enrichment factors (ε) obtained from cultures with known degradation pathways (16). For instance, the reported enrichment factors determined for cometabolic aerobic *cis*- and *trans*-DCE isomers degradation (17,18) were found to significantly differ from those determined for anaerobic degradation *via* reductive dechlorination (19,20). Attributing these variations to mechanistically different degradation pathways, CSIA can help distinguishing DCE oxidation from reductive dechlorination in complex environmental systems experiencing spatial and temporal variability of redox conditions.

This study focuses on the spatial and temporal development of biogeochemical processes in a model wetland treating *cis*- and *trans*-1,2-dichloroethene contaminated groundwater with emphasis on the characterization of degradation pathways. While the system as a whole is dynamic, specific geochemical processes may dominate on a spatial and/or temporal scale and control the contaminant transformation mechanism. Therefore, the variability of hydrogeochemical indicators and carbon stable isotope composition of *cis*- and *trans*-DCE in pore water was assessed at high spatial resolution across the wetland over 430 days. Multivariate analysis was used in order to retrieve additional insight into the spatial and temporal trends of biogeochemical development and the relationships between the hydrogeochemical variables. To our knowledge, this is the first report of detailed characterization of biogeochemical processes associated with degradation DCE compounds in a wetland system.

2. Material and Methods

System design. A general scheme of the model constructed wetland and detailed experimental design information are provided in Fig. S1 and S2. The model horizontal subsurface flow wetland consisted of a stainless steel tank (201 x 60 x 5 cm), filled with quartz sand and planted with the common rush (*Juncus effusus*, L.). The supplied groundwater was collected bimonthly from a local, well-characterized contaminated aquifer (21,22) in 50 L tanks and maintained under anaerobic conditions at constant N_2 pressure. The hydrogeochemistry of the groundwater was constant over time and *cis*- and *trans*-1,2-DCE were the dominant contaminants. Detailed information regarding the groundwater quality is provided in SI (section 1.2 and table S1). Additional amounts of contaminant were spiked into the tanks during the groundwater sampling to reach average concentrations of 6.5 and 1.5 mg L^{-1} for *cis*- and *trans*-DCE, respectively. During five months preceding the investigation as well as during the investigation period, the system was operated in a flow-through mode with a rate of 1.8 L d^{-1}

contaminated groundwater, corresponding to a retention time of approximately 15 days.

Sampling procedure. Pore water samples were collected 18 times at regular intervals (two or three weeks) over 430 days for hydrogeochemical investigation, concentration and isotopic composition analysis of chlorinated ethenes. Pore water samples were retrieved at the inflow chamber, at 12 sampling ports across the sand compartment and at the pond (SI, Fig. S1 and section 1.3). Four vertical profiles across the sand compartment located at 6, 49, 94, 139 cm from the inflow, each consisting of 3 sampling ports at 20, 32, and 44 cm depth below the surface, were systematically investigated. *In situ* oxygen measurements were performed in parallel at discrete resolution (Δ ~ 6 cm) along the four vertical profiles across the soil compartment.

Pore water geochemistry and contaminant concentration analysis. The sampling and analytical methods are described in the supporting information (section 1.4.).

Compound specific isotope analysis and definitions. Stable carbon isotope compositions of the chlorinated ethenes were measured using a gas chromatography-combustion-isotope ratio mass spectrometry system (GC-C-IRMS) as described earlier (22,23). The detailed procedure of stable isotope analysis and definitions are provided in SI (section 1.5). Briefly, estimates of the wetland isotope fractionation factor α were derived from the slope of the standard linear regression plot applying the log-based Rayleigh equation according to Mariotti *et al*. (24). Fractionation factors were then converted into carbon enrichment factors (ε). Two data treatment were performed. First, a single and integrative carbon enrichment factor, further referred as “mean isotope enrichment factor”, was derived for each DCE isomer at each sampling date, taking the inflow chamber values as the initial values. For this purpose, discrete-depth concentrations and isotopic composition values retrieved at each of the four vertical profiles across the sand compartment of the wetland were separately averaged before a mean enrichment factor was calculated. Second, assuming laminar flow in the system, isotope composition and concentration data were also evaluated separately for each investigated depths. Three enrichment factors were then retrieved for each specific flow path in the sand compartment per sampling date and per DCE isomer, taking the inflow chamber values as initial values.

Statistical analysis. The significance of the correlation of the standard linear regression for further derivation of the carbon enrichment factor ε was evaluated based on the coefficient of determination (R^2) of the linear regression curve and on *F*-statistics (ANOVA).

The hydrogeochemical and isotope data from day 0 to 127 and from day 127 to 430 were separately subjected to principal component analysis (PCA). PCA were performed on the basis of the correlation matrix and the numerical data matrices were converted using the program R (R: Copyright 2005, The R Foundation for Statistical Computing, Version 2.1.1).

3. Results and Discussion

3.1. Biogeochemical development of the wetland

Two main phases could be clearly distinguished over the investigation period based on the hydrogeochemical analysis (Table S1). During the first phase (from day 0 to day 127), oxic conditions prevailed in the system, as inferred from the mean dissolved oxygen concentration > 1.5 mg L^{-1} and low concentrations of reduced redox-sensitive constituents. The oxygen concentration globally decreased over depth and increased over the flow path, indicating the occurrence of spatial gradients within the system (Fig. S2). Oxygen diffusion and/or advection through the water column and sand combined with oxygen transport in the subsurface *via* plant tissues likely contributed to the establishment of the aerobic-anaerobic interfaces. During the second phase (from day 127 to 430), lower mean dissolved oxygen concentrations (< 0.5 mg L^{-1}) in the system indicated overall anoxic conditions. Moreover, the increase of reduced species concentration values (NH_4^+, Fe^{2+}, sulphide and CH_4) suggested parallel activity of several microbially mediated redox reactions in the wetland. This period also coincided with intense precipitation of iron sulphide minerals. Since day 199, the precipitates formed a front of black patches in the sand compartment. The front progressed from the inflow region along the flow path at about 1.5 cm d^{-1} (Fig. S2). This clearly points to the prevalence of anoxic conditions in the sand compartment.

To highlight the spatio-temporal variability of hydrogeochemical patterns across the wetland and to support the interpretation of biogeochemical processes, the hydrogeochemical data sets corresponding to each phase were separately subjected to principal component analysis (Fig. 1). In the two analyses, the percent of variance amounted within the data for the first (PC1) and second (PC2) principal components was > 50%. Isotopic composition values of DCE showed negative correlation with DCE concentration values (Pearson's product-moment correlation < -0.34). This strongly suggests a mechanistic relationship between stable isotope fractionation and contaminant depletion. Moreover, the enrichment of ^{13}C in the residual *cis*- and *trans*-DCE fractions provides a qualitative evidence of microbiological degradation as a mechanism contributing to DCE removal in the model wetland over time.

The PCA plot of variables corresponding to the first phase (day 0 to 127) permitted to highlight geochemical variables associated with changes in the isotopic signatures (Fig. 1A). $\delta^{13}C$ values for *trans*- and *cis*-DCE are positively correlated with chlorine, sulphate, redox potential and oxygen. This suggests a possible linkage between these geochemical indicators and degradation activity. At wetland zones displaying relatively higher redox potential, DCE oxidation reaction may preferentially be linked to oxygen or, at lower redox conditions, to sulphate, with concomitant release of chlorine. However, high background concentration of chlorine in the supplied groundwater (127 ± 9 mg L^{-1}) hindered a concentration-based sensitive analysis of the fraction released during DCE degradation. Furthermore, significant isotope fractionation under prevailing oxic geochemical conditions (Table S1) with no detection of reductive dechlorination metabolites (*i.e.* VC, ethene) supports the occurrence of oxidative degradation processes. In the PCA plot of sampling locations (Fig. 1B), no clusters could be distinguished and samples mainly grouped close to the origin of the biplot, independently on their spatial or temporal origin. This emphasises no clear spatio-temporal trends in the evaluated biogeochemical processes and relatively homogeneous hydrogeochemical conditions between day 0 and 127.

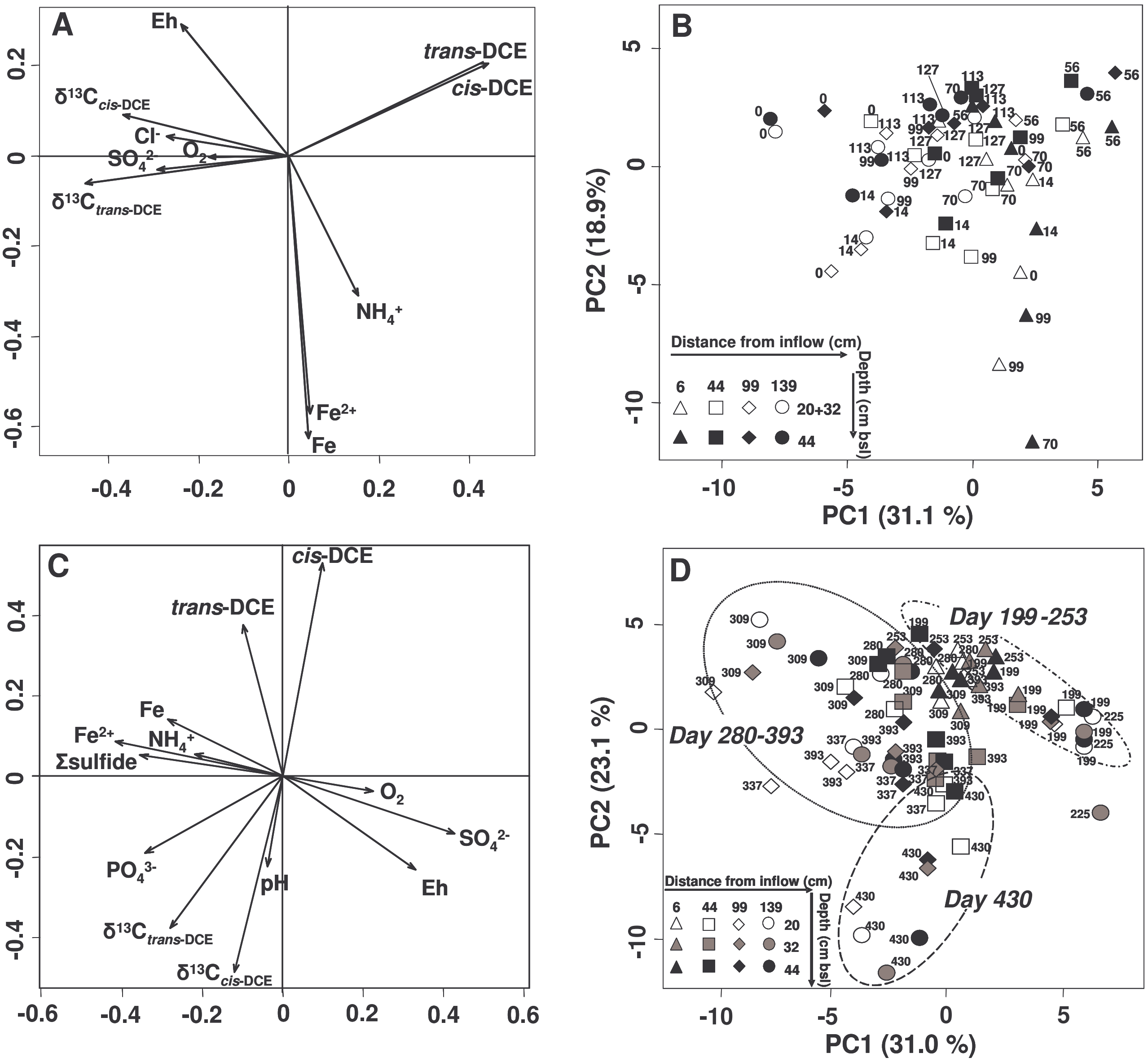

Figure 1: PCA ordination plot of hydrogeochemical characteristics and isotope composition values of porewater samples collected from the model constructed between day 0 and 127 (A, B) and between day 127 and 430 (C, D) of the investigation period. Values on the axes indicate % of total variation explanation by the corresponding axis (PC 1, principal component axis 1; PC 2, principal component axis 2). Description vectors correspond to: $\delta^{13}C_{DCE}$=carbon isotope signature of *cis*- or *trans*-DCE; Fe^{2+}=ferrous iron; Fe=tot. Total iron; SO_4^{2-}, sulphate; Σsulphide= total sulphide; Cl^-, chloride; O_2=oxygen; NO_3^-= nitrate; NH_4^+=ammonium; PO_4^{3-}= phosphate; Eh= redox potential. Objects corresponds to the samples retrieved at different sampling days from sampling ports at 6 (Δ), 44 (□), 99 (◊) and 139 (○) cm from the inflow of the wetland and at 20 (white) and/or 32 (grey) and 44 (black) cm below the surface level (cm bsl).

In the PCA plot of sampling locations corresponding to the second phase (day 127 to 430), sampling locations from the early anoxic (day 199 to 253), intermediate (day 280 to 393) and late sampling (day 430) periods could be distinguished (Fig. 1D). This suggests an overall temporal variation of the hydrogeochemical patterns. Higher PC1 and PC2 scores indicated lower extent of fractionation and less reducing conditions. Scores of PC1 correlated positively to sulphate, redox potential and oxygen (loadings > 0.2) and negatively to ferrous iron, sulphide and ammonium (loadings < -0.2) in the plot of variables (Fig. 1C). This emphasizes the importance of redox sensitive indicators to distinguish clusters of sampling locations and substantiates the expected negative correlation over space and time between reduced and oxidized redox species. In the second phase, sampling locations from day 199 to 253 globally clustered positively along PC1 and PC2 and were associated with higher contaminant concentrations and indicators of less-reducing conditions (higher O_2 concentrations and redox potential values). Indeed, this transient period corresponded to the beginning of iron- and sulphate-reduction processes and moderate reducing conditions. From day 225 to 430, reductive dechlorination occurred in the system as inferred from increasing vinyl chloride and ethene concentrations (< 5 to 518 ± 13, and < 5 to 102 ± 5 μg L^{-1}, respectively). Microbial reductive dechlorination guilds require a sustainable source of electron donors such as fermentable organic compounds (25). Average outflow/inflow COD, TOC and DOC concentration ratios higher than 2 in the second phase (Table S3) indicated the production of organic compounds in the wetland. This was likely due to plant leached organic carbon and supports the occurrence of conducive conditions for reductive dechlorination. Sampling locations from day 280 to 393 clustered negatively along PC1 and were associated with larger amounts of reduced species (Fe^{2+}, NH_4^+ and sulphide). During this period, enrichment in ^{13}C coincided with reductive dechlorination of DCE and the simultaneous occurrence of various reduction processes. Finally, samples from day 430 clustered separately and were associated with higher $\delta^{13}C_{DCE}$ values. This emphasizes a substantial change in the magnitude of the isotope fractionation coinciding with evidences for reductive dechlorination activity in the system. Overall, a predominant temporal variability of biogeochemical processes over spatial variability was observed in the second phase. However, various biogeochemical reactive zones occurred across the system putatively influencing transformation of DCE over the flow path. It mainly explains the differences between hydrogeochemical patterns within each cluster of sampling locations. Nevertheless, the converging geochemical evolution of discrete wetland zones over time towards more reducing conditions favoured a prevailing degradation mechanism at the system scale.

PCA were performed on the basis of complex datasets consisting of a large number of samples and variables collected at multiple regular intervals over a reasonably long period. This strategy permitted a robust and integrative evaluation of dynamic biogeochemical processes occurring in the wetland system. In particular, the combination of both hydrogeochemistry and isotope data in a single analysis allowed proposing mechanistic interpretation of contaminant degradation.

3.2. $\delta^{13}C$ Values

To interpret in more details the observed hydrogeochemical changes with respect to DCE biodegradation, isotope fractionation accompanying contaminant depletion over the flow path was analysed. The relation between isotope fractionation and DCE removal over the flow path is exemplified for two representative sampling dates (day 14 and 430) in Fig. 2. During the investigation period, the removed fraction of DCE was similar for both isomers and generally increased across the flow path with respect to the initial concentrations. The removed fraction of DCE ranged between 0 and 60 % in the sand compartment (Fig. S3), whereas it reached 74 ± 16 % (n=18) at the pond. A parallel significant isotopic shift for *trans*- and *cis*-DCE ($\delta^{13}C$ > 0.5 ‰) could be observed between the inflow and the end of the sand compartment for 9 and 11 sampling campaigns, respectively. The largest shifts in the DCE isotopic composition were observed at day 393 and 430, and reached 8.1 ± 3.0 ‰ and 28.8 ± 1.5 ‰ for *trans*- and *cis*-DCE, respectively. These values were 7 to 50 fold larger compared to previous sampling dates for a similar fraction of DCE removal. This change in the fractionation patterns coincided with the detection of VC and ethene since day 225, but concentrations were generally too low for determining the isotopic composition. However, at day 430, a broad range of isotopic signatures for vinyl chloride existed across the wetland (VC = -43.8 ± 0.1 to -23.1 ± 0.2 ‰). This suggested various stages of DCE conversion *via* reductive dechlorination across the wetland. Indeed, the newly formed VC would be initially lighter than non-degraded DCE (*cis*-DCE = -22.5 ± 0.2; *trans*-DCE = -17.9 ± 0.2 ‰) and may then become heavier in the course of

DCE degradation as well as through further dechlorination to ethene (20).

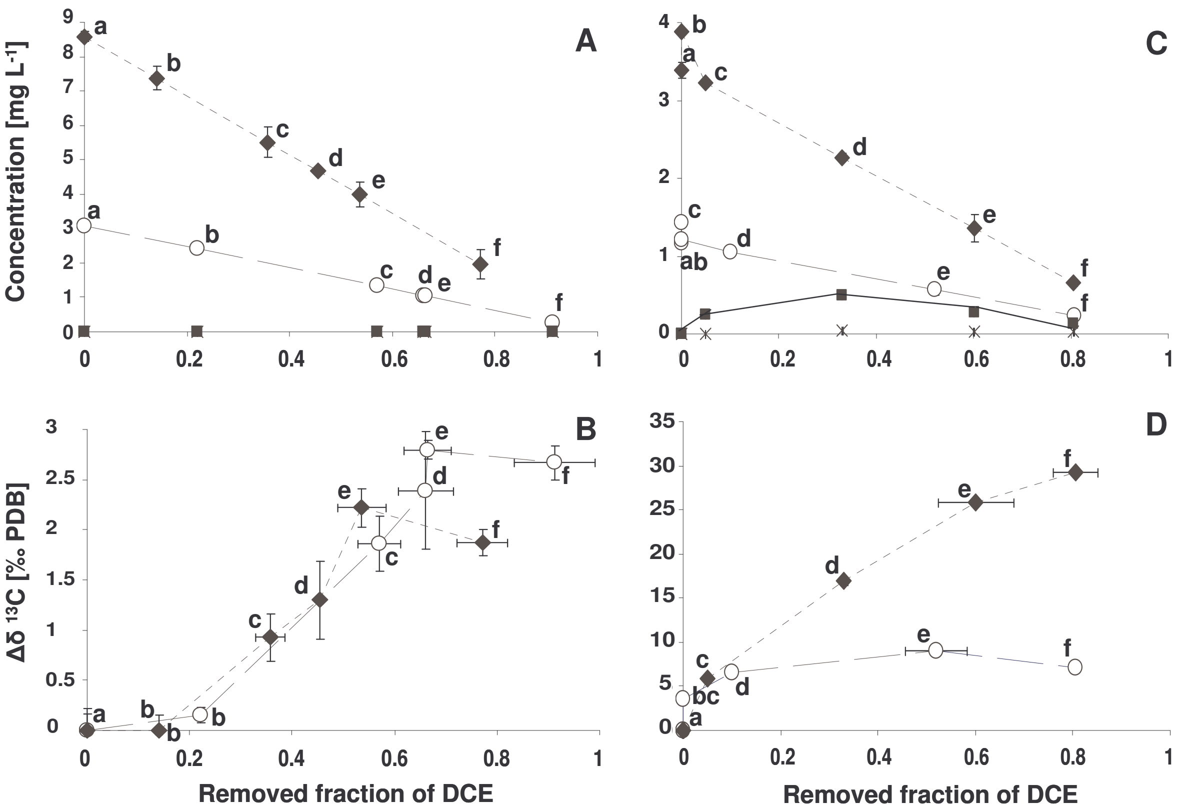

Figure 2: Change in concentrations (A, C) of *cis*-DCE (♦), *trans*-DCE (○) and their products vinyl chloride (■) and ethene (X) and shift in carbon isotopic composition (B, D) of *cis*-DCE (♦), *trans*-DCE (○) in the model wetland as a function of the removed DCE fraction over the flow path at 44 cm depth. Figures A and B correspond to day 14 (first phase) and figures C and D to day 430 (second phase) of the investigation period. Letters *a* to *e* correspond the investigated distances across the wetland: a: 0 cm (inflow); b: 6 cm; c: 49 cm; d: 94 cm; e: 139 cm and f: 175 cm (pond).

The ability of CSIA to distinguish between mass losses due to biodegradation *versus* mass loss due to non-degradative processes permitted assessing DCE biodegradation in the wetland. Indeed, non-destructive processes such as dissolution, volatilization, and sorption of chlorinated ethenes were reported to cause no significant isotope fractionation (26-29). Moreover, biological degradation of DCE is very likely to largely predominate in the wetland and hypothetical abiotic transformation to proceed at very low rate (30). However, mass balancing of contaminants in wetland system is often complicated, because the sorbed, volatilized and biodegraded fractions of DCE can hardly be differentiated *in situ*. The relative contribution of sorption to the overall contaminant removal is assumed to be negligible, as DCE display a relatively low K_{ow} and very likely reached sorption equilibrium after five months of groundwater inoculation preceding the investigation. Maximal DCE removal was observed at the pond and amounted to about ¾ of the initial contaminant load. The removed fraction integrates both volatilization and degradation losses. However, very low DCE volatilization rates are likely occurring in the sand compartment due to very slow flow regimes (4) and slow diffusion rates through the unsaturated zone. Furthermore, a wetland study based on the more volatile TCE suggests low transfer of DCE in the atmosphere (2). This is also supported by the absence of substantial DCE removal at day 56, 70, 113 and 127 (Fig. S3) and the distribution of removed DCE fraction over the depth profiles (Table S4).

3.3. Carbon Enrichment Factors

Carbon isotopic enrichment factors were derived over the investigation period to integrate the isotope composition changes as a function of contaminant mass depletion over the flow path in the wetland. The mean isotope enrichment factors for *cis*- and *trans*-DCE ranged from -1.7 ± 0.3 ‰ to -32.6 ± 2.2 ‰ and became progressively larger over time (Fig. 3 and Table S5). While factors for *cis*- and *trans*-DCE were generally in the same range during the first investigation period, fractionation patterns of *trans*-DCE more rarely fitted a Rayleigh model. Differences in aerobic degradation patterns between similar isomeric forms of chlorinated compounds have also been noted previously (31,32). From day 0 to 225, stable isotope enrichment factors for *cis*-DCE reproducibly ranged between -1.7 ± 0.3 ‰ to -4.4 ± 0.8 ‰. This corresponds to the range of enrichment factor values obtained under aerobic cometabolic degradation for DCE in laboratory experiments (17,18). Together with the data of the hydrogeochemical analysis, these results indicate prevailing occurrence of degradation *via* oxidative pathways during this period. Since day 225, gradually larger mean enrichment factors for *cis*-DCE were retrieved. Since day 337, the mean isotope enrichment factors were in the characteristic range of values obtained under reductive dechlorination of *cis*- and *trans*-DCE by enrichment or pure cultures (range from -14.1 to -30.1‰ (19,20,33)). The occurrence of reductive dechlorination was further evidenced by the systematic detection of *Dehalococcoides* sp. related DNA in the sand compartment between day 225 and 430 (data not shown). Considering depth-discrete flow paths, larger enrichment factors for *cis*-DCE were found at lower depths at days 225, 337 and 393, whereas an opposite pattern was found at day 430 (Fig. S4 and Table S6). This can be explained by the occurrence of more pronounced oxic-anoxic interfaces before day 430. Indeed, dissolved oxygen concentration values were slightly higher than 0.5 mg L^{-1} until day 393 at about one third of the 32 measured locations of the wetland (data not shown). In the CW, stronger fractionating reductive dechlorination may occur in anaerobic zones adjacent to aerobic zones where less fractionating oxidative processes occur. Indeed, oxygen diffusion may longer contribute to DCE degradation *via* oxidative pathways in the upper layers of the CW. This results in lower enrichment factors than in deeper sediment layers.

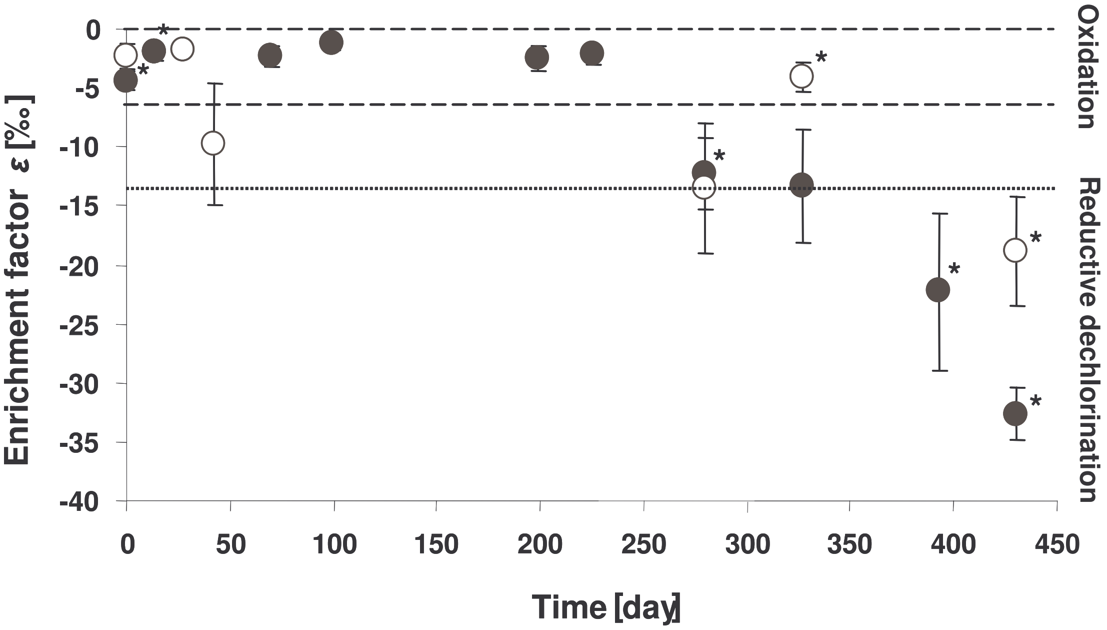

Figure 3: Temporal changes in carbon enrichment factors of *cis*-1,2-DCE (●) and *trans*-1,2-DCE (○) obtained by fitting a Rayleigh model to concentration and isotope composition data. The enrichment factors correspond to mean data retrieved from transects across the wetland at 20, 32 and 44 cm depth below the surface. The plotted enrichment factors were retrieved when the coefficient of determination (R^2) of the linear regression curve of the respective Rayleigh model was > 0.5 and (*) indicates values significantly fitting the model at $P < 0.05$. Dashed lines delimit the ranges of enrichment factors reported in the literature for DCE degradation *via* oxidation pathway (---) and reductive dechlorination (…). Error bars show the standard error of the regression.

Overall, the progressive change towards more pronounced isotope fractionation over the investigation period or with increasing depth mirrored the development of geochemical conditions. It also emphasized the succession of degradation mechanism of DCE on both a spatial and temporal scale. The range of kinetic isotope fractionation in the wetland may offer further characterization on the mechanism of the degradation reaction. One the one hand, the reductive dehalogenation reaction includes a C-Cl bond cleavage, which generally results in significant isotope effects (23,33). On the other hand, the oxidative degradation of DCE and vinychloride is suggested to be initiated by an epoxidation reaction of the C=C bond (19,34,35), which results in lower fractionation factors (36). Thus, the observed fractionation pattern in complex systems would mainly depend on the respective contribution of degradation reactions occurring in oxic and anoxic compartments intercepting the flow path. However, a quantitative estimation of the relative contribution of each DCE degradation pathway based on an intermediate enrichment factor integrating both reactions remains limited. Indeed, preliminary knowledge about the enrichment factors corresponding to each pathway before their co-occurrence would be required. Furthermore, the existence of micro- and macroheterogeneity sustaining strongly varying local reaction conditions in the wetland affects the relationship between measured isotope signatures and the extent of contaminant degradation. This hinders a direct quantitative estimation of biodegradation by the mean of the Rayleigh equation holding for homogenous reaction media.

CSIA allow distinguishing degradation pathways if the respective ranges of enrichment factors associated with oxidative degradation and with reductive dechlorination of DCE differ from each other under field conditions. However, wetlands may sustain dynamic microbial communities with a widely distributed potential among a variety of microorganisms for both metabolically or cometabolically DCE mineralization and reductive dechlorination (37,38). Therefore, when one single pathway predominates in the system, diverse DCE degrading microbial populations may generate different fractionation patterns. This would result in an intermediate enrichment factor integrating the co-occurring fractionation patterns. For instance, large differences in carbon isotope fractionation were already observed during aerobic cometabolic degradation of TCE (18,39) as well as during reductive dechlorination of PCE and TCE by different dehalorespiring bacteria (23). These examples emphasize the possible extent of the ranges of variation of DCE enrichment factors for oxidative degradation and reductive dechlorination. An overlapping between these ranges would complicate the interpretation and would require detailed insight into the hydrogeochemistry, intrinsic degrading community, and/or reaction mechanism. Moreover, stable isotope fractionation patterns potentially associated with the hypothetical oxidative transformation of chloroethenes under anaerobic conditions is not currently understood and may differ to that associated with oxidative dechlorination under aerobic conditions. Further studies are clearly necessary to understand and delineate the possible range of variation of enrichment factors associated with each major DCE degradation pathway. Nevertheless, our results support the relevance of CSIA coupled with hydrogeochemical analysis to assist in identifying over space and time the prevailing DCE degradation pathway in hydraulically well-characterized engineered or natural field settings subjected to variation of geochemical conditions. In this case, the change of isotope enrichment factors characterizes the transition from aerobic to anaerobic conditions and reflects a compartmentalized environment leading to heterogeneous reaction conditions.

3.4. Characterizing Degradation of Organic Contaminants in Wetlands

Wetland systems may sustain on a spatial and/or temporal scale degradation of organic chemicals subjected to both anaerobic and aerobic pathways. However, the potential for organic chemicals degradation over the system lifespan is determined by the respective contribution and significance of occurring elimination pathways. Therefore, differentiating pathways is crucial as transformation rates and toxicities of the intermediate metabolites may vary according to the prevailing pathway. In this study, CSIA permitted capturing information on biogeochemical variability occurring in the saturated zone of a model wetland. This provided an opportunity to elucidate possible underlying causes. In particular, CSIA permitted identifying and tracing the prevailing mechanism of DCE degradation over space and time in a complex environmental system. It supports that degradation of DCE occurs in wetlands *via* both oxidative and reductive pathways, with relative contribution mainly depending on the variability of hydrogeochemical conditions. Application strategies including quantification aspects could be conceived in environmental systems if future research permits delineating mechanism-specific ranges of enrichment factors for DCE degradation. Furthermore, the combination of hydrogeochemical analysis and CSIA aided with multivariate statistics represents a promising approach for interpreting wetlands biogeochemical development with respect to organic chemicals degradation. Indeed, the evaluation of spatial and temporal trends is critical for evaluating and predicting the biodegradation potential over the system lifespan since the hydrogeochemistry may control and/or reflect specific patterns of contaminant degradation. Finally, coupling isotope fractionation and hydrogeochemical analysis is of interest to understand factors affecting the transformation of contaminant in natural or constructed wetlands. Knowledge about processes may then support optimization of wetland systems with respects to natural attenuation of organic contaminants.

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Supporting Information Available

Detailed schemes of the model wetland. Pore water geochemical analysis procedure and hydrogeochemical analysis. Detailed carbon isotope enrichment factors over the investigation period.

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Section 3: Characterization of microbial communities in a model constructed wetland treating 1,2-dichloroethenes contaminated groundwater

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Abstract

The bacterial community and the dehalogenating guild of bacteria were characterized in a model wetland supplied with *cis*- and *trans*-1,2-dichloroethenes (DCE) contaminated groundwater. PCR-DGGE analysis revealed that changes of the bacterial community structures coincided with an evolution of the hydrogeochemistry, from oxic towards reducing conditions. During this transition, detection of vinyl chloride and ethene indicated reductive dechlorination of DCE, which correlated with the simultaneous presence of putative dehalogenators (*Dehalococcoides*, *Geobacter* and *Dehalobacter*). 16S rRNA gene libraries revealed changes of the bacterial composition between the supplied groundwater and the model wetland pore water. *Proteobacteria* accounted for > 50 % of 16S rRNA genes clone libraries, whereas about 17 % of the sequences from the wetland could be related to sulphate reducers. This study demonstrated that a shift of the DCE degradation mechanism at the model wetland scale were associated with concomitant changes of geochemical conditions, bacterial communities and composition of the putative dechlorinating guild.

1. Introduction

Lower chlorinated solvents often accumulate in anoxic aquifer systems (MaymoGatell et al., 1997), whereas they may be subjected to destructive natural attenuation processes in biogeochemically heterogeneous wetlands intercepting groundwater discharges of contaminant plumes (McCarty and Semperini, 1994; Lorah and Olsen, 1999; Amon et al., 2007). For instance, in strictly anoxic plumes, dichloroethenes (DCE) generally originate from sequential biotic dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), and tend to be recalcitrant to further degradation (MaymoGatell et al., 1997; McCarty and Semperini, 1994; Vogel, 1994). In wetlands, the bulk of water quality improvement is generally attributed to microbial activity (Reddy and DAngelo, 1997; Stottmeister and Wiessner, 2003), which may play a critical role in limiting transport of chlorinated solvents to surface water bodies. Indeed, at wetland areas located down gradient a contaminant plume, several degradation pathways are expected to simultaneously contribute to DCE degradation. These degradation processes are assumed to be controlled by a complex and dynamic assemblage of adjacent aerobic and anaerobic zones (Armenante et al., 1992; Master et al., 2002; Meade and D'Angelo, 2005). For instance, in aerobic zones, oxidation of DCE can occur metabolically (Bradley and Chapelle, 2000; Coleman et al., 2002) or cometabolically (Kim and Semprini, 2005). Alternatively, anaerobic oxidation of DCE under Mn(IV)-, Fe(III)- or SO_4-reducing conditions has been demonstrated in laboratory experiments (Bradley and Chapelle, 1998; Hata et al., 2004). Under reducing conditions, DCE can also be subjected to biotic reductive dechlorination and further transformed up to non-toxic ethene, *via* carcinogenic vinyl chloride (VC) as an intermediate compound (Maymo-Gatell et al., 1997). However, information on microbial diversity and processes in sub-surface wetlands treating organic chemicals is scarce. In particular, little work has been done on wetland-inherent microbial community structure dynamics and functional composition associated with the *in situ* biodegradation of chlorinated solvents.

Simulating natural wetlands, constructed wetlands may capitalize intrinsic physical, chemical and biological processes for the purpose of water remediation. For instance, constructed wetlands have been recently investigated with respect to their potential function of chlorinated solvents removal, based on the spatiotemporal analysis of contaminant and degradation metabolites concentrations and/or their isotopic compositions (Kassenga et al., 2003; Keefe et al., 2004; Amon et al., 2007; Braeckevelt et al., 2007; Imfeld et al., 2008). This approach can be efficiently coupled to direct knowledge on the indigenous microbial community in order to gain better understanding of biogeochemical processes related to contaminant removal. For instance, culture-independent molecular approaches offer additional insights into bioremediation processes in wetlands hydraulically connected with groundwater systems by providing key-information about phylogenetic diversity, composition or/and structure of autochthonous wetland microbial communities (Nocker et al., 2007; Weiss and Cozzarelli, 2008). In particular, DNA fingerprinting techniques, such as PCR-denaturing gradient gel electrophoresis (DGGE) as well as 16S rRNA gene clone libraries enabled characterizing microbial diversity and community structure dynamics in constructed wetlands treating domestic and dairy wastewater (Ibekwe et al., 2003; Ibekwe et al., 2007; Truu et al., 2005) or acid coal mine drainage (Nicomrat et al., 2008; Nicomrat et al., 2006), as well as assessing microbial community composition of wetland leachate (Walsh et al., 2002) and sediments (Lloyd et al., 2004). Additionally, potential members of contaminant degrading guilds of microorganisms can be targeted. For instance, specialized bacteria affiliated to the genera *Dehalobacter*, *Dehalococcoides* or *Geobacter* have been reported to use chlorinated ethenes as terminal electron acceptors and can then be used as indicators of potential dechlorinating activity (Duhamel et al., 2004; Hendrickson et al., 2002; Imfeld et al., 2008; Löffler et al., 2000). Microbial community diversity, structure and degradation activity are likely to considerably vary in wetland system as a function of spatial or temporal geochemical variability. Therefore, statistical tools may allow gaining more consistent insight into contaminant attenuation potential by relating microbial community structures to hydrochemical processes across ecological scales of interest (Ramette, 2007).

This study examined the microbial community structure dynamics and composition as well as the distribution of the dehalogenating guild of bacteria in a model system for transition wetland zones between anoxic*,* DCE contaminated groundwater, and oxic surface water. Previous compound-specific isotopic and hydrogeochemical analyses revealed both the occurrence of biodegradation activity as well as major changes of the prevailing geochemical conditions over time from oxic to strongly reducing conditions in the model wetland (Imfeld et al., 2008a). These changes were associated with a parallel shift of the dominant degradation mechanism at the system scale, in a sequence initiated by DCE oxidation and ending with biotic reductive dechlorination. Therefore, the first objective was to characterize the wetland microbial community during the transition phase from a prevailing aerobic to an anaerobic regime. Indeed, changes of the microbial diversity may affect the prevailing degradation mechanism in the model wetland, which in turn influences the system functioning with respect to organic contaminant removal over time. The second objective was then to identify the potential contribution of the microbial community to the biogeochemical processes influencing DCE transformation as well as specific microorganisms putatively involved in the degradation process. For these purposes, the wetland microbial community structures were assessed by means of PCR-DGGE as well as clone libraries. Putative bacterial members of the dehalorespiring guild were screened in the system using classical 16S rRNA gene biomarkers. Multivariate analyses were applied to highlight the relationship between selected hydrogeochemical variables and the bacterial community structures over spatial and temporal scales.

2. Materials and methods

2.1. System design and sampling procedure

A complete description of the system design has been previously reported (Imfeld et al., 2008a). A general scheme of the model constructed wetland is provided in Figure 1. Briefly, the model horizontal subsurface flow wetland consisted of a stainless steel tank (201 x 60 x 5 cm), filled with quartz sand and planted with the common rush (*Juncus effusus*, L.). The contaminated groundwater was collected bimonthly from a local, well-characterized aquifer and maintained under N_2 atmosphere when supplied to the wetland. The hydrogeochemistry of the collected groundwater remained constant over time and *cis*- and *trans*-1,2-DCE were the dominant contaminants (refer to Supplementary Information (SI), section 1.1). Additional amounts of contaminant were spiked into the tanks during the groundwater sampling to reach average concentrations of 6.5 and 1.5 mg L^{-1} for *cis*- and *trans*-DCE, respectively. The system was equipped with a permanent cooling system that maintained over the investigation period the tank and the wetland at 11°C ± 2 °C (yearly mean groundwater temperature). The system was operated in a flow-through mode with a rate of 1.8 L d^{-1} contaminated groundwater, corresponding to a retention time of approximately 15 days.

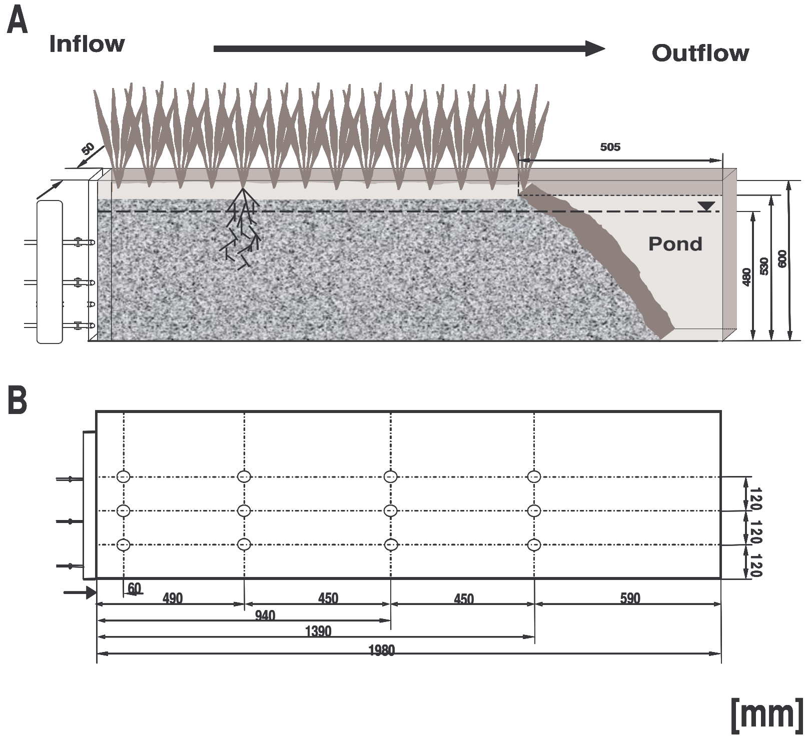

Figure 1. Detailed scheme of the model constructed wetland (A), and location of the pore water sampling devices (B). The dimensions are provided in [mm]. The model horizontal subsurface flow wetland was filled to an average depth of 54 cm with quartz sand ($kf_{average} = 2.27 \pm 0.14 \times 10^{-4}$ m s^{-1}; grain size = 0.40-0.63 mm) and planted with the common rush (*Juncus effusus*, L.). A 50 cm long water pond at the pond side remained in direct contact with the atmosphere. Groundwater was continuously pumped from the 50 L tank and injected at equal flow rate (0.4 mL min^{-1}) by means of three channel pipes before it reached the inflow chamber, passed through the model wetland and was drained away at the pond (175 cm from the inflow). The supplied groundwater in 50 L tanks and maintained under anaerobic conditions at constant N_2 pressure (0.5 mbar). (B) Pore water sampling devices are located at 6, 49, 94, 139 cm from the inflow. At each of these distances, the sampling ports are displayed at 20, 32, and 44 cm depth from the surface (○).

The biogeochemical development of the wetland was followed up over 430 days (Imfeld et al., 2008a). For the detailed microbial investigations reported in this study, water samples were retrieved at days 199, 227, 255, 283 and 311, which corresponded to the transition phase from a prevailing aerobic to an anaerobic regime at the system scale, as well as at day 430 (end of the investigation period). At each sampling date, samples were collected from the tank, the inflow chamber, the pond, and from four vertical profiles across the sand compartment for further hydrogeochemical and microbial analysis. The vertical profiles at the sand compartment were located at 6, 49, 94, 139 cm from the inflow and consisted of three sampling ports at 20, 32, and 44 cm depth below the surface (Fig. 1).

For the molecular investigations, two parallel integrative pore water samples were retrieved using sterile syringes from the sand compartment *via* the sampling ports (Fig. 1). Both integrative samples consisted of six 20 mL sub-samples, which were separately pooled in sterile vials. The first series of pore water sub-samples was retrieved from the two vertical profiles at the inflow side of the sand compartment (6 and 49 cm from the inflow) and the second series of sub-samples was retrieved from the two vertical profiles at the pond side of the sand compartment (94 and 139 cm). This integrative sampling procedure accounted for the dominant spatial development of the hydrogeochemical gradients within the model wetland that occurred along the flow path across the wetland rather than over depth, as documented in SI Figure S1. In parallel, 0.75 L water samples were collected in cleaned and sterile Schott bottles from the tank, the inflow and the pond. The sampled material was immediately cooled at 4°C and filtered within less than 1 hour.

2.2. Hydrogeochemical analysis and compound specific carbon isotope analysis

Quantification of *cis*-, *trans*-1,2-dichloroethene, vinyl chloride and ethene was performed with a gas chromatograph equipped with a flame ionisation detector (Varian Chrompack CP-3800, Middelberg, The Netherlands) as previously described (Nijenhuis et al., 2007), with detection limits of 50, 30, 5 and 5 µg L^{-1}, respectively. Geochemical parameters (pH, redox potential, Cl^-, NH^{4+}, PO_4^{3-}, Fe^{2+}, total Fe, O_2, SO_4^{2-}, total sulphide) were determined using DIN and DIN EN ISO standards and laboratory procedures as previously described (Imfeld et al., 2008a). Stable carbon isotope compositions of DCE were measured using a gas chromatography-combustion-isotope ratio mass spectrometry system (GC-C-IRMS) as described earlier (Imfeld et al., 2008b; Nijenhuis et al., 2007). The carbon isotope ratio for an individual compound is reported in δ-notation [‰] relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna) (Coplen *et al.*, 2006). The analytical error is ± 0.5 δ unit and incorporates both the accuracy and the reproducibility on replicate measurements of the sample.

2.3. Molecular analysis

2.3.1. DNA extraction

Preparation of the samples for DNA extraction from the water samples was carried out within 1 hour after water collection. Water samples were filtered through a sterile 0.2 µm membrane (MoBio Water DNA kit, Carlsbad, CA, USA) and stored at -20°C until extraction. DNA from cells was extracted with the bead beater technique (Fast Prep System, Qbiogene, Irvine, CA, USA) using a FastDNA spin kit for DNA extraction (BIO101, La Jolla, CA, USA) according to the manufacturer's instructions and eluted in nuclease-free water (50 µl).

2.3.2. 16S rRNA gene-targeted PCR

PCR was used to partly amplify 16S rRNA genes from *Bacteria*. PCR mix per reaction contained 1X PCR + $MgCl_2$ buffer (Qiagen, Hilden, Germany), 0.2 mM (each) deoxynucleoside triphosphate (Qiagen), 0.5 µM (each) forward and reverse primer (Invitrogen), 1.5 U of HotStar*Taq* DNA polymerase (Qiagen), 1:10 v:v of DNA template and molecular grade water (Promega). Eubacterial primers 27f (Lane, 1991) and 1378r (Heuer et al., 1997) were used to amplify almost the complete 16S rRNA gene using the following amplification program: 95°C (15 min), followed by 30 cycles of 95°C (30 s), 51°C (30 s), and 72°C (50 s), completed with an additional 30 min at 72°C. The second round PCR for DGGE analysis employed universal primers GC968f (Nubel et al., 1996) and 1378r. The conditions for PCR amplifications were as follows: 95 °C (15 min) followed by 30 cycles of 95 °C (30 s), 60 °C (1 min), 60 °C to 55 °C (0.5 °C min^{-1}), 72 °C (1 min), and a final extension for 30 min at 72 °C. In parallel, to test the presence of the genera *Dehalobacter* (Schlotelburg et al., 2002) and *Geobacter* (Duhamel and Edwards, 2006) in the model system, a second round of PCR with specific primers was performed using the PCR products from the universal 16S rRNA gene amplification as template, as previously described (Imfeld et al., 2008b). A Dehalococcoides-specific amplification protocol was used for detecting *Dehalococcoides*-affiliated bacteria as described previously (Hendrickson et al., 2002).

2.3.3. Cloning-sequencing

Two samples, one representative for the originally collected groundwater further supplied to the wetland between day 200 and 315 as well as an integrative pore water sample retrieved at day 227 from the sand compartment at the vertical profiles located at 6 and 49 cm from the inflow were selected for setting-up 16S rRNA gene libraries. The integrative pore water sample from the wetland was selected in order to assess the bacterial composition associated with the developing front of iron sulphide mineral precipitates in the wetland (SI Fig. S2). Amplified DNA obtained after the above described first amplification round was ligated into a pGEM-TEasyTM (Promega, Madison, WI). Ligated vectors and inserts were transformed into competent *E. coli* JM109 cells using the manufacturer protocol with recombinant transformations selected by blue-white screening. Following plasmid extraction and amplification using M13f and M13r primers, the PCR products of clones were separately digested with 1 U of the tetrameric site restriction endonuclease *Hin*6I and *Bsu*RI (Fermentas, St. Leon-Rot, Germany). Clones displaying the same pattern were grouped into an operating taxonomic unit (OTU) (Massol-Deya et al., 1995). The 16S rRNA genes of the OTU representative clones were purified prior to sequencing using an ABI PRISM BigDye Terminator Cycle Sequencing Kit V. 3.0 (Applied Biosystems) according to the manufacturer's protocol and sequencing was carried out in an ABI PRISM 3100 DNA analyzer (Applied Biosystems, Foster City, CA). Putative chimeric sequences were retrieved from the database after detection analysis performed using the Chimera Detection Program of the RDP-II (Cole et al., 2003). The examination of phylogenetic relatedness and taxonomic assignments (confidence level of 80%) was performed using the naïve Bayesian rRNA Classifier and the Sequence Match tools of the RDP-II (release 9.61, http://rdp.cme.msu.edu/), respectively (Cole et al., 2007; Wang et al., 2007). Bacterial nucleotide sequence data are available in the EMBL database under accession numbers FM205929 to FM206115.

2.3.4. Molecular fingerprinting analysis of microbial communities

PCR products were separated using the DCode Universal Mutation Detection System (BioRad, Munich, Germany) denaturing gradient gel electrophoresis (DGGE) system as previously described (Imfeld et al., 2008b). The DGGE fingerprints were normalized according to the reference patterns and compared using the GelComparII software (Applied Maths, Kortrijk, Belgium). DGGE banding patterns were then converted to a binary numerical matrix based on presence or absence of bands and subsequently used for statistical analysis.

2.4. Data analysis

Data analyses were performed using the R software (R Development Core Team, 2008). Principal component analysis (PCA) based on the *z*-scores and performed on the correlation matrix was used to assess the gradients in the hydrogeochemical data collected at day 227, 255, 283 and 311 and to gain insight into the relationships between the hydrogeochemical variables (pH, Eh, Cl^-, NH^{4+}, PO_4^{3-}, Fe^{2+}, O_2, SO_4^{2-}, total sulphide, *cis*- and *trans*-DCE concentrations and isotopic composition). Correspondingly, non-metric multidimensional scaling (nMDS) was use to evaluate the changes in bacterial community structures from day 227 to 311. The binary numerical matrix based on presence or absence of DGGE bands was converted to a distance matrix using the Euclidean dissimilarity index, which displayed the highest rank-order similarity with gradient separation after indices comparison (data not shown). The relationship between the community profiles and the hydrogeochemical variables was interpreted by fitting *a posteriori* the environmental vectors onto the nMDS as a result of their calculated correlations. The significance of the fitted vectors was assessed using a permutation test (1000 permutations).

3. Results and discussion

3.1. Hydrogeochemical processes

The hydrogeochemical development of the system over 430 days was described in details previously (Imfeld et al., 2008). The results of the hydrogeochemical measurements performed at discrete intervals across the system between day 199 and 430 are provided in SI Table S1. Figure 2 summarizes the main changes occurring from day 199 to 430 in the system with respect to both pore water geochemistry and degradation mechanisms. This period was characterized by a progressive decrease of dissolved oxygen concentrations (SI Table S1) accompanied by an increase over time of both ferrous iron (Fig. 2A) and sulphide (Fig. 2B) concentrations. This indicated that anoxic conditions progressively prevailed in the sand compartment of the wetland, with several microbially mediated redox reactions occurring in parallel. Vinyl chloride and ethene concentrations increased between day 225 and 430 from < 5 to 518 ± 13 and < 5 to 102 ± 5 $\mu g\ L^{-1}$, respectively, indicating reductive dechlorination activity (Imfeld et al., 2008a). The concentration increase of these metabolites coincided with significant and increasing isotopic enrichment of both *trans*- and *cis*-DCE ($\Delta\delta^{13}C > 0.5$ ‰) between the inflow and the end of the sand compartment since day 225 (Fig. 2 C and D). These progressive changes indicated that less fractionating DCE oxidation was progressively replaced over time by reductive dechlorination, associated with a more pronounced isotopic effect (Imfeld et al., 2008a).

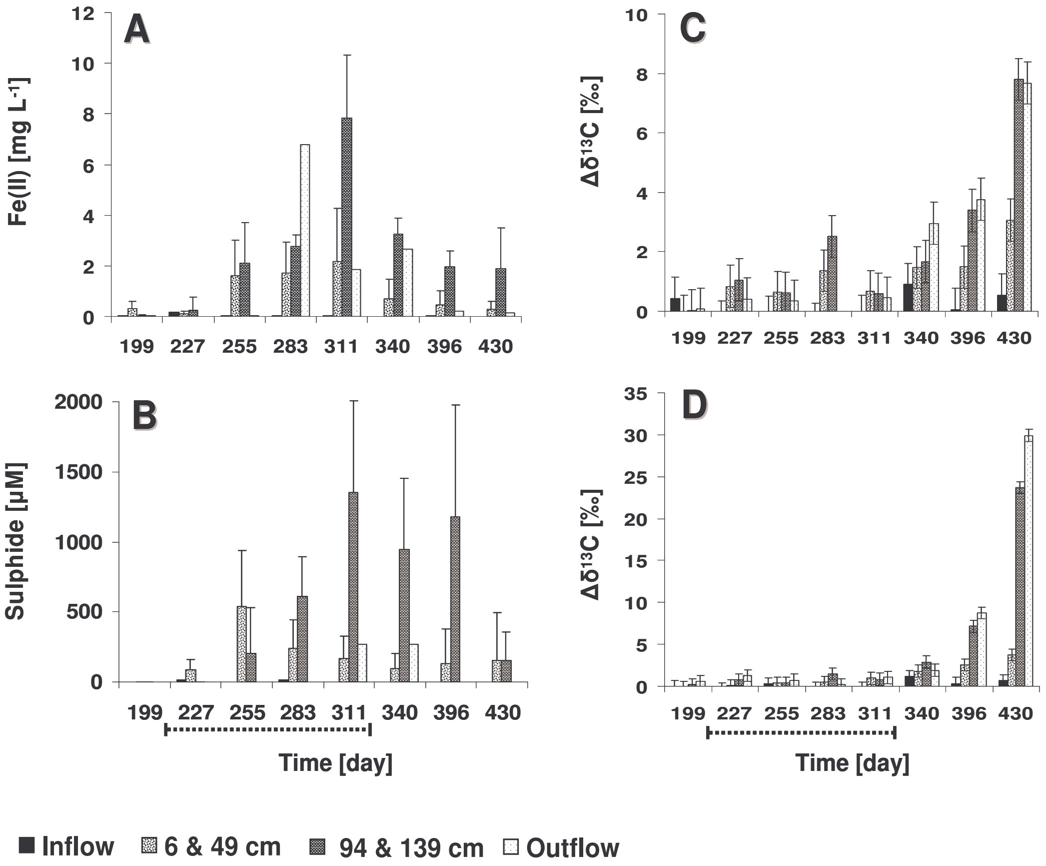

Figure 2. Iron(II) (A) and sulphide (B) concentration values, as well as mean carbon isotopic signature of *trans*- (C) and *cis*- (D) 1,2-dichloroethenes at the inflow, the sand compartment (6, 49 and 94, 139 cm from the inflow) and the outflow in the model wetland between day 199 and 430. Error bars for iron(II) and sulphide mean concentration values indicate each the standard deviation of the six concentration values retrieved at the sampling ports from the sand compartment (at 6, 49 and 94, 139 cm from the inflow, respectively). The dashed lines indicate the time period of microbial community structures analysis.

Principal component analysis illustrated the major hydrogeochemical trends in the model wetland from days 227 to 311 (Fig. 3), which corresponds to the period of microbial community structures characterization. Hydrogeochemical profiles of the tank and the inflow clustered together over time and scored negatively on the *x*-axis (PC1). The similarity of these hydrogeochemical profiles reflected the constancy of the supplied water quality over the investigation period. However, hydrogeochemical profiles of the sand compartment or the pond did not form homogenous clusters and differed from the previous ones. A change of the hydrogeochemical profiles over time was particularly visible at the pond. Overall, the higher the distance from the inflow at which a particular sample was collected, the higher the variation from one sampling date to another with respect to the prevailing hydrochemistry. On the plot of the variables (Fig. 3B), scores of PC1 correlated positively to sulphide, phosphorus, *cis*- and *trans*-DCE carbon isotope composition shifts ($\Delta\delta^{13}C$), and ferrous iron, in association with time. Hence, these variables mostly explained the hydrogeochemical changes occurring in the wetland during the investigation period. Furthermore, hydrogeochemical profiles from the tank and the inflow scored positively along the *y*-axis (PC2), and could be distinguished from the profiles of the sand compartment and the pond that scored negatively (Fig. 3A). This infers changes of the hydrogeochemical profiles over the flow path across the wetland. For instance, mobilization of ferrous iron was revealed by concentration values increasing at increasing distance from the inflow between day 283 and 311. PC2 correlated positively to DCE concentrations and negatively to oxygen and chloride (Fig. 3B). This underscored that progressive contaminant depletion from the inflow to the pond coincide with increasing of oxygen and chlorine concentrations over the flow path.

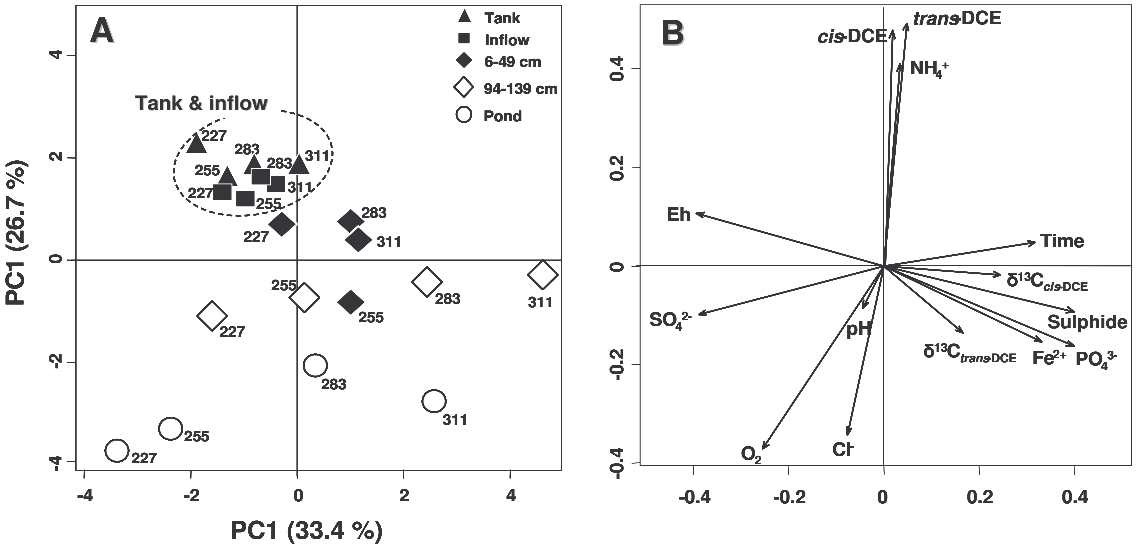

Figure 3. PCA ordination plot of (A) hydrogeochemical characteristics of water samples and of (B) hydrogeochemical variables collected in the model wetland between day 227 and 311 of the investigation period. (A) Values on the axes indicate % of total variation explanation by the corresponding axis (PC 1, principal component axis 1; PC 2, principal component axis 2). The first and second principal component accounted for > 50 % of the variance in the data set. Objects are labeled according to the section of the wetland they were collected from (▲: Tank; ■: Inflow; black and white ◊: sand compartment at 6-49 cm and 94-139 cm from the inflow, respectively; ○: Pond) and numbered according to the sampling date (days 227, 255, 283, 311). (B) Description vectors correspond to: *cis*-DCE, 1,2-*cis*-dichloroethene; *trans*-DCE, *trans*-1,2-dichloroethene; $\delta^{13}C_{trans\text{-}DCE}$, trans-DCE isotopic composition; PO_4^{3-}, P-phosphate; Fe^{2+}, ferrous iron; $\delta^{13}C_{cis\text{-}DCE}$; Cl^-, chloride; O_2, oxygen; SO_4^{2-}, sulphate; Eh, redox potential; NH_4^+, ammonium.

Until day 255, ferrous iron concentration remained generally one order of magnitude lower than total iron concentrations (Fig. 2 and SI Table S1), suggesting that iron prevailed in the ferric form in the model wetland. Although the quality of the supplied groundwater remained constant, and ferrous iron or sulphide were undetectable in the tank, ferric iron progressively underwent reductive dissolution in the course of the experiment. Severely reduced conditions in the sediments may also result in the release of phosphorus (Mann, 1990). In particular, since Fe(III)-hydroxide are known to have high binding affinities to P (Buffle et al., 1988; Kalbitz et al., 2000), the progressive reduction of Fe(III) may have resulted in the release the bound phosphate ion back into solution, resulting in the increase of phosphate in the pore water of the model wetland since day 227. Similar phenomena have also been reported in natural wetlands (Gosselink & Turner, 1978).

Since day 199, a front of iron sulphide mineral precipitates could be observed within the sand compartment and progressively developed from the inflow towards the outflow (SI Fig. S2). This evidenced sulphate reduction activities. However, the excess of dissolved Fe likely led to the immediate precipitation of sulphide from the solution in the form of iron sulphide minerals, and thus hampered the detection of sulphide until day 227. Principal component analysis also underlined that stepwise increase of sulphide concentrations from day 227 to 311 was inversely correlated to decline with sulphate over the flow path (Fig. 3B), which consistently indicated sulphate reduction. Since day 311, ferrous iron accounted for a large part of the total iron present within the system and sulphide was concomitantly detected. From day 199 to 227, ferrous iron and sulphide concentrations likely exceeded the equilibrium solubility threshold and both reduced species were only partly removed from the solution by precipitation of iron sulphide. This then led to considerable iron sulphide formation and subsequent accumulation of both soluble species within the wetland. Enzymatic microbial reduction coupled to organotrophy and hydrogen consumption by dissimilatory reducing bacteria is assumed to be a dominant mechanism in anaerobic soils and sediments (Holmer et al., 2001). However, the simultaneous occurrence of iron and sulphide reduction suggests that these processes were not totally controlled by the electron acceptor availability. High availability of electron donor due to decaying plant material, evidenced by high DOC concentrations in the system (Imfeld et al., 2008a), may explain the co-existence of these processes, as already described in freshwater wetlands (Alewell et al., 2007). The development of zones of iron sulphide precipitation can be explained by i) the maintenance of reducing conditions created by continuous supply of anoxic groundwater, and ii) the high availability of electron donors in the form of decaying plant organic matter within the wetland. Thus, the progression of the front of iron sulphide minerals occurred horizontally across the model wetland, at an approximate progression rate of 1.5 cm day^{-1}, likely controlled by the depletion of organic substrates at local zones of intense ferric iron and sulphate reduction.

3.2. Analysis of the bacterial community structures

DGGE of PCR-amplified partial 16S rRNA genes was applied to investigate the changes in bacterial community structures both over the flow path across the wetland and during the transition phase from a predominantly oxic to an anoxic regime. DGGE patterns from wetland samples collected between day 227 and 311 provided complex and heterogeneous microbial fingerprints (SI Fig. S3). The mean number of 16S rRNA bands ranged between 12 and 31 and slightly decreases over the flow path (SI Fig. S4). As a mean to analyze changes in bacterial community structures, a non-metric multidimentional scaling of 16S rDNA-DGGE patterns was carried out (Fig. 4). In the nMDS plot, the distance between the points reflects the degree of similarity of the DGGE profiles and samples displaying a similar community structure are found closer. The 16S rDNA-DGGE fingerprints of the tank samples were found together, which emphasizes the similarity of community structures in the groundwater supplied to the wetland over time. However, the analysis revealed substantial changes in the bacterial community structures at the inflow, the sand compartment and the pond from one sampling date to another. For instance, sand compartment and pond bacterial fingerprints from days 227 and 255 scored negatively along the *y*-axis (nMDS 2), and were separated from bacterial fingerprints from days 283 and 311, which scored positively. In the sand compartment, changes in the bacterial community structures seem larger between the sampling days 255 and 283 than between other sampling days.

To obtain insights into the relationship between the bacterial community dynamics and the geochemical development in the model wetland, variables significantly associated with the observed changes in the bacterial community structures were revealed by fitting them *a posteriori* onto the nMDS. Statistically significant variables were represented on the plot (Fig. 4). The time, phosphate, and sulphide vectors positively correlated with the observed changes in the microbial community structures, whereas sulphate correlated negatively ($P<0.05$). Thus, the observed changes of the microbial community structures over time coincided with sulphate reduction activity in the wetland (Fig. 2B).

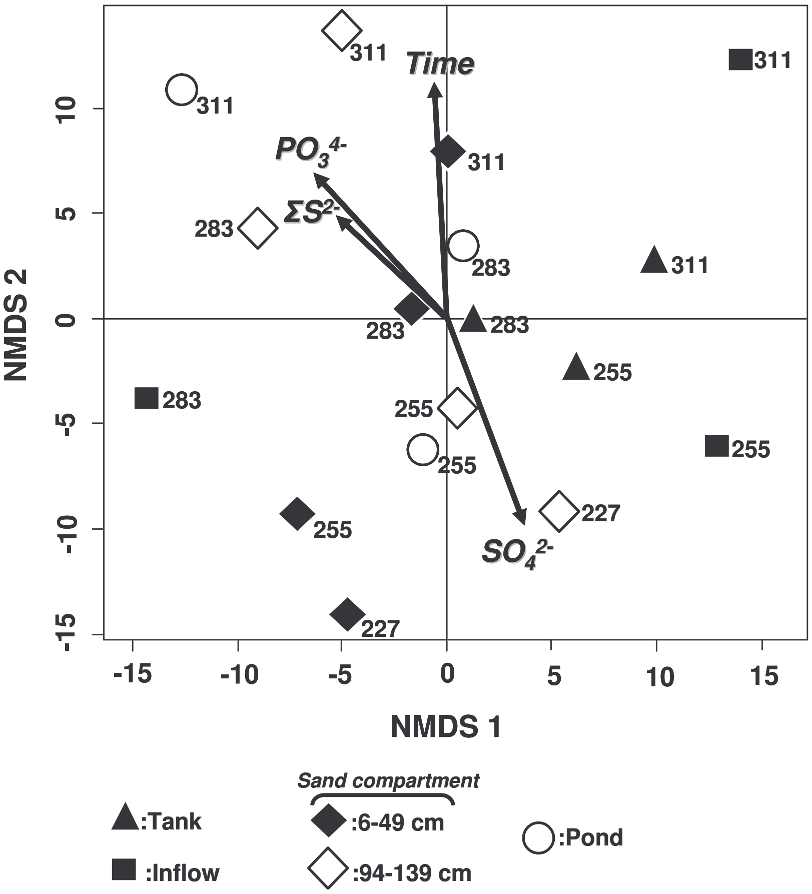

Figure 4: Non-metric multidimentional scaling plot (nMDS – 2 dimensional) representation of the 16S rDNA DGGE patterns of water samples from the model wetland, showing the community changes over time (stress value: 18.5 %). Objects are labeled according to the section of the wetland they were collected from (▲: Tank; ■: Inflow; black and white ◊: sand compartment at 6-49 cm and 94-139 cm from the inflow, respectively; ○: Pond) and numbered according to the sampling date (days 227, 255, 283, 311).

The analysis of microbial fingerprints dynamics partly reflected the observed hydrochemical changes and revealed that a diverse community was associated with the development of prevailing biogeochemical conditions at the system scale (Fig. 4 and SI Fig. S3 and S4). This analysis showed that relatively large changes in the bacterial community structures occurred during the first investigation period (day 227 to 253) whereas thereafter, smaller changes were constantly observed in the model wetland. These initial changes in the bacterial community corresponded to the mobilization of both ferrous iron and sulphide within the system, and thus reflected major variations of the prevailing redox conditions. Changes can be attributed to an acclimatation of the bacterial community to the relatively fast evolution of the hydrochemical framework towards stronger reducing conditions. During the investigation period, stable, climaxic community structure were not established within the sand compartment and the outflow, in consistence with the fluctuation of ambient variables. Indeed, changes of the wetland hydrochemical properties over both spatial and temporal scales may have severely shaped the ecological habitat within the porous sand matrix. Such changes were directly underscored by the spatial evolution of the dynamic front of iron sulphide precipitates. For instance, the accumulation of decaying plant organic material and the deposits of iron sulphite mineral may have produced chain effects such as reduction of hydraulic conductivity and void space of the porous medium, oxygen supply and increased dispersion (Tanner and Sukias, 1995; Garcia et al., 2004). These changes in both hydrochemistry and hydrodynamic properties of the porous matrix might have in turn affected both the structures and diversity of the microbial community by changing the flux and transport pattern of organic substrate and nutrients.

Hence, microbial community in the model wetland was highly dynamic and these variations also corresponded to changes in the degradation mechanism over the investigation period. Though change of the degradation mechanism was possibly associated with modifications of the system physico-chemical properties, DCE degradation activities were maintained within the system over the investigation period. The occurrence of substantial bacterial community structure fluctuation with concomitant maintenance of biodegradation capacity was also observed in bioreactors during activated sludge treatment (Mirua, et al., 2007). However, changes of microbial community structures may mirror variations at the contaminant degrading guild or population levels, which in turn can affect the degradation mechanism.

3.3. Distribution of potential members of the dehalorespiring guild

The presence of *Dehalobacter*, *Dehalococcoides*- and *Geobacter* DNA at the inflow, the sand compartment and the pond of the model wetland was tested from day 199 to day 430 (Table 1). Indication of the presence of a complex guild of reductive dehalogenators was provided by the double step PCR-based taxon-specific assays. *Geobacter* spp. DNA was systematically detected over the investigation period in all samples retrieved from the model system. For instance, *Geobacter* sp. was widely distributed over the investigation period. These iron-reducing and metabolically versatile microorganisms are obligate anaerobes capable of reductive dechlorination. Furthermore, active populations of *Geobacter* sp. may play a critical role in the dechlorination of chlorinated ethenes during the iron reduction phase, as the process may inhibit reductive dechlorination by other species (Sung *et al.*, 2003; Sung *et al.*, 2006). Actually, the wetland rhizosphere and is a site of unusually active microbial Fe cycling (Weiss et al., 2003), whereas *Geobacter* sp. in wetland sediment were found capable of both dissimilatory Fe(III) reduction and oxidation of Fe(II) with concomitant reduction of NO_3^- to NH_4^+ (Weber et al., 2006). Presence of *Dehalobacter* spp. DNA was observed since day 311 and systematically coincided with the detection of *Dehalococcoides* spp. DNA. DNA of the all targeted genera could be concomitantly detected across the system at day 430, suggesting the establishment of a complex guild of dechlorinating bacteria. The presence of *Dehalococcoides*-like bacteria was detected since day 253 in the sand compartment and correlated with detection of vinyl chloride and ethene as well as larger shift in the carbon isotopic composition of both *cis*- and *trans*-DCE (Fig. 2C and 2D). The correlation between the presence of *Dehalococcoides* sp. DNA and the detection of VC and ethenes further substantiated the occurrence of microbially mediated reductive dechlorination in the model wetland (Hendrickson et al., 2002). Although members of the genera *Geobacter*, and *Dehalobacter* are capable of partial dechlorination of PCE and TCE to *cis*-DCE, *Dehalococcoides* is the only group known today to completely dechlorinate PCE/TCE to ethene, and is highly evolved to utilize halogenated organic compounds and hydrogen (MaymoGatell *et al.*, 1997; Seshadri *et al.*, 2005).

Table 1. Detection of *Dehalococcoides*, *Dehalobacter* and *Geobacter* DNA in the model wetland using taxon-specific assays. (-) indicate the absence of the targeted genera. Letters correspond to the part of the wetland at which samples were retrieved: a, inflow; b, sand compartment at 6 and 49 cm from the inflow; c, sand compartment at 94 and 139 cm from the inflow; d, pond.

Taxon-specific assay	Time [day]				
	225	253	280	309	430
Geobacter spp.	a,b,c,d	a,b,c,d	a,b,c,d	a,b,c,d	a,b,c,d
Dehalobacter spp.	-	-	-	a,b	a,b,c,d
Dehalococcoides spp.		a,b	a	a,b,c	a,b,c,d

The detection of a complex guild of dehalogenating bacteria provided a supplementary line of evidence for the occurrence of reductive dechlorination as a key-mechanism for contaminant removal, which may ultimately influence the final effluent water quality. The detection of both VC and ethene since day 225 indicated that dechlorinating microorganisms were directly involved in the DCE degradation observed to non-toxic products in the model wetland. The degrading function of the dechlorinating guild may have become more important over time, until it completely overcome the contribution of oxidative degradation, as suggested by the larger enrichment in ^{13}C of DCE (Imfeld et al., 2008). Relatively high DCE concentrations and decreasing availability of preferred electron acceptors for anaerobic oxidation (i.e. Fe^{3+}) likely progressively limited mineralization of DCE or VC in the course of the investigation period (Bradley and Chapelle, 1998). Reciprocally, the detection of ethene in the presence of putative dehalogenators and prevailing strongly reducing conditions underscored the relevance of reductive dechlorination as a key DCE degrading mechanism in the later stage of the investigation period. Hence, the results indicated a narrow interaction between the microbial community, including the dechlorinating guild, and the TEAPs. This was particularly emphasized by the stepwise detection of several potential members of the dechlorinating guild over time, which mirrored the temporal hydrogeochemical variation in the model wetland.

3.4. Microbial community composition

Two 16S rRNA clone libraries were constructed to gain insight into the bacterial communities composition and composition changes of between the original supplied groundwater and the model wetland at the beginning of the transition phase from oxic to anoxic conditions (day 227) (Tables 2 and SI Tables S2 and S3). A total of 138 and 165 clones were obtained and restriction pattern analysis further refined theses sequences, with a resulting 89 and 97 OTUs for the groundwater and wetland samples, respectively. Although the rarefaction curves indicated a tendency of saturation, discovery of additional sequences is expected by increasing the number of investigated clones (Fig. 5).

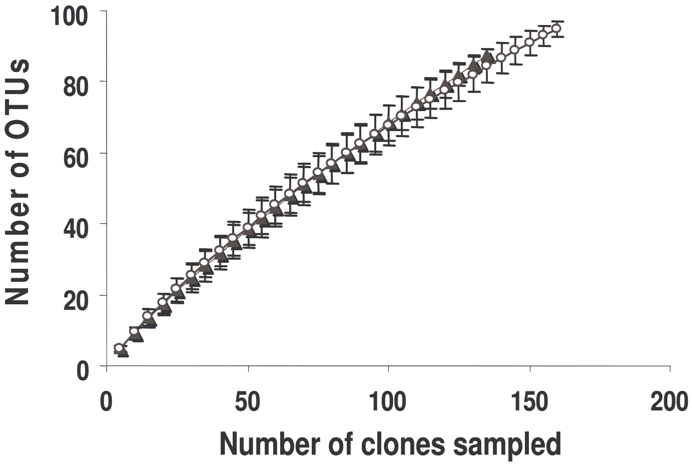

Figure 5. Rarefaction curve of bacterial 16S rRNA gene clones recovered from groundwater (▲) and from the wetland (○). Error bars show the upper and lower 95% confidence limits.

Clone sequences from the groundwater and the wetland could be affiliated with 8 and 11 classes of the domain *Bacteria*, respectively (Tables 2; SI Tables S2 and S3). Overall, ribosomal sequences affiliated to the *Proteobacteria* largely dominated, and accounted for about the half of both clone libraries. About ¼ of the clones could not be assigned to any bacterial phyla at a confidence level of 80%. Sequences affiliated to the *β*- and *δ-Proteobacteria* were retrieved in higher numbers than the other groups in both libraries. However, the relative frequency of *δ-Proteobacteria* sequences was higher in the wetland, whereas the relative frequency of *β-Proteobacteria* affiliated sequences was higher in the groundwater library (Table 1). The majority the sequences within the *β*-subgroup in the groundwater were found similar to sequences affiliated to the neutrophilic and chemolithotrophic iron-oxidizing *Gallionella ferruginea* (Seqmatch score = 0.43 to 0.86). The occurrence of this bacterium is constistent with the hydrogeochemistry of the supplied groundwater (SI, section 1.1.). Other *β*-subgroup-affiliated sequences displayed a high level of similarity with an *Acidovorax* sp. related clone isolated from a naphthalene-contaminated soil (unpublished; Seqmatch score = 1), *Hydrogenophaga* clones found in benzene enrichment cultures from BTEX-contaminated groundwater (unpublished; Seqmatch score > 0.9), as well as a ferrous iron oxidizing bacterium *Thiobacillus* sp. sequence (Seqmatch score = 0.79).

In the wetland, *Gallionella* sp. accounted for only 0.6 % and *β-Proteobacteria* sequences were closely related to *Malikia* sp. sequences obtained from microcosms prepared with lake water (unpublished; Seqmatch score = 0.88 to 1). Most of the sequences within the *δ*-subgroup retrieved from the wetland were found close to *Desulfovibrio* sp. (Seqmatch score = 0.86 to 0.97), and some of those sequences were found closely related to clones from an *in situ* reactor system treating monochlorobenzene contaminated groundwater (Alfreider et al., 2002). Overall, about 17 % of the sequences retrieved from the wetland shared close homology with sulphate reducing organisms commonly found in oligotrophic aquatic environments (*Desulfovibrio* sp., 15.2 %; *Desulfosporosinus* sp. 1.2 %; *Desulforhopalus* sp., 0.6 %). *Geobacter* sp. (Seqmatch score = 0.75 to 0.89) accounted for 2.4 % of the clone library. Sequences affiliated to *Geobacter* sp. had close relationship to sequences retrieved from Fe(II)-reducing enrichment cultures based on heavy metal and radionuclide contaminated river sediments (Scala et al., 2006) and petroleum-contaminated iron-reducing sediments (Holmes et al., 2004). Moreover, one clone was closely related to an uncultured bacterium sequence associated with PCE dechlorination recently retrieved from anoxic river sediment by RNA-based stable isotope probing (Kittelmann and Friedrich, 2008; SI Table S3, clone 26, Seqmatch score = 0.89). This further supports a possible involvement of *Geobacter* sp. in the observed dechlorinating activities, in agreement with the results of the taxon-specific assay. Some α-*Proteobacteria* clone sequences from the wetland were affiliated to the microaerophilic, fresh water and sediment bacterium *Magnetospirilum* sp. (Seqmatch score = 0.75 to 0.82), but could not be detected in groundwater. Sequences affiliated to *Verrumicrobia*, *Chloroflexi* and *Spirochaetes* were exclusively detected in the constructed wetland. Within the phylum *Chloroflexi*, one clone sequence was affiliated to the *Levilinea* genus (*Caldilineacea* class) at a high confidence level and was closely related to a clone isolated from the chemocline of a meromictic alpine lake (Seqmatch score = 0.94) (Bosshard et al., 2000). This observation suggests that non-Dehalococcoides *Chloroflexi* populations potentially involved in reductive dehalogenation may have been present in the model wetland (Watts *et al.*, 2005). In contrast, no *Dehaloccoides*-affiliated sequences could be retrieved, which underscores the higher sensitivities of the taxon-specific PCR assay over the cloning-sequencing procedure (Hendrickson *et al.*, 2002). Noticeably, sequences related to the TM7 candidate division accounted for about 9 % of the groundwater clone library, but could not be retrieved from the wetland. TM7 clones have been recovered previously from a TCE- and *cis*-DCE-contaminated site (Lowe et al., 2002) and some members of this division were suggested to be associated with TCE and *cis*-DCE cometabolic oxidation (Connon et al., 2005). *Firmicute*, *Bacteroides*, *Lentisphaerae, Actinobacteria* and *Acidobacteria* were found in similar proportions in both clone libraries. Some members of the *Actinobacteria* are iron-oxidizing, heterotrophic, acidophilic bacteria capable of autotrophic growth.

Table 2. Relative clone frequencies in major phylogenetic groups of the clone libraries from the original groundwater (supplied to the wetland between day 200 and 310) and the wetland (sampling day 227). The various sub-phyla of *Proteobacteria* are indicated by Greek letters.

Group	Relative frequency [%]	
	Groundwater	**Wetland**
Proteobacteria	53.9	53.3
α-*Proteobacteria*	0.7	6.7
Magnetospirillum spp.	0	1.8
β-*Proteobacteria*	36.2	18.8
Gallionella spp.	16.3	0.6
Malikia spp.	0	7.3
δ-*Proteobacteria*	2.8	23.0
Geobacter spp.	0	2.4
Desulfovibrio spp.	1.4	15.2
ε-*Proteobacteria*	0.7	1.8
γ-*Proteobacteria*	10.6	1.8
Dokdonella	1.4	1.2
Thiothrix spp.	1.4	0.6
Unclassifed *Proteobacteria*	2.8	1.2
Firmicute		
"Clostridia"-Clostridiales	3.5	5.5
TM7 Division	8.5	0
Bacteroidetes	2.8	4.2
Lentisphaerae		
Victivallis spp.	1.4	4.2
OD1 Division	1.4	1.8
Actinobacteria	0.7	
Actinomycetales		1.8
Verrucomicrobia	0	3.0
Cloroflexi		
Anaerolineae	0	2.4
Acidobacteria		
Gp6	0.7	0.6
Spirochaetes	0	0.6
Unclassifed *Bacteria*	25.6	21.9
Number of clones	**138**	**165**
Number of OTUs	**89**	**97**

Analysis of 16S rRNA gene-based clone libraries showed a striking correlation of the bacterial population of samples from both the groundwater and the wetland with processes of iron reduction (genus *Geobacter*), iron oxidation (genus *Gallionella*), sulphate reduction (genus *Desulfovibrio)* and sulfur oxidation (genus *Thiothrix*). The results showed the occurrence of a high diversity of bacteria in the model wetland, which may form a suitable habitat for iron and sulfur cycle bacteria, in agreement with the hydrogeochemical observations. However, retrieved sequences were mostly affiliated with obligatory, anaerobic bacteria, and several types of identified organisms have been previously described in hydrocarbons- and chlorinated solvents contaminated anoxic aquifers.

Interaction among the various bacterial populations present in the model system may have influenced the DCE degrading capacity of the system. In particular, competition for available electron donor and acceptors is likely to affect both the microbial community composition and the preferential occurrence of one mechanism *versus* another, which ultimately may control the quality of the effluent water. In the model wetland, declining health status of the plants over the investigation period (data not shown) may have progressively led to a decrease of the oxygen input and an increase of decaying organic matter in the porewater, which supported the activity of several anaerobic populations and the hydrochemical development towards reducing conditions. This in turn created favorable conditions for cometabolic and dehalorespiration processes. However, though dechlorination of *cis*-1,2-DCE to ethenes is assumed to be linked to H_2, several identified bacterial groups may compete for the available H_2. The competition for electron donor or its fermentation products (e.g. H_2, lactate) is a crucial factor in determining the rate and extent of dechlorination, the amount of electron donor necessary for dechlorination (Aulenta et al., 2002; Heimann et al., 2006). The large proportion of 16S rDNA sequences affiliated to suphate-reducing bacteria in the clone library of the sand compartment during the beginning of the transition from an oxic to a prevailing anoxic regime brings-up the eventually that this guild interfered with the DCE degrading processes, since both sulphate reducing and dechlorinating microorganisms are able to thrive at very low and similar H_2 levels (Heimann et al., 2005). However, dechlorinating activity was observed concurrently with sulphate reducing activities, and even progressively increases over the investigating period. This suggests that dechlorinators can effectively compete with sulphate reducers for the electron usage, and/or possibly benefit from the H_2 production by sulphate reducing bacteria, in consistence with previous observations (Aulenta et al., 2007). However, if dehalorespirers do not display enzymatic capabilities to use iron or sulphate, competitive exclusion may influence populations of organisms responsible for the cometabolic dechlorination of *cis*-DCE. These populations include methanogens and acetogens, both of which are highly influenced by H_2 consumption. Though considered as ubiquitous in anaerobic systems, cometabolic dechlorinating processes are generally incapable of mediating complete reduction to ethene (McCarty and Semprini, 1994), and potential candidate microorganisms could not be directly identified in the model wetland.

4. Conclusion

This study aimed at characterizing the microbial community associated with the degradation of 1,2-dichloroethenes in a model wetland for groundwater-surface water interfaces. Though many studies have been performed with synthetic water and relatively short operating times, the bacterial community of the model system was investigated during a relatively long-term operation using culture-independent tools. The characterization was performed during a hydrogeochemical transition at the system scale, from mostly oxic toward reducing conditions, which coincided with changes of the prevailing DCE biodegradation mechanism. The following conclusions can be drawn:

- Iron and sulphate reduction co-occurred and were the dominant terminal electron-accepting processes, resulting in a progressing front or iron sulphide minerals in the sand matrix of the model wetland. In turn, changes of the TEAPs coincided with a shift of the predominant DCE degrading mechanism in the system, from oxidation reactions towards reductive dechlorination.

- The transition between the oxic and the anoxic phases was associated with major changes of both the bacterial community structures and the composition of the putative dechlorinating guild of bacteria. Hence, changes in the microbiology mirrored the evolution of the hydrochemical conditions and were indicative of the shift of the prevailing DCE biodegradation mechanisms.

- The presence of putative dehalogenators in association with evidences of dechlorinating processes indicated favorable conditions for DCE reductive dechlorination within the wetland. *Geobacter* sp. likely dominated at the beginning of the transition period, when iron reduction occurred, whereas the complexity of the putative dechlorinating guild, characterized by the stepwise occurrence of *Dehalococcoides* sp. followed by

Dehalobacter sp., increased over time.

- 16S rRNA clone library experiments indicated that the model wetland supported dynamic habitats reflecting interfaces between varying TEAP, and characterized by a diverse bacterial community. A large proportion of recovered microorganisms were putatively involved in iron and sulphate reduction processes, and may substantially affect the system functioning with respect to DCE degradation mechanism, both by changing the hydrochemical framework and interacting with members of the dechlorinating guild.

- Although the DCE degradation mechanism varied over time, the degrading function of the model wetland sustain by the microorganism was maintained. Though coupling reduction and oxidation processes in wetland systems to reach an efficient transformation of some chlorinated solvents and their transformation products is of interest, changes of degradation mechanism over the wetland system lifespan needs to be carefully considered.

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3

Assessment of in situ biodegradation of chlorinated solvents in aquifers

Section 1: Integrative approach to delineate Natural Attenuation of chlorinated benzenes in anoxic aquifers

Section 2: Assessment of in situ degradation of chlorinated ethenes and bacterial community structure in a complex contaminated groundwater system

Section 3: Variability of in situ biodegradation of chlorinated ethenes and microbial communities along a vertical profile in a heterogeneous groundwater system

Section 1: Integrative approach to delineate natural attenuation of chlorinated benzenes in anoxic aquifers

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Abstract

Biodegradation of chlorobenzenes was assessed at an anoxic aquifer by combining hydrogeochemistry and stable isotope analyses. In situ microcosm analysis evidenced microbial assimilation of MCB derived carbon and laboratory investigations asserted mineralization of MCB at low rates. Sequential dehalogenation of chlorinated benzenes may affect the isotope signature of single chlorobenzene species due to simultaneous depletion and enrichment of ^{13}C, which complicates the evaluation of degradation. Therefore, the compound specific isotope analysis was interpreted based on an isotope balance. The enrichment of the cumulative isotope composition of all chlorobenzenes indicated in situ biodegradation. Additionally, the relationship between hydrogeochemistry and degradation activity was investigated by principal component analysis underlining variable hydrogeochemical conditions associated with degradation activity at the plume scale. Although the complexity of the field site did not allow a straightforward assessment of natural attenuation processes, the application of an integrative approach appeared relevant to characterize the in situ biodegradation potential.

Capsule

Evidence of in situ biodegradation of chlorinated benzenes in an anoxic aquifer was obtained by a multi step approach including hydrogeochemical analysis, stable isotope tools, and multivariate statistics.

1. Introduction

Chlorinated aromatic compounds are worldwide intensively used to synthesize pesticides and other chemicals leading to ubiquitous distribution in the environment (Field and Sierra-Alvarez, 2008). Chlorinated benzenes (CBs) can also be formed during anaerobic microbial transformation of hexachlorocyclohexane, of which the γ-isomer (Lindane) is worldwide used as pesticide (Phillips et al., 2005;Van Agteren et al., 1998). Due to their toxicity, persistence and accumulation in the food chain CBs are of great environmental concern.
CBs may be subjected to both aerobic and anaerobic microbial degradation (Adrian and Gorisch, 2002; Field and Sierra-Alvarez, 2008; Van Agteren et al., 1998). With increasing chlorination CBs may undergo reductive dehalogenation either as cometabolic reaction or energy yielding halorespiration (Adrian and Gorisch, 2002; Van Agteren et al., 1998). The occurrence of reductive dehalogenation under methanogenic and sulfate reducing conditions has been observed in a variety of anaerobic mixed cultures (Adrian and Gorisch, 2002; Van Agteren et al., 1998), but only one bacterial strain (*Dehalococcoides* strain CBDB1) capable to couple energy conservation with reductive dehalogenation of CBs (≥ 3 chlorine substituents) has been isolated so far (Adrian et al., 2000b;Jayachandran et al., 2003). Reductive dehalogenation generally results in the transformation of higher chlorinated CBs but may lead to an accumulation of lower chlorinated CBs such as monochlorobenzene (MCB) under strongly reducing conditions. Preliminary indications for reductive dehalogenation of MCB have been presented (Nowak et al., 1996). Kaschl et al. suggested that anaerobic MCB degradation may lead to significant stable carbon isotope fractionation and applied compound-specific isotope analysis (CSIA) to further indicate in situ biodegradation of MCB in an anoxic aquifer (2005). Recently, complete mineralization of MCB has been proven using stable isotope tracer techniques, which enabled very sensitive detection of low rate degradation processes (2007).
CSIA has been established for monitoring the biodegradation of pollutants at contaminated field sites (Meckenstock et al., 2004a). This method was successfully applied for monoaromatic hydrocarbons (Fischer et al., 2007;Griebler et al., 2004b;Mancini et al., 2002;Vieth et al., 2005), fuel oxygenates (Kuder et al., 2005;Zwank et al., 2005) and chlorinated ethenes (Hunkeler et al., 1999a;Imfeld et al., 2008;Nijenhuis et al., 2005;Sherwood Lollar and Slater, 2001) but, to our knowledge, beside the work of Kaschl et al. (2005) no further field studies were performed for CBs. Moreover, except a few studies on chlorinated ethenes, most studies were conducted to characterize degradation of single contaminants instead of addressing complex scenarios such as sequential degradation mechanisms, multiple contaminants and/or various degradation pathways.

Microbial degradation of organic contaminants is associated with isotope fractionation, leading to an enrichment of heavier isotopes in the residual non degraded fraction. The magnitude of isotope fractionation mainly depends on the reaction mechanism. As shown for reductive dehalogenation of chlorinated ethenes (Cichocka et al., 2008; Nijenhuis et al., 2005) and trichlorobenzenes (TCB) (Griebler et al., 2004a), degradation of CBs should also be associated with significant isotope fractionation, if the reduction of a C-Cl-bond is involved. Similar to anaerobic degradation of monoaromatic hydrocarbons, the anaerobic oxidation of the benzene ring, for which cleavage of a C-H-bond is expected, should lead to isotope fractionation (Fischer et al., 2008; Mancini et al., 2003). However, if the fate of a contaminant is simultaneously controlled by its production (associated with depletion in ^{13}C) and further degradation (leading to enrichment in ^{13}C), as expected from sequential dechlorination, changes in the isotope signature of a contaminant can not be attributed to a single process (i.e. biodegradation) which is limiting the use of the Rayleigh approach (Hunkeler et al., 1999a; Nijenhuis et al., 2005).

At the study site, release of CBs resulted in a severe contamination of the groundwater. It is hypothesized that reductive dehalogenation and/or anaerobic oxidation may govern the removal of CBs under anoxic conditions in the aquifer. Because active remediation technologies were recognized as technically and economically not feasible (Thullner and

Schäfer, 1999), this study aimed at evaluating the efficacy of natural attenuation (NA) processes as remedial option with an emphasis on most persistent MCB, since its accumulation seems to be the limiting factor to apply NA.

For this purpose, following US-EPA guidelines (US-EPA, 1999), we developed an integrative approach which includes the assessment and monitoring of chlorobenzene degradation by means of CSIA and tracer studies under field and laboratory conditions and the delineation of major hydrogeochemical trends associated with observed in situ biodegradation activity. To overcome complications associated with the Rayleigh concept for interpretation of the isotope data, an isotope balance was calculated to assess biodegradation of CBs.

2. Material and Methods

Study site and sampling

The field site is located at a former chemical plant where the production of mostly pesticides caused an intensive release of chlorinated organic contaminants to the subsurface. The direct source zone characterized by high loads of contaminants and dense nonaqueous phase liquid (DNAPL) was completely encapsulated and hydraulically disconnected from the surrounding natural groundwater system to prevent further release of contaminants. The initial contamination was dominated by higher chlorinated benzenes and is preserved inside the containment whereas the resulting plume mainly consists of MCB and dichlorobenzene (DCB) with total concentrations up to 2840 µg L^{-1}. A detailed site description is given in supporting information (SI) (section 1.1).

Groundwater samples for hydrogeochemical and isotope analyses were taken in 2005 and 2006 from 22 monitoring wells (Fig. 1). The sampling, extraction procedure and analytical methods are described in SI. A table summarizing the geochemical data collected during sampling in 2005 is given in SI (Tab. S2).

Chemicals

The [$^{13}C_6$] MCB was purchased from Chemotrade Leipzig (Germany) with chemical and isotopic purity of > 99%. All other chemicals used were obtained in p.a. quality or higher.

Field study with in situ microcosms

In February 2005 a field experiment was performed using an in situ microcosm test system as described elsewhere (Geyer et al., 2005;Kästner et al., 2006;Nijenhuis et al., 2007;Peacock et al., 2004;Stelzer et al., 2006a). Briefly, Bio-Sep® beads were loaded with [$^{13}C_6$] MCB to a concentration of about 100 mg g^{-1} beads. The in situ microcosms were deployed in 6 different wells at the low (H, L), medium (D, E) and high contaminated (A, B) area of the plume in the lower strictly anoxic part of the aquifer (Fig. 1 and Fig. S1). After 72 days in situ microcosms were removed from the aquifer and the carbon isotope signature of total lipid fatty acids (TLFA) was analyzed. Detailed information about the extraction procedure and analytical methods can be found in SI (section 1.4).

Laboratory study with enrichment cultures

Material of in situ microcosms amended with natural abundance MCB as described above were used as inoculum for the laboratory enrichment cultures. From each selected well (A, B, D, E, H, L) four enrichment cultures were prepared in 38 ml vials. Each vial finally contained approx. 27 ml of groundwater and 4-6 Bio-Sep® beads. MCB was added to the vials: always two cultures were amended with 1 µl natural abundant and another two with [$^{13}C_6$] MCB. Cultures were incubated stationary at 20°C in the dark and were sampled at regular time intervals to determine the carbon isotope signature of possible mineralization products (CO_2, CH_4). A more detailed description of the methods is given in SI (section 1.5).

Determination of δ^{13}C values for the total chlorinated benzenes

The isotope signatures of individual CBs ($\delta^{13}C_i$) were analyzed by GC-C-IRMS and are given in δ-notation (per mill) (for details see SI, section 1.6).
The isotope signature (per mill) of the total CBs ($\delta^{13}C_{CB}$) was calculated by multiplying the molar concentration of each compound (C_i) with its respective carbon isotope signature ($\delta^{13}C_i$), adding all contributions and dividing by the total molar concentration of all chlorinated benzenes (C_{CB}) (Eq. 1).

Equation (1)
$$\delta^{13}C_{CB}[‰] = \frac{\sum (C_i * \delta^{13}C_i)}{C_{CB}}$$

In this paper we refer to the term isotope balance to distinguish between the calculated isotope signature of the total CBs ($\delta^{13}C_{CB}$, isotope balance) and the measured isotope signatures ($\delta^{13}C_i$) of the single species of CBs, such as MCB and DCB isomers.
The uncertainty associated with the isotope balance was calculated based on the error propagation using the standard deviation of the isotope measurements ($\Delta\delta^{13}C_i$) and a precision for the concentration analysis of 10 % ($\Delta C_i = 0.1 * C_i$) for each chlorobenzene species (Eq. 2).

Equation (2)
$$\Delta_{tot}\delta^{13}C_{CB} = \frac{\sqrt{\sum \left(C_i * \Delta\delta^{13}C_i\right)^2 + \sum \left(\left[\delta^{13}C_i - \delta^{13}C_{CB}\right] * \Delta C_i\right)^2}}{C_{CB}}$$

Using equation 2, the error for the value of the isotope balance (Δ_{tot} $\delta^{13}C_{CB}$) was ≤ 0.4 ‰ for each sample.

Principal Component Analysis

Principal component analysis (PCA) based on the z-scores was used to assess the gradients in the hydrogeochemical and isotope data and to gain insight into the relationship between variables. The numerical data matrices were converted and the correlation analyses carried out using the program R (R: Copyright 2005, The R Foundation for Statistical Computing Version 2.1.1). Two data sets were subjected to PCA: (i) the hydrogeochemistry data (Tab. S2), isotope composition of MCB and of total CBs corresponding to the year 2005 (Tab. S4), as well as (ii) concentrations of MCB and DCB isomers, isotope composition of MCB and the total CBs corresponding to years 2005 (Tab. S4) and 2006.

3. Results and discussion

3.1. In situ microcosms

The patterns of total lipid fatty acids (TLFA) extracted from in situ microcosms are provided for one representative well located at the low (H), medium (E) and high contaminated (A) area of the plume (Fig. S1). All samples showed significant amounts of saturated hexadecanoic (C16:0) and octadecanoic (C18:0) acids as well as the unsaturated hexadecenoic (C16:1) and octadecenoic (C18:1) acids. Further, an octadienoic acid (C18:2) was identified in all samples. Comparison of the three samples revealed only minor differences in the fatty acid (FA) compositions. While sample H was dominated by C18:0, the most abundant FA in the other two samples was a C18:1 isomer. In sample A, octadecanoic acid was one order of magnitude less abundant compared to E and H (Fig. S1). TLFA profiles showed low taxonomic value similar to previous findings (Geyer et al., 2005; Stelzer et al., 2006a). The total concentrations of TLFA varied between 4000-12000 pmol per microcosm (data not shown) suggesting a significant microbial colonization in the bead material similar to previously published results (Geyer et al., 2005).

Phospholipid fatty acids (PLFA) may offer a sensitive measure to characterize viable bacterial community structures whereas TLFA fraction can additional comprise lipids of dead biomass (Green and Scow, 2000;Kaur et al., 2005;Lu et al., 2007). Basically, the microbial community trapped on in situ microcosms may consist of both viable and non-living organisms as well as degraders and non-degraders. Therefore extraction of TLFA was favored over PLFA to investigate more sensitively the incorporation of ^{13}C into total biomass as an indicator for in situ activity of the bacterial community.

Compared to unlabeled controls (δ^{13}C = -30 ± 5 ‰, data not shown), the TLFA methyl ester fraction of labeled samples showed a clear enrichment in ^{13}C up to 4500 ‰ in individual fatty acids (Tab. 1). Fatty acids with 16 carbons generally represented the highest enrichment in ^{13}C and unsaturated FA were higher labeled than the saturated ones as similarly observed by Nijenhuis et al. (Nijenhuis et al., 2007). FA with odd chain length (C17) showed the highest δ^{13}C value of 4500 ‰ which corresponded to 6 atom % incorporation, but was only found in sample A in very low quantity. For some FA (C18:2, C18:1) no label was found (-31 to -23 ‰) indicating part of the microbial community were not involved in monochlorobenzene (MCB) degradation.

Tab. 1: Carbon isotope signature of TLFA methyl esters $\delta^{13}C_{FAME}$ [‰] extracted from in situ microcosms amended with [$^{13}C_6$] MCB and incubated in the contaminated aquifer at the study site.

Well **[C_{MCB} in µg L^{-1}]**	**A** **[1400]**	**E** **[430]**	**H** **[120]**
C16:1	2600	2000	2000
C16:1	nd	nd	2900
C16:0	1600	70	10
iC17:0	4500	nd	nd
C18:2	-28	-31	-31
C18:1	-28	-31	-31
C18:1	360	43	1
C18:0	50	-25	-31
C21:0	-23	-26	-30

nd - not detectable

However, the transformation of the labeled carbon from the [$^{13}C_6$] MCB into bacterial fatty acids provided evidence of microbial degradation of MCB under ambient aquifer conditions although an enrichment of ≤ 6 atom percent indicated that microorganisms colonizing the in situ microcosms mainly used other carbon sources than the labeled MCB. Overall, the labeling was comparable in all three samples but slightly higher at A suggesting that the presence of a more MCB adapted microbial community correlated with high MCB concentrations.

3.2. Laboratory enrichment cultures

To analyze whether the microflora colonizing the in situ microcosms is capable of MCB mineralization, cultivation-dependent methods were used in the laboratory. Surfaces are very often required for successful cultivation and pre-incubation in the field may improve a later laboratory cultivation procedure (Herrmann et al., 2008). Therefore, material of in situ microcosms, which were directly incubated at the field site, served as pre enriched-inoculum. Application of ^{13}C-labeled substrates in enrichment cultures is one of the few approaches suitable to sensitively detect complete mineralization of a single contaminant in a complex mixture determining the evolution of labeled CO_2 (Morasch et al., 2007). Addition of [$^{13}C_6$] MCB allowed exclusive detection of anaerobic MCB degradation, a supposedly slow process (Nijenhuis et al., 2007).

After 197 days highest enrichment of ^{13}C in CO_2 was observed for the samples from well L with values of 629 ± 85 ‰ and lowest for wells H and D with values of 91 ± 59 ‰ and 130 ± 40 respectively. Analyses of enrichment cultures from A, B and E showed comparable results with $\delta^{13}C_{CO2}$ of 250 ± 91 ‰, 222 ± 19 ‰ and 296 ± 122 ‰, respectively (Tab. S3). Labeled methane could not be detected indicating that MCB degradation was not coupled to methanogenesis. All unlabeled controls showed no ^{13}C enrichment in CO_2 during the course of the experiment.

The extent of MCB mineralization was calculated according to the method of Morasch et al. (Morasch et al., 2007) based on the ^{13}C-CO_2 production in the course of the experiment with respect to the total amount of CO_2 initially analyzed from the groundwater of the respective wells (Tab. S3). Mineralization rates varied between 0.1 to 1.1 nmol per day, indicating very slow MCB mineralization underlining the difficulties associated with cultivation of such organisms. The results are in good agreement with data obtained from Nijenhuis et al. (Nijenhuis et al., 2007) for a similar experiment at another field site. An electron balance to link MCB degradation to iron or sulfate reduction was not feasible due to too high background concentrations of potential electron donors (Tab. S2). In all samples MCB mineralization was further maintained over time up to 1000 days (data not shown).

In summary, both the in situ microcosm and laboratory investigations confirmed anoxic degradation of MCB by proving assimilation of MCB-derived carbon and mineralization of MCB. In situ microcosms provide a very promising tool to directly test in situ biodegradation of recalcitrant contaminants within reasonable time and this technique opens prospects for detailed analysis of the microbial key-players in future.

3.3. Isotope balance computation

Compound-specific isotope analysis (CSIA) of MCB and dichlorobenzene (DCB) was performed to investigate the relevance of degradation processes at the field site. Occurrence of these contaminants in the plume is presumably related to reductive dehalogenation of initially spilled higher chlorobenzenes (CBs) and hexachlorocyclohexane, which are still present in the containment today (SI, sections 1.1, 2.4).

Due to relatively low trichlorobenzene (TCB) concentrations, reductive dehalogenation of TCB is not a relevant process within the plume and other sources for DCB production are lacking (SI, section 2.4). In the plume the carbon isotope signatures of DCBs were in the range of -25.5 to -15.6 ‰, -28.1 to -22.3 ‰ and -28.1 to -22.0 ‰ for 1,2-; 1,3- and 1,4-DCB, respectively (Tab. S4, Fig. S5). Although concentrations did not systematically correlate with the isotope signatures, the high variability observed in the isotope data is supposedly related to microbial DCB degradation. Furthermore, enrichment of ^{13}C in the residual DCB fraction up to 4-5 ‰ compared to the source area (represented by well K) was observed with increasing distance from the containment. The highest isotopic shift (10 ‰ for 1,2-DCB and 4 ‰ for 1,3-DCB) was recorded in the area defined as contaminant source of the plume close to wells A, B, D suggesting that this part of the aquifer may experience reductive dehalogenation of DCB possibly associated with an accumulation of MCB. Further, our data supported the hypothesis of a preferential degradation of 1,2- over 1,3- and 1,4-DCB presumably due to higher yields in Gibbs free energy (Dolfing and Harrison, 1993) since lowest concentrations were determined for 1,2-DCB accompanied by the highest enrichment in ^{13}C and accordingly 1,4-DCB showed highest concentration and lowest isotope fractionation.

Under anoxic conditions reductive dehalogenation is the known degradation pathway for DCB leading to the formation of MCB, which consequently should be depleted in ^{13}C. Indeed in most of the wells the isotope signature of MCB was isotopically lighter compared to DCB. The high abundance of MCB in the plume further indicated that apparently reductive dehalogenation of DCB and probably TCB led to the formation of MCB. Since no significant amounts of higher chlorinated benzenes were detectable in the plume, we suppose that the isotope signatures of DCB were only affected by its degradation.

The isotope signatures of MCB in the plume ranged between -29.6 ‰ and -25.3 ‰. Highest enrichments were found at the N and SE fringe of the plume, where rather low MCB concentrations were detected (Fig. S5). The ^{13}C-enrichment in combination with low MCB concentrations indicated MCB biodegradation in this zone of the aquifer. In the central and western part, presumably representing the former centerline of the plume, $\delta^{13}C$ values of MCB were more depleted indicating microbial mediated production of MCB (Tab. S4, Fig. S5).

As previously suggested, two main mechanisms for MCB degradation should be considered: (i) reductive dehalogenation of MCB to benzene and its subsequent degradation or (ii) anaerobic oxidation of MCB which in this case would serve as electron donor (Braeckevelt et al., 2007a;Nijenhuis et al., 2007;Nowak et al., 1996). As previously observed during reductive dehalogenation of TCB or anaerobic degradation of benzene (Fischer et al., 2008;Griebler et al., 2004a;Mancini et al., 2003), it is expected that microbial degradation of MCB under anoxic conditions would also lead to significant isotope fractionation. However, at the site the situation appears more complex. Indeed MCB could be the product of DCB dehalogenation and simultaneously be subjected to microbial degradation. As both processes are associated with an opposite isotope fractionation effect, the reductive dehalogenation of DCB may result in isotopically lighter MCB whereas the MCB degradation would lead to an enrichment of ^{13}C in the residual MCB fraction. For this reason, the isotope signature of MCB may reflect both processes and the Rayleigh concept is not applicable to quantify biodegradation of MCB hampering a direct interpretation of the MCB isotope data.

In order to investigate whether or not degradation of MCB occurred, an isotope mass balance including all CBs detected was performed (Eq. 1) based on the following assumptions: First, although reductive dehalogenation of DCB to MCB should be associated with isotope fractionation (Griebler et al., 2004a) this fractionation will not affect the cumulative isotope composition of all CBs ($\delta^{13}C_{CB}$). Consequently, only further MCB degradation can lead to ^{13}C enrichment in the isotope balance of all CBs. Therefore significant enrichment of $\delta^{13}C_{CB}$ can be considered as indicative of MCB biodegradation. This assumption is valid regardless to which extent a particular degradation reaction in the reductive sequence (i.e. DCB dechlorination) is associated with isotope fractionation. However, the last reaction step of the reduction sequence should be linked to isotope fractionation to document MCB biodegradation.

Second, if alternative degradation pathways, for example mineralization of a DCB isomer without formation of MCB, occur in parallel and affect concentration or isotope signature of one species of the hypothetical sequence, the isotope mass balance is not closed. In this case, DCBs and MCB will not form a complete reaction sequence and the relative enrichment of individual species reflects degradation. Theoretically, a direct mineralization of DCB to CO_2 would thermodynamically be feasible, but so far this has only been described for aerobic biodegradation (Van Agteren et al., 1998). However, isotope enrichment of DCBs will suggest in situ biodegradation in any case.

The spatial distribution of concentration and respective carbon isotope signatures for the total CBs ($\delta^{13}C_{CB}$) is presented in Fig. 1. The $\delta^{13}C_{CB}$ values ranged between -27.6 ‰ up to -24.8 ‰. The lowest $\delta^{13}C_{CB}$ value of -27.2 ‰ inside the plume was found at well A (Fig. 1), located nearby the containment and defined as the centre of the plume due to highest contaminant concentrations and a cumulative isotope signature of CBs comparable to well K inside the containment ($\delta^{13}C_{CB}$ = -27.6 ‰). The decrease in total CB concentration was associated with significant enrichment of ^{13}C by ≥ 1 ‰ (Fig. 2) indicating in situ biodegradation. Highest ^{13}C-enrichment of total CBs was mainly observed in the west (well O and N) and north (well J) of the plume, suggesting biodegradation of CBs (Fig. 1, 2). Overall, with the exception of well B and I, the data obtained from the isotope balance computation indicated a destructive removal of total CBs from the aquifer and demonstrated that chlorobenzene degradation is a relevant process in most parts of the plume.

The source zone (A, D, B) and the western fringe of the plume, where DCB is still present in higher amounts, were characterized by more enriched $\delta^{13}C_{DCB}$ and $\delta^{13}C_{CB}$ values suggesting microbial degradation of DCB leading to the formation of MCB more depleted in ^{13}C. MCB isotope signatures did not reflect MCB degradation in the western part of the plume. Nevertheless, the isotope balance of total CBs showed ^{13}C enrichment suggesting that chlorobenzenes are destructively removed from the aquifer. At the SE fringe, higher $\delta^{13}C_{MCB}$ values directly indicated MCB degradation. DCB is not very abundant at this part of the aquifer and consequently significant formation of MCB due to DCB degradation is unlikely (Fig. S5).

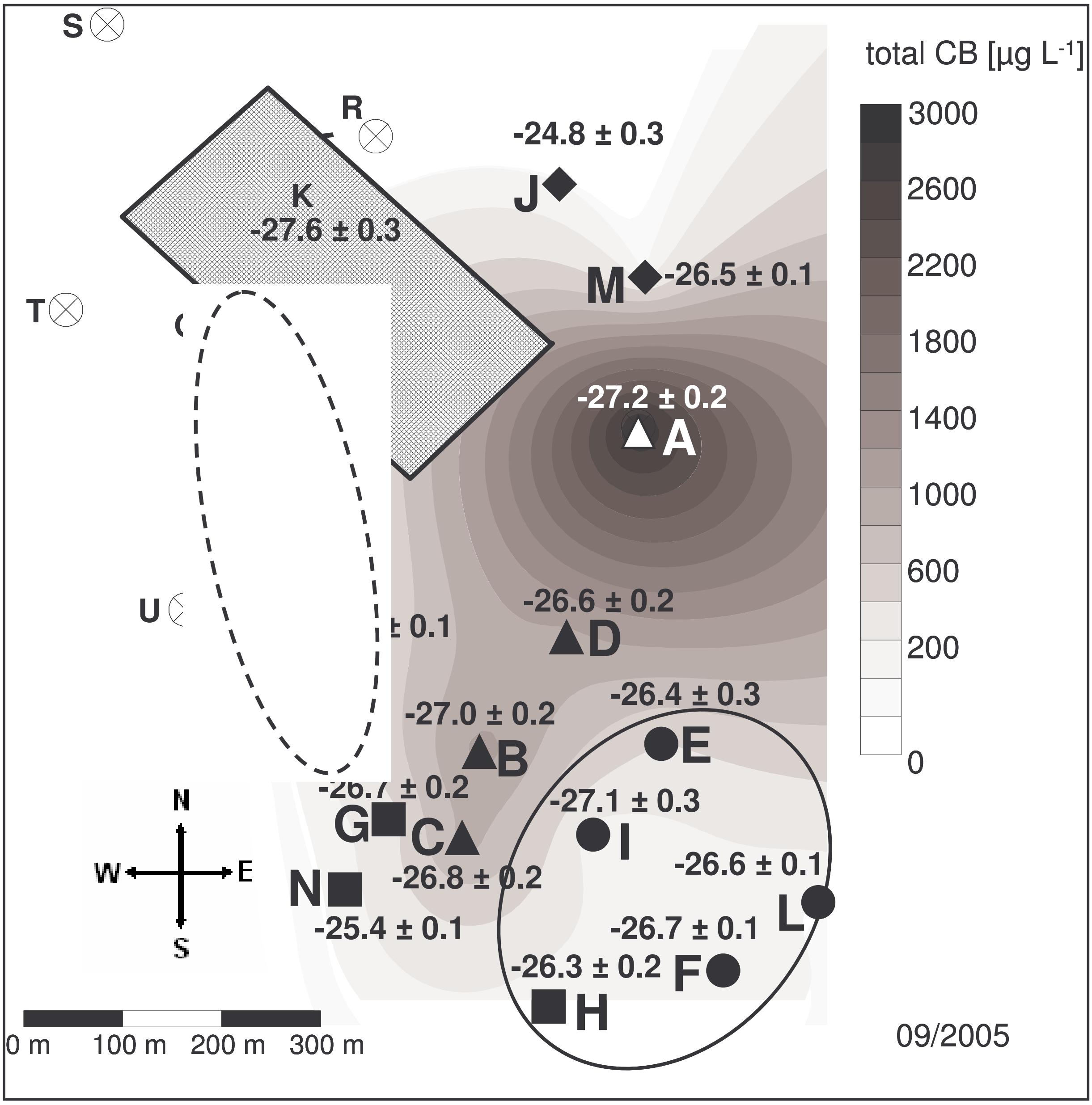

Fig. 1: Concentrations [µg L^{-1}] and carbon isotope signatures [‰] of total chlorinated benzenes (numbers) at the study site for sampling in 2005. The letters indicate the name of the wells and the rectangle in diagonal crosses surrounds the area of the containment, which is hydraulically disconnected from the plume. Additionally the dashed (- - -) black line shows the area were the ratio between MCB and DCB is < 0.3 and the solid (——) line indicates where the ratio between MCB and DCB is > 0.9. The symbols indicate the samples which belong to a common cluster as obtained by PCA: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M) (see fig. 3). Wells which were not considered for PCA are indicated by ⊗.

The isotope balance approach might be challenged, if the different contaminants vary significantly with respect to their physico-chemical properties which may cause a preferential transport of contaminant species between source and down gradient the plume. For DCB and MCB no considerable differences concerning their retardation are expected, since adsorption coefficients are almost identical (Thullner and Schäfer, 1999). Abiotic processes such as dilution were generally not expected to cause considerable isotope effects, but they can influence the contaminant concentration (Fischer et al., 2006;Kopinke et al., 2005) (Fig. 2). As for all isotope based analysis of in situ biodegradation, also the application of an isotope balance requires a relatively stable isotope signal of the contaminant source. The similarity of the $\delta^{13}C_{CB}$ values inside (K) and outside (A) the containment suggests that isotope values of well A can be taken as a source signal at this site.

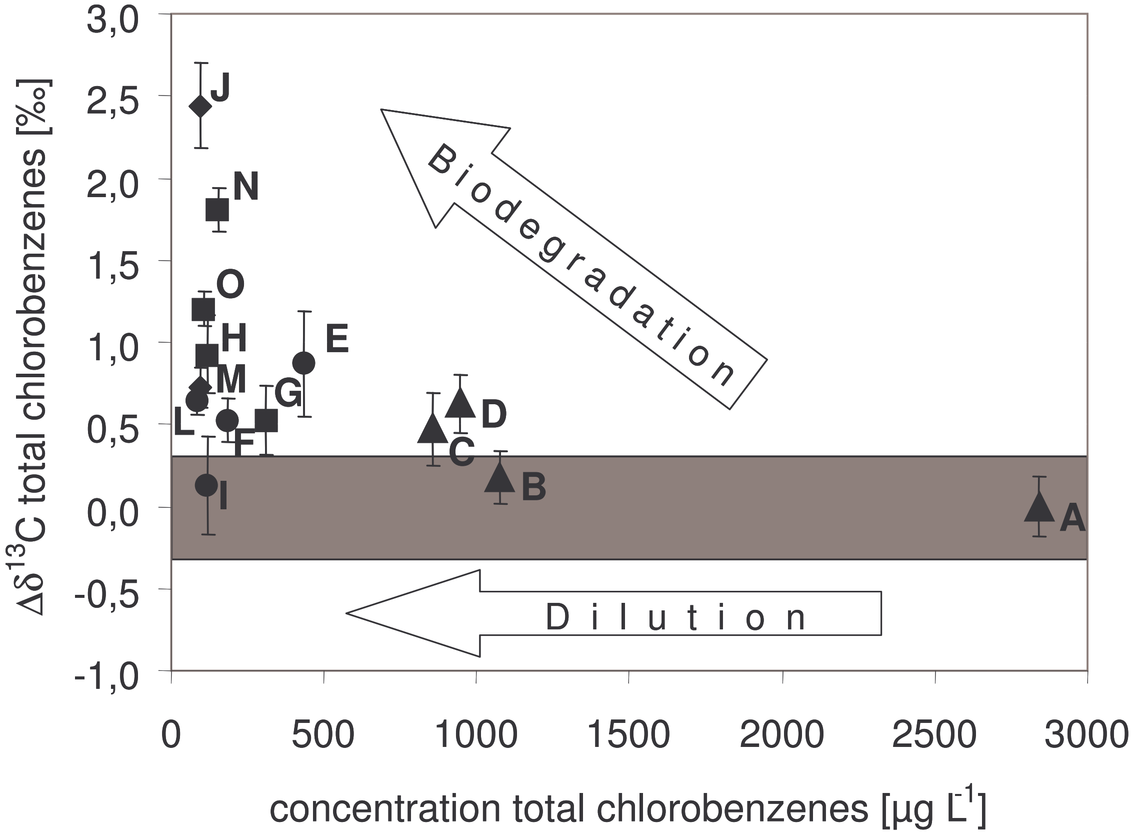

Fig. 2: Correlation between concentration [µg L^{-1}] and isotopic enrichment [‰] of total chlorinated benzenes. The symbols indicate the sampling locations which belong to a common cluster as obtained by PCA: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M) (see fig. 3).

3.4. Statistical analysis of the field data

Principal Component Analysis (PCA) was used to illustrate the main hydrogeochemical variations in the plume and to highlight possible correlations between the assessed hydrogeochemical variables (Andrade et al., 2008;Lee et al., 2001).

In the PCA, carried out on the data obtained in the year 2005, the percent of variance within the data accounted for by the first (PC1) and second principal component (PC2) was high (55 %; Fig. 3). Overall, hydrogeochemical profiles corresponding to wells where the occurrence of degradation activity was clearly noticeable (cluster II and IV) could be distinguished along the PC1 or PC2 from profiles of apparently less active locations (cluster III) or sampling locations where the activity could hardly be detected (cluster I) (Fig. 3A). Higher PC1 and PC2 scores indicated higher biodegradation activity in a well. Indeed, on the variable plot (Fig. 3B), scores of PC1 correlated positively to $\delta^{13}C_{MCB}$, total organic carbon (TOC), electric conductivity, ferrous iron, methane and $\delta^{13}C_{CB}$ (loadings > 0.3) and negatively to total CBs and MCB concentrations. This emphasizes the significance of these variables in distinguishing clusters of similar hydrogeochemical profiles. Interestingly, isotopic composition values of both MCB and CBs were negatively correlated with their concentration values (Pearson's product-moment correlation, $P<0.05$). This supported the results of the isotope balance illustrating a linkage between the shift in the stable isotope signature values and the concentration data as a function of microbial chlorobenzene degradation.

A detailed interpretation of the PCA results revealed four different clusters of hydrogeochemical profiles which correspond to different geographical locations within the plume area (Fig. 3A, Fig. 2 for location of the wells). Cluster I encompasses profiles of wells A, B, C, D located within the more heavily contaminated zone of the plume and consequently scoring negatively along both principal component axes. However, the absence of significant enrichment in ^{13}C for MCB or total CBs in this area does not imply the absence of in situ biodegradation activity in this part of the aquifer. Indeed, CSIA suggested DCB degradation as revealed by relatively high isotopic enrichment of ^{13}C accompanied by MCB formation (Tab. 3, Fig. S5). In this case, the significant DCB enrichment was masked in the isotope balance possibly due to the accumulation of relatively ^{13}C depleted MCB which may point to reductive dehalogenation of DCB. Moreover, the results of the in situ and laboratory tracer studies showed that in situ biodegradation of MCB is feasible under the ambient aquifer conditions, but could not be determined by means of CSIA.

Profiles of cluster II, encompassed wells located at the SW fringe (G, H, N, O), grouped positively along PC2, which is associated with manganese, sulfate and $\delta^{13}C_{CB}$ emphasizing relatively higher values for these variables in this zone. This raises the eventuality of a linkage between anaerobic oxidation of MCB and the presence of sulfate or manganese although reductive dehalogenation is likely to occur in this zone (see section above). Cluster III includes profiles of wells located at the SE fringe of the plume (E, F, I, L), which grouped close to the origin and thus show no extreme values in one or more variables included in the analysis. Alternatively, cluster IV includes profiles of wells located at the northern fringe of the plume (J, M). These samples clustered positively along PC1 and were mostly characterized by relatively higher values of $\delta^{13}C_{MCB}$, ferrous iron, methane, TOC and electric conductivity. At this area, MCB is present > 60% of total CB and isotope enrichment of CB and MCB is likely to mirror MCB degradation. Because $\delta^{13}C_{MCB}$ values were positively correlated with ferrous iron, TOC and electric conductivity ($P<0.05$), a linkage between these geochemical indicators and degradation activity may exist. Indeed, TOC may serve as a source of electron donors to support reductive dehalogenation of CBs. Moreover, positive correlation of ^{13}C enriched MCB with increased ferrous iron concentrations may suggest anoxic oxidation of MCB during ferric iron reduction.

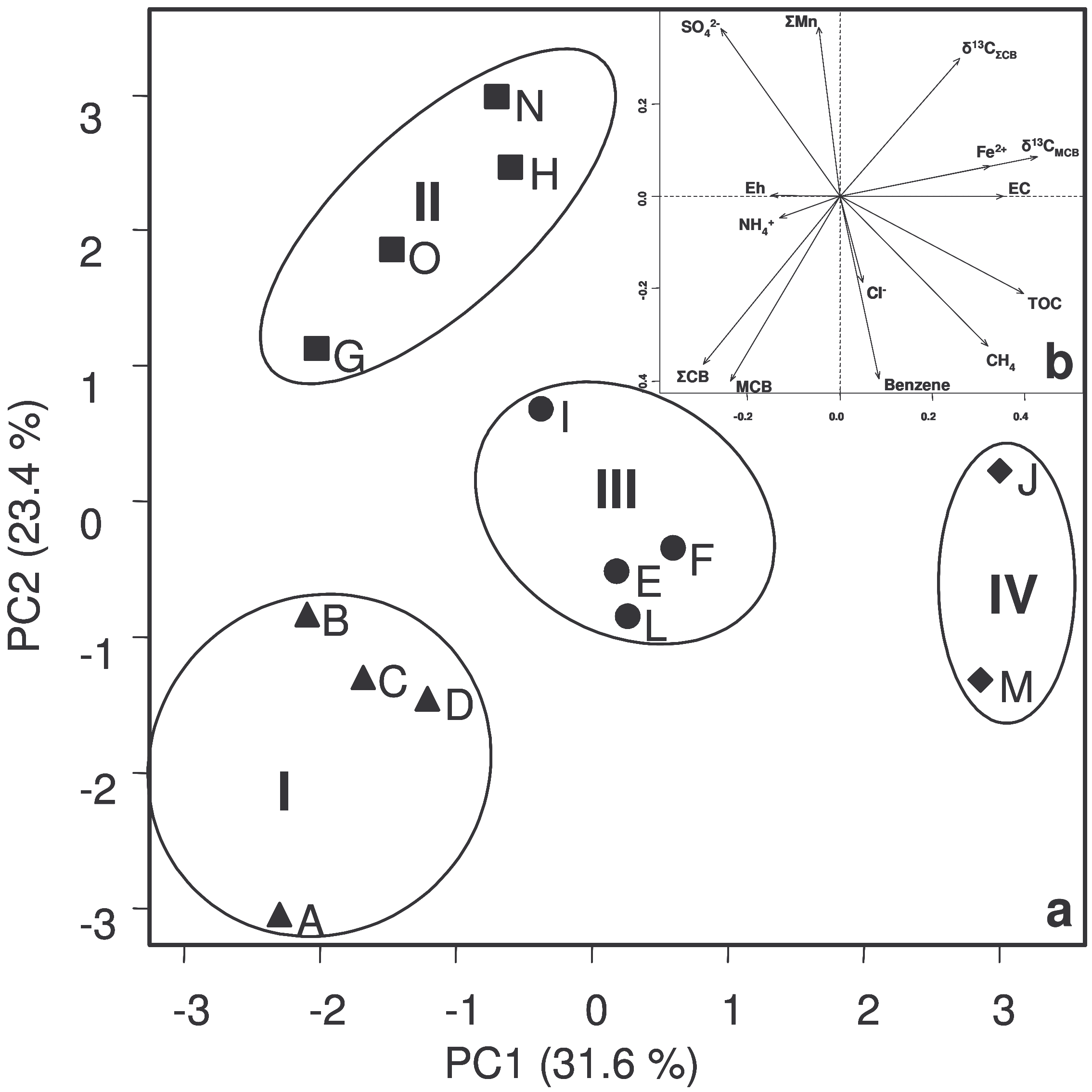

Fig.3a: PCA ordination plot of geochemistry, contaminant concentrations, carbon isotope composition of MCB and total CBs in groundwater samples obtained during sampling in 2005. Values on the axes indicate % of total variation explanation by the corresponding axis (PC1, principal component axis 1; PC 2, principal component axis 2). The symbols indicate the samples which belong to a common cluster: ▲-cluster I (A, B, C, D); ■-cluster II (G, H, N, O); ●-cluster III (E, F, I, J); ♦-cluster IV (J, M).

Fig. 3b: The figure in the upper right corner of the plot depicts the relative contribution of the descriptors in the reduced space. Description vectors correspond to: $\delta^{13}C_{\Sigma CB}$, isotope balance of total CBs; $\delta^{13}C_{MCB}$, carbon isotope signature of MCB; Fe^{2+}, ferrous iron; EC, electric conductivity; TOC, total organic carbon; CH_4, methane; Cl^-, chloride; MCB, monochlorobenzene; ΣCB, total chlorobenzenes; NH_4^+, ammonium; Eh, redox potential; SO_4^{2-}, sulphate; ΣMn, total manganese.

Overall, the statistical analysis revealed clear hydrogeochemical variations within the contaminant plume area, reflecting its inherent heterogeneity. Various zones (cluster I to IV) could be distinguished based on both, the prevailing hydrogeochemical conditions and the stable carbon isotope signatures of MCB and total CBs suggesting chlorobenzene degradation activity under the different geochemical conditions at the site. Moreover, while a combination of tools may be necessary to reliably assess the in situ biodegradation potential of apparently recalcitrant contaminants, interest exists in combining isotope values along with hydrogeochemical variables to determine relevant biogeochemical processes correlating with biodegradation activity.

To further confirm that monitored natural attenuation might be suitable as remediation method at the study site, the stability of the plume as well as of NA processes constitutes a prerequisite (US-EPA, 1999). The comparative analysis of contaminant and isotope signature patterns of sampling locations in year 2005 and 2006 by PCA did not reveal substantial changes (Fig. S3). Additionally, the temporal evolution of contaminant concentration (2000-2007) and corresponding isotope signature (2005-2007) were analyzed in more detail for wells H, F, L at the southern fringe of the plume (Fig S7-9). In all three wells, concentrations varied significantly while the isotope signatures remained relatively stable over time. Although minor temporal variations within the data occurred, the constant enrichment of heavy isotopes compared to central parts of the plume indicated that the biodegradation potential was sustained. This indicates that plume stability may be controlled by microbial activity (see also SI, section 2.5).

4. Conclusions

The integrated approach enabled delineating the in situ degradation potential of recalcitrant chlorinated benzenes under anoxic conditions inside a contaminated heterogeneous aquifer system. Tracer studies with ^{13}C-labelled MCB were performed under both, field and laboratory conditions providing evidence of MCB assimilation and mineralization, respectively. To overcome limitations of isotope data interpretation for sequential degradation pathways such as reductive dehalogenation, the application of an isotope balance was found useful to clearly demonstrate sustainable microbial contaminant removal by CSIA within the plume. The hydrogeochemical analysis using PCA revealed various hydrogeochemical zones within the plume associated with microbial degradation potential. This further suggested that various processes may be involved in contaminant removal and underlined the complexity and heterogeneity of the field site. Especially at such complex field sites characterized by multiple contaminants, sequential degradation and/or different potential pathways one single method can hardly elucidate the relevant biogeochemical processes associated with degradation activity. Therefore, coupling of several techniques may lead to a more robust assessment and allows verifying plausibility of lines of evidence for in situ biodegradation processes as required by authorities (US-EPA, 1999). Even for recalcitrant contaminants (e.g. MCB) with unknown degradation pathways, an appropriate methodology may provide evidence of in situ biodegradation. Future research is necessary to identify the microorganisms involved in chlorobenzene degradation as well as the underlying degradation mechanism and environmental conditions.

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Supplementary Information Available

Detailed description of the field site, sampling, experimental set up, analytical methods, hydrogeochemical data, chromatograms of in situ microcosm analysis, $\delta^{13}C_{CO2}$ of laboratory enrichment cultures, isotope analysis of chlorinated benzenes, plume stability is available free of charge via the Internet.

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Section 2: Assessment of in situ degradation of chlorinated ethenes and bacterial community structure in a complex contaminated groundwater system

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Abstract

The occurrence of in situ degradation of chlorinated ethenes was investigated using an integrated approach in a complex groundwater system consisting of several geological units. The assessment of hydrogeochemistry and chlorinated ethenes distribution using principal component analysis (PCA) in combination with carbon stable isotope analysis revealed that chlorinated ethenes were subjected to substantial biodegradation. Shifts in isotopic values up to 20.4‰, 13.9‰, 20.1‰ and 31.4‰ were observed between geological units for tetrachloroethene (PCE), trichloroethene (TCE), cis-dichloroethene (cDCE) and vinyl chloride (VC), respectively. The use of specific biomarkers (16S rRNA gene) indicated the presence of *Dehalococcoides* sp. DNA in 20 of the 33 evaluated samples. In parallel, the analysis of changes in the bacterial community composition in the aquifers using canonical correspondence analysis (CCA) indicated the predominant influence of the chlorinated ethene concentrations (56.3% of the variance, $P<0.005$). The integrated approach may open new prospects for the assessment of spatial and temporal functioning of bioattenuation in contaminated groundwater systems.

1. Introduction

The Bitterfeld/Wolfen region (Sachsen-Anhalt, Germany) comprises a contaminated area of about 25km^2 as a result of the former chemical industry (Wycisk et al., 2003).Chlorinated ethenes are among the main contaminants in this area and they belong to the most common contaminants observed in groundwater systems worldwide (Vogel, 1994; McCarty and Semperini, 1994). Tetrachloroethene (PCE) and trichloroethene (TCE) are initially predominant compounds in this groundwater system, whereas dichloroethenes (DCEs) and vinyl chloride (VC) occur mainly as the result of in situ microbial transformation (Nijenhuis et al., 2007). Under anaerobic conditions, the main mechanism resulting in chlorinated ethenes transformation to non-toxic ethene is reductive dechlorination (MaymoGatell et al., 1997).

Monitored natural attenuation (MNA) is an approach gaining increased attention to manage sites contaminated with chlorinated hydrocarbons. However, successful implementation of MNA requires a monitoring strategy relying on several lines of evidence, which can provide an insight into the processes underlying contaminant depletion. In particular, combining several complementary methods may be highly profitable to substantiate lines of evidence for in situ biodegradation in geologically complex large-scale contaminated sites displaying a restricted number of monitoring wells. The analysis of the geochemistry and the dominating terminal electron acceptors of contaminated aquifers is thought to be critical and may be improved using multivariate statistics (Lee et al., 2001; Palumbo et al., 2004). Compoundspecific isotope analysis (CSIA) is a tool to monitor in situ biodegradation over space and time (Slater et al., 1999; Hunkeler et al., 1999; Meckenstock et al., 2004). Carbon isotope fractionation results from slightly different activation energies needed to break chemical bonds involving the light (12C) versus heavy isotope (13C). This means that the bonds formed by the light isotope are weaker and more readily reacted than those with the heavy isotope (Hoefs, 1997). Isotope composition and concentration of the residual, not yet degraded substrate fraction, is analysed and enrichment in the heavy isotope indicates biodegradation. Substantial carbon isotope fractionation of chlorinated hydrocarbons during reductive dechlorination was observed in both laboratory (Nijenhuis et al., 2005) and field studies (Hunkeler et al., 2005; Morrill et al., 2005; Nijenhuis et al., 2007). An application of the stable isotope fractionation concept to quantify in situ degradation of chlorinated ethenes usually requires information concerning the presence and activity of degrading microorganisms in the field (Nijenhuis et al., 2005).

Nucleic acid-based tools offer insights into bioremediation by exploring in situ microbial community dynamics during biotransformation (Eyers et al., 2004). Specific molecular markers can be used to determine the presence of potential degraders. In particular, attention has been focused on the relationship between complete conversion of PCE and TCE to ethene and the involvement of *Dehalococcoides* populations in this process (Hendrickson et al., 2002; Major et al., 2002; Lendvay et al., 2003; Lu et al., 2006). In addition, DNA fingerprinting techniques, such as PCR-denaturing gradient gel electrophoresis (DGGE), may further elucidate the bacterial community structure and allow assessment of the changing composition of bacterial communities in contaminated aquifers subjected to natural attenuation (Zhuang et al., 2005; Feris et al., 2004). For this purpose, multivariate ordination methods can be used to highlight the possible environmental descriptors governing the ordering of information, such as microbial community structure variation. In addition, these methods present the major advantage of condensing the information on a simple scheme (Legendre and Legendre, 1998; Fromin et al., 2002). Indeed, so far, the diversity and stability of bacterial communities in contaminated subsurface environments undergoing biodegradation remain poorly characterized. For instance, very little is known about the changes in genetic diversity and the spatial distribution of bacterial communities in response to the variations in geology, groundwater geochemistry and contaminant concentrations. Few studies have focused on the characterization of in situ bacterial community structure at chlorinated ethene-contaminated sites, and the relationship between structure and function remains poorly understood.

In this study, we investigated the distribution of hydrogeochemical variables and chlorinated ethenes within the Quaternary and Tertiary Bitterfeld aquifers using multivariate analysis. The carbon stable isotope composition of the chlorinated ethenes was also analysed to assess the in situ contaminant degradation. In parallel, molecular techniques were applied to retrieve information on the bacterial guild associated with in situ biodegradation. Biomarkers (16S rRNA gene) were employed to detect the presence of potentially degrading microorganisms, and DGGE analysis was applied to address the total community composition changes in the contaminated aquifers. Geochemical variables and contaminant concentrations were used as explanatory variables to determine their influence on the observed bacterial community changes.

2. Materials and methods

Field site. The study site is located in the city of Bitterfeld in eastern Germany and covers an area of approximately 2 km^2 (Fig. 1 and Fig. S1 (Supplementary Material)). The geological setting and the hydrogeological situation have been described previously by Nijenhuis et al. (2007), Heidrich et al. (2004a, b) and Wycisk et al. (2002, 2005). Detailed information concerning the hydrogeological situation and the geological setting is provided in Fig. S2 (Supplementary Material).

Selection of wells and sampling procedure. The sampling procedure is summarized in Table 1 and wells' characteristics are provided in Table S1 (Supplementary Material). Three sampling campaigns were carried out over 13 months to collect the groundwater samples for further analysis: campaign 1: 04/2005, campaign 2: 12/2005 and campaign 3: 05/2006. Geochemical and contaminant concentration data corresponding to the first campaign were previously described (Nijenhuis et al., 2007). For the geological and contaminant survey, 60 wells were selected for sampling; of these, 24 and 36 wells were chosen to investigate the Quaternary and the Tertiary aquifers, respectively. For the taxon-specific investigation, 33 wells were selected. 14 representative wells were chosen for the total bacterial community analysis. The relative locations of the wells listed in Table 1 are shown in Fig. 1. The groundwater samples were taken using submergible pumps (Type MP1, Grundfos, Bjerringbro, Denmark). To ensure representative sampling, several volumes of the groundwater well were pumped before sample collection. During pumping, pH, temperature, redox potential and oxygen concentration were monitored. Geochemical variables and contaminant concentrations were determined by the laboratory of the SGS Institute Fresenius GmbH in Espenhain, Germany (Tables S2–S5, Supplementary Material). For the molecular investigation, at least two replicate samples (1 L each) were collected in clean autoclaved glass bottles. Samples were stored on ice in the field and during transport to the laboratory.

Analytical methods. Gas chromatography combustion isotope ratio mass spectroscopy (GC-C-IR-MS) was applied to determine the stable carbon isotope composition in 200 mL groundwater samples. Stable isotope samples were measured in triplicate and the analyses were carried out within 30 days after sampling. Samples were heated for at least 2 h at 60 ℃ prior to measurement to enhance partitioning of analytes into the head space. The carbon isotope ratio measurements for the chlorinated ethenes were carried out via headspace analysis. Aliquots (50–1000 µL) of head space samples were injected into a gas chromatograph (Agilent 6890; Palo Alto, USA) in split mode connected via a combustion line to a Finnigan MAT 252 isotope ratio mass spectrophotometer according to Nijenhuis et al. (2007). Split ratio was set at 1:3 to 1:100, using a split/splitless injector at 250 ℃. For carbon isotope analysis of the chlorinated ethenes, a Poraplot Q column (30m x 0.32mm ID, 1 µm film; Chrompack, Middelburg, The Netherlands) was used for the separation. The injection temperature was 250 ℃. The oven temperature program was: 40 ℃ (5min); 27 ℃min^{-1} to 150 ℃; 5 ℃min^{-1} to 245 ℃; increase with 35 ℃ min^{-1} to 260 ℃ and hold isotherm for 2 min. Further analysis of data revealed that the standard deviation of each triplicate measurement was systematically lower than the typically defined analytical error of 0.5 ‰ associated with carbon isotope analysis (Dempster et al., 1997). The carbon isotope composition is reported in the delta notation as $\delta^{13}C$ values (‰) relative to Vienna Pee Dee Belemnite Standard (V-PDB, IAEA Vienna) (Coplen et al., 2006).

Molecular biology investigation

DNA extraction. Preparation of the samples for DNA extraction was carried out within 24h after groundwater collection. Groundwater samples from the first sampling campaign were centrifuged (40 mL sample, 14,000*g*, 30 min) and DNA was isolated from the obtained pellets. In order to extract cells from samples corresponding to the second and the third sampling campaigns, 1 L of each sample was filtered through a sterile 0.2 µm membrane (MoBio Water DNA kit). DNA from cells was extracted with a bead beating technique (Fast Prep System, Qbiogene, Irvine, CA) using a FastDNA spin kit for DNA extraction (BIO101).

Taxon specific detection. Taxon-specific 16S rRNA-based PCR amplification was used to test for the presence of the genera *Dehalococcoides* (Hendrickson et al., 2002), *Dehalobacter* (Schlotelburg et al., 2002) and *Desulfuromonas* (Löffler et al., 2000). To increase the sensitivity of our molecular detection, a nested PCR approach was used with *Dehalococcoides*-specific primers. Instead of using universal bacteria primer sets, *Dehalococcoides*-specific primers (Fp DHC 1 and Rp DHC 1377) were used for the first round of PCR, and the second PCR was carried out with a nested *Dehalococcoides*-specific primer set (Fp DHC 774 and Rp DHC 1212) using 2 µL product from the previous PCR as a template in a final volume of 50 µL. For the detection of the other genera, due to the lack of the nested primer approach, a second PCR was carried out using the same primers set. Direct sequencing of PCR product from the same site in a previous study resulted in unambiguous specific sequences (Nijenhuis et al., 2007).

Table 1 – Summary of the sampling scheme

Well	Geological unit	Sampling campaign	Geochemistry	Chlorinated ethenes conc.	Taxon-specific analyses	DGGE profile	CSIA
BVV588	Upper quaternary	2	Yes	Yes	No	No	Yes[a]
BVV589		2	Yes	Yes	Yes[a]	No	Yes[a]
BVV595		2	Yes	Yes	No	No	Yes[a]
BVV593		2	Yes	Yes	No	No	Yes[a]
BVV591		2	Yes	Yes	No	No	Yes[a]
BVV3050		2	Yes	Yes	No	No	Yes[a]
BVV599		2	Yes	Yes	Yes[a]	No	Yes[a]
RT03		3	Yes	Yes	Yes[a]	Yes	Yes[a]
RT07		3	Yes	Yes	Yes[a]	Yes	Yes[a]
RT02		3	Yes	Yes	Yes[a]	Yes	Yes[a]
RT12		3	Yes	Yes	Yes[a]	Yes	Yes[a]
BVV5891	Lower quaternary	2	Yes	Yes	No	No	Yes[a]
BVV5881		2	Yes	Yes	Yes[a]	No	Yes[a]
BVV5951		2	Yes	Yes	No	No	Yes[a]
BVV3063		2	Yes	Yes	Yes[a]	No	Yes[a]
BVV5931		2	Yes	Yes	No	No	Yes[a]
BVV607		2	Yes	Yes	No	No	Yes[a]
BVV5961		2	Yes	Yes	No	No	Yes[a]
BVV3073		2	Yes	Yes	No	No	Yes[a]
BVV606		2	Yes	Yes	No	No	Yes[a]
RT021		3	Yes	Yes	Yes[a]	Yes	Yes[a]
RT031		3	Yes	Yes	Yes[a]	Yes	Yes[a]
RT071		3	Yes	Yes	Yes[a]	Yes	Yes[a]
RT121		3	Yes	Yes	Yes[a]	Yes	Yes[a]
BVV1242	Upper tertiary	3	Yes	Yes	Yes[b]	No	Yes[b]
BVV092		3	Yes	Yes	Yes[a]	Yes	Yes[a]
LK19		3	Yes	Yes	Yes[a]	Yes	Yes[a]
BVV1252		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV3032		1	Yes	Yes	No	No	Yes[b]
BVV3042		1	Yes	Yes	No	No	Yes[b]
BVV3052		1	Yes	Yes	No	No	Yes[b]
BVV3062		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV3062		3	Yes	Yes	Yes[a]	Yes	Yes[a]
BVV3072		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV5232		1	Yes	Yes	No	No	Yes[b]
BVV5242		1	Yes	Yes	No	No	Yes[b]
BVV5252		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV5262		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV5262		3	Yes	Yes	Yes	Yes	Yes[a]
BVV6052		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV6082		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV1241	Lower tertiary	1	Yes	Yes	Yes[b]	No	Yes[b]
BVV1241		3	Yes	Yes	Yes[a]	Yes	Yes[a]
BVV3051		1	Yes	Yes	No	No	Yes[b]
BVV3051		3	Yes	Yes	Yes[a]	Yes	Yes[a]
BVV1251		1	Yes	Yes	No	No	Yes[b]
BVV3031		1	Yes	Yes	No	No	Yes[b]
BVV3041		1	Yes	Yes	No	No	Yes[b]
BVV3061		1	Yes	Yes	No	No	Yes[b]
BVV3071		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV5231		1	Yes	Yes	No	No	Yes[b]
BVV5241		1	Yes	Yes	No	No	Yes[b]
BVV5251		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV5261		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV5261		3	Yes	Yes	Yes[a]	Yes	Yes[a]
BVV538		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV6051		1	Yes	Yes	Yes[b]	No	Yes[b]
BVV6083		1	Yes	Yes	Yes[b]	No	Yes[b]
GOI898		1	Yes	Yes	No	No	Yes[b]
LK342		1	Yes	Yes	No	No	Yes[b]

Listed are wells, corresponding geological unit, sampling campaign and presence/absence of geochemical analysis, contaminant concentration analysis, DGGE profile and CSIA. CSIA = compound-specific isotope analysis; DGGE = denaturing gradient gel electrophoresis.

[a] Indicate analysis performed in this study.

[b] Indicate analysis performed by Nijenhuis et al. (2007).

DGGE analysis. DGGE was carried out using the DCode Universal Mutation Detection System (BioRad, Munich, Germany). Two PCR-DGGE analyses were performed separately for each of the 14 selected wells. A two-step PCR was used to amplify a fragment of about 400 bp of the bacterial 16S rRNA gene by using HotStar Taq polymerase (Qiagen) with buffers supplied by the manufacturers. The detailed amplification and gel preparation protocols are provided in the Supplementary Material. The DGGE fingerprints were normalized according to the reference patterns and were compared using the GelComparII software (Applied Maths, Kortrijk, Belgium). DGGE banding patterns were then converted to a numerical matrix, to be subsequently used for statistical analysis. The matrix was composed of rows of objects representing the selected wells and columns of species representing the DGGE bands position along the vertical gel gradient. A binary matrix corresponding to the presence/absence of each band, further defined as species, was obtained. Bands with an average relative contribution of <1% were discarded fromthe analysis and rare species (<2) were discarded from the matrix.

Statistical analysis

Monte Carlo permutation, Pearson's correlation tests and ordination methods (principal component analysis (PCA) and canonical correspondence analysis (CCA)) were applied on the basis of numerical data matrices converted using the program R (R: Copyright 2005, The R Foundation for Statistical Computing Version 2.1.1). The hydrogeochemical and contaminant concentration data were subjected to PCA to display the correlations among those descriptors and to investigate geological, spatial and temporal interrelation between the groundwater samples. Correlation between descriptors was analysed using the Pearson's product moment correlation coefficient, and considered significant at $P<0.05$. To enhance the readability and interpretation of the results by avoiding a too high level of congruence of the samples, the data sets corresponding to the Quaternary and the Tertiary aquifers were analysed separately. For the same reasons, geochemical and contaminant concentrations data sets corresponding to the Tertiary aquifers were analysed separately. First, Mantel tests were carried out to determine the correlation between the community matrix obtained from the DGGE matrix and environmental data measured on the same samples (Mantel, 1967). The tests were carried out on the basis of dissimilarity matrices, calculated using the Jaccard index and Euclidian distances, for the DGGE profiles matrix and the environmental matrices, respectively. The statistic was evaluated as a moment correlation, using 1000 permutations to assess its significance. Second, the classical Sørensen similarity index was used to evaluate β-diversity based on pairwise comparisons of bacterial species composition (Jongman et al., 1995). CCA was further applied to test and quantify the effects of various sets of explanatory variables on the DGGE profiles variation, assuming unimodal distribution of species (ter Braak, 1986). Thus, the purpose of this method was to find the combination of explanatory environmental variables that best explained the variation of the DGGE matrix. The significance of the constrained ordination process was tested with the Monte Carlo permutation test (999 permutations).

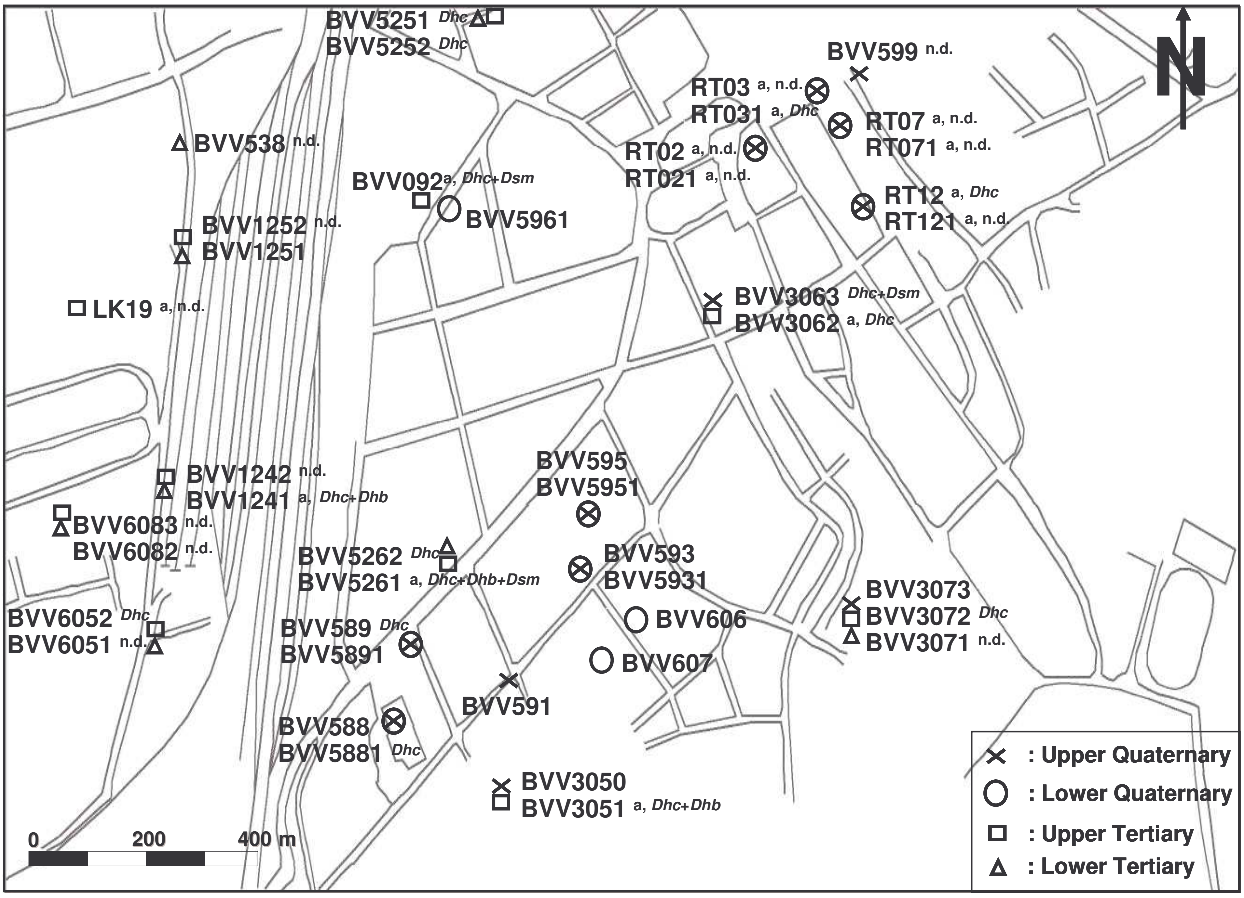

Figure 1: indicates monitoring well numbers, relative locations and type molecular investigations carried out. The location of the wells is indicated: (×) upper, (○) lower Quaternary aquifer, (□) upper, and (Δ) lower Tertiary aquifer. Labels next the markers indicate well number with the well reaching the upper Quaternary aquifer listed on the top, and the well reaching the under layers at the bottom.
[a] Bacterial community patterns generated by 16S rRNA gene DGGE analysis
[Dhc, Dhb, Dsm] Taxon-specific detection trial: *Dhc* = *Dehalococcoides* sp., *Dhb* = *Dehalobacter* sp., *Dsm* = *Desulfuromonas* sp., n.d. = not detected.

3. Results

3.1. Quaternary aquifer

Geochemistry and contaminant concentrations analysis. The geochemical and chlorinated ethene concentration data sets were combined and analyzed by principle component analysis. The PCA ordination plot of groundwater samples retrieved from the Quaternary aquifer is shown in Fig. 2. The PCA was performed by combining both the geochemistry and the contaminant concentration data sets. However, samples BVV3073 and BVV3063 display particularly high TCE and DCE concentrations, respectively. Therefore, they were removed from the analysis because they caused the rest of the objects to cluster together and hindered a good readability of the data (data not shown). The amount of variation explained by the first and second principal components represented 49.6% of the total variation. Samples from the upper and lower Quaternary aquifer originating from the northeast zone of the investigation area clustered together and were associated with PCE, TCE, ethene, pH and nitrite, suggesting that nitrate reduction might be prevalent in this zone. The vectors representing variables such as electric conductivity, ammonium, redox potential and dissolved oxygen were positively correlated ($P<0.05$). These vectors were found in the opposite direction with respect to vectors representing PCE, TCE, ethene, pH and nitrite, themselves positively correlated ($P<0.05$), indicating no correlation or negative correlation ($P<0.05$) with these variables. Samples from the other wells were mainly associated with ammonium, electrical conductivity, dissolved oxygen, redox potential and iron (II). Samples fromthe upper and lower Quaternary aquifer did not display substantially different geochemical patterns. Moreover, the PCA suggests a coincidence of various terminal electron-accepting processes (TEAPs) in the Quaternary aquifer, as depicted by the relationship between geochemical descriptors.

Isotope composition. Generally, low amounts of chlorinated ethenes (<5 µg L^{-1}) were observed in the wells of the upper layer, which hindered the determination of an isotope composition (Table S4, Supplementary Material). In the lower Quaternary aquifer, the plumes of the two DCE isomers overlapped and the trans isomer systematically accounted for less than 20% of the DCEs. The lower Quaternary samples from the N-E zone displayed high amounts of PCE and TCE, and showed similar isotope signatures (δ[PCE] = -27.5‰ to -18.7‰; δ[TCE] = -26.6‰ to -23.9‰). In the same wells, lighter cis-DCE (δ = -30.7‰ to -25.0‰) signatures compared with the corresponding TCE signatures suggest that the dechlorination process started without further transformation of the DCEs. Moreover, VC and ethene concentrations were systematically below the detection limit of the GC-C-IRMS. Conversely, PCE and TCE concentrations and signatures were under the detection limit in the other samples from the lower Quaternary, whereas DCEs, VC and ethene were detectable. The DCEs were particularly enriched in ^{13}C (δ[cis-DCE] = -18.1‰ to -16‰; δ[trans-DCE] = +0.7‰ to +4.8‰) in several wells (BVV606, BVV5961, BVV607 and BVV5931), suggesting a further dechlorination confirmed by the corresponding presence of VC and ethene. The highest concentration of VC was observed in the southern part of the investigated area (BVV606), corresponding to the lightest isotope signature (δ[VC] = -26.7‰). Relatively heavy VC was observed in other wells (δ[VC] up to -7.4‰), which indicates a further degradation. Ethene concentrations ranged from 98 to 568 µg L^{-1} and were systematically lighter than that of VC (δ[ethene] = -37.8‰ to -24.5‰), suggesting further degradation of VC.

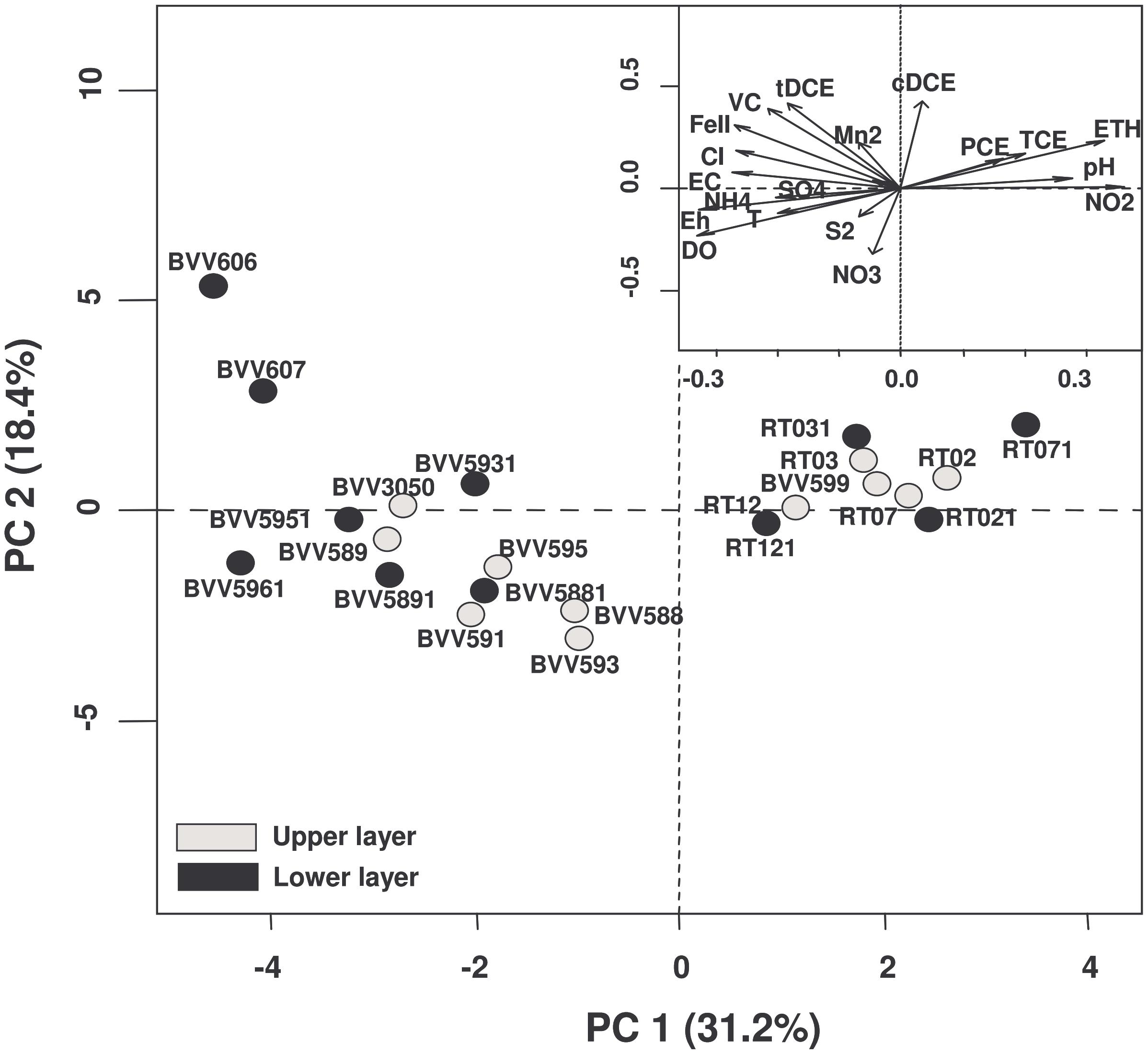

Fig. 2: PCA ordination plot of the groundwater samples according to the geochemistry and chlorinated ethenes concentrations in the Quaternary aquifer. Description vectors correspond to: NO3, nitrate; S2, sulphide; DO, dissolved oxygen; T, temperature; Eh, redox potential; NH4, ammonium; SO4, sulphate; EC, electric conductivity; Cl, chloride; FeII, ferrous iron; VC, vinyl chloride; tDCE, *trans*-DCE; Mn2, manganese(II); cDCE, *cis*-DCE; PCE, tetrachloroethene; TCE, trichloroethene; ETH, ethene; NO2, nitrite. Objects correspond to the wells. Values on the axes indicate % of total variation explanation by the corresponding axis (PC 1, principal component axis 1; PC 2, principal component axis 2).

3.2. Tertiary aquifer

Geochemistry and contaminant concentrations analysis. Two principal component analyses were carried out to investigate separately the main trends of variation of the geochemistry and the contaminants in the Tertiary aquifer (Fig. 3). In the analysis of the geochemistry (Fig. 3a), the amount of variation explained by the first and second principal components represented 61.8% of the total variation.

The vectors representing the Fe(II), electric conductivity and sulphate variables were orientated in the same direction, indicating a positive correlation ($P<0.05$). These vectors were in the opposite direction with regard to the vector representing pH, indicating no correlation (between sulphate and pH) or negative correlation ($P<0.05$) with the previous variables. Samples from the upper and lower Tertiary aquifer did not display substantially different geochemical patterns. Some samples of the lower Tertiary aquifer displayed exceptionally high ferrous iron and sulphate concentrations (BVV1252, BVV538 and BVV1242). Samples LK19, BVV1242 and BVV3052 display particularly high concentration of PCE, TCE and DCEs, respectively (Table S5, Supplementary Material). Therefore, they were removed from the analysis because they caused the rest of the objects to cluster together when included in the same PCA (data not shown). In the analysis of the contaminant concentration (Fig. 3b), the amount of variation explained by the first and second principal components represented 71.7% of the total variation. A positive correlation was observed between PCE and TCE variables ($P<0.005$), which displayed no correlation with ethene. This emphasizes the relationship between decreasing concentrations of the higher chlorinated ethenes and the production of ethene, characterizing the main trend of variation of the samples along the second component (PC 2). The variable characterizing the main trends of variation of the samples along the first component (PC 1) were the intermediates trans-DCE and, to some extent, cis-DCE. A majority of wells displayed a similar composition of contamination patterns and thus clustered together. Wells outside this original cluster display uncommon contaminant composition patterns as they showed relatively higher concentrations of at least one species of chlorinated ethene.

Isotope composition. The changes of both concentration patterns and isotope signatures of the chlorinated ethenes between the first and third sampling campaigns were compared in five wells of the Tertiary aquifer (BVV3051, BVV5261, BVV5262, BVV1241 and BVV3062) (Table S5, Supplementary Material). A shift in the concentration patterns of some of these samples occurred over time (Fig. 3b). Ethene, the final reductive dechlorination product, was detected over time in all the investigated wells, whereas PCE concentrations increased in wells BVV1241, BVV5262 and BVV3062. Similarly, TCE concentrations increased in the wells BVV3062, BVV5262 and BVV3051. However, no clear variation of the isotope signature was observed (δ[PCE] = -39.1‰ to -32.7‰; δ[TCE] = -29.0‰ to -25.6‰), but PCE and TCE were lighter in the Tertiary than in the Quaternary aquifer, indicating their vicinity to the contaminant source of the Tertiary aquifer wells (Nijenhuis et al., 2007). TCE in wells BVV5262 and BVV3062 was heavier than that in the heaviest industrially produced TCE (δ = -27.8‰), suggesting that degradation has taken place (van Warmerdam et al., 1995). For wells BVV3051 and BVV3062, the cis-DCE isomer dominated over time (>80%), whereas the isotope signature of TCE did not change in the other wells. Slightly heavier cis-DCE was only observed in wells BVV1241 and BVV5261 over time (δ[cis-DCE] = -24.6‰ to -22.6‰, and -25.4‰ to -18.3‰, for sampling campaigns 1 and 3, respectively) and corresponds to the presence of VC as a dechlorination product, suggesting a further microbial transformation. In the other wells, VC became enriched over time (well BVV5262) or did not display significantly different changes (wells BVV5261, BVV1241, BVV3051 and BVV3062). Finally, ethene concentrations decreased over time, except in well BVV1241, and ethene became systematically lighter in all the wells ($\delta 1^{3}$C-ethene= -63.9‰ to -29.1‰, and -76.2‰ to -57.9‰, for campaigns 1 and 3, respectively).

3.3. Investigation of the groundwater bacterial community using taxon-specific detection

Positive PCR signals were yielded in 20 of the 33 selected groundwater samples (5 of 12 and 15 of 21, in the Quaternary and Tertiary aquifers, respectively). Interestingly, the presence of *Dehalobacter* and *Desulfuromonas*-like bacteria systematically coincided with the presence of *Dehalococcoides*-like bacteria detected in all positive samples. The sample from well BVV5261 was the only one in which the presence of all three genera was observed. The detection of *Dehalococcoides*-like bacteria and the presence of ethene and VC generally coincided. Wells BVV5881 and BVV589, both reaching the Quaternary aquifer, constituted exceptions and displayed very low chlorinated ethene concentrations. However, in 10 wells where ethene and VC production was observed, no target microorganisms were detected with the applied PCR strategy. In general, the geochemical milieu, as defined by the measured variables, in which

DNA of the evaluated dehalorespiring genera was detected, widely varied. In the 20 samples where *Dehalococcoides* DNA was detected, the pH ranged from 3.7 to 8, the electrical conductivity was in the range of 545–6960 µS cm^{-1}, the redox potential ranged from -476 to 192mV and the dissolved oxygen concentration was
in the range of 0 to 2.7mg L^{-1}.

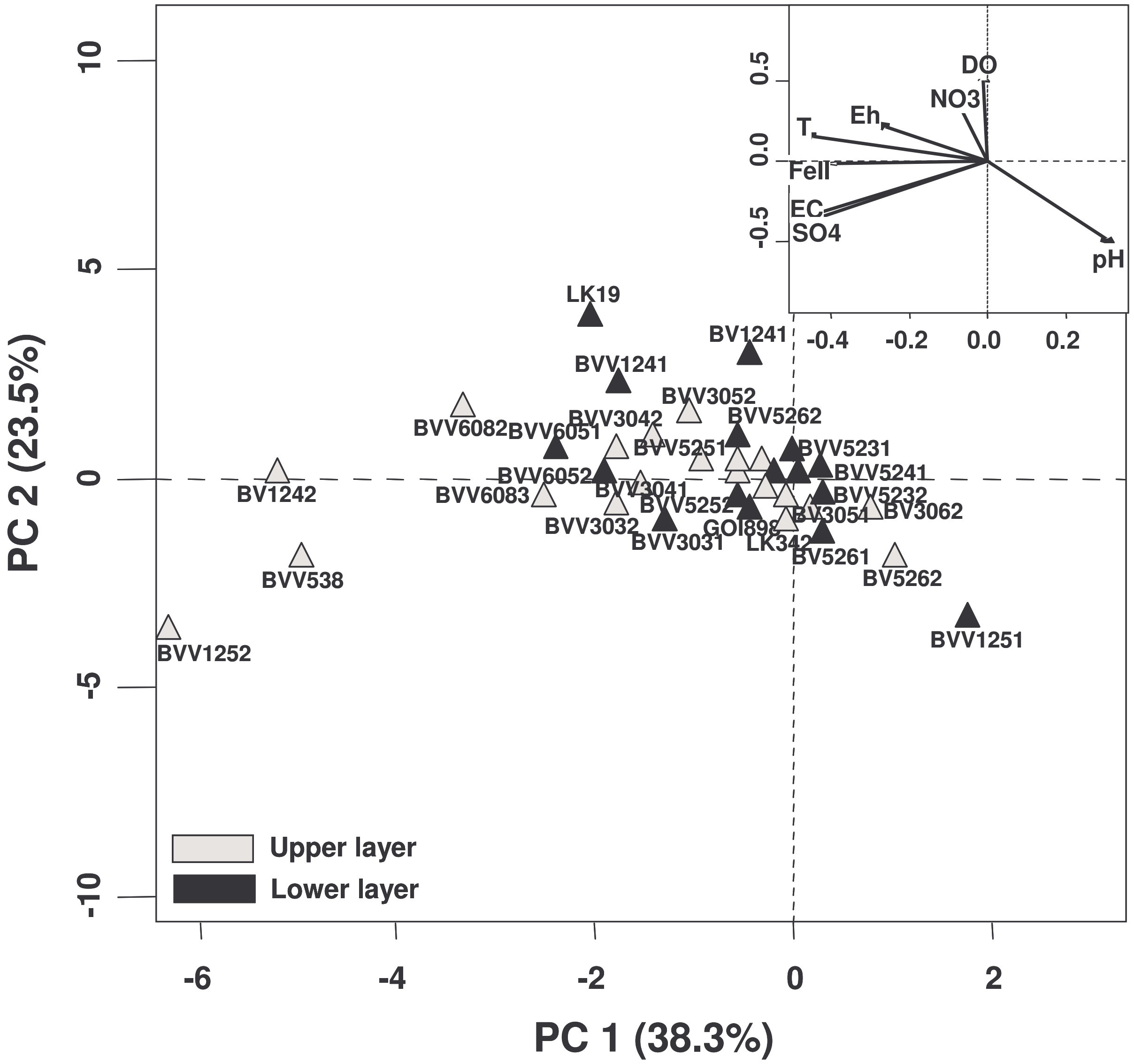

Fig. 3a: PCA ordination plot of the groundwater samples according to the geochemistry of the Tertiary aquifer. Description vectors correspond to: NO3, nitrate; DO, dissolved oxygen; T, temperature; Eh, redox potential; SO4, sulphate; EC, electric conductivity; FeII, ferrous iron. Objects correspond to the wells. Values on the axes indicate % of total variation explanation by the corresponding axis (PC 1, principal component axis 1; PC 2, principal component axis 2).

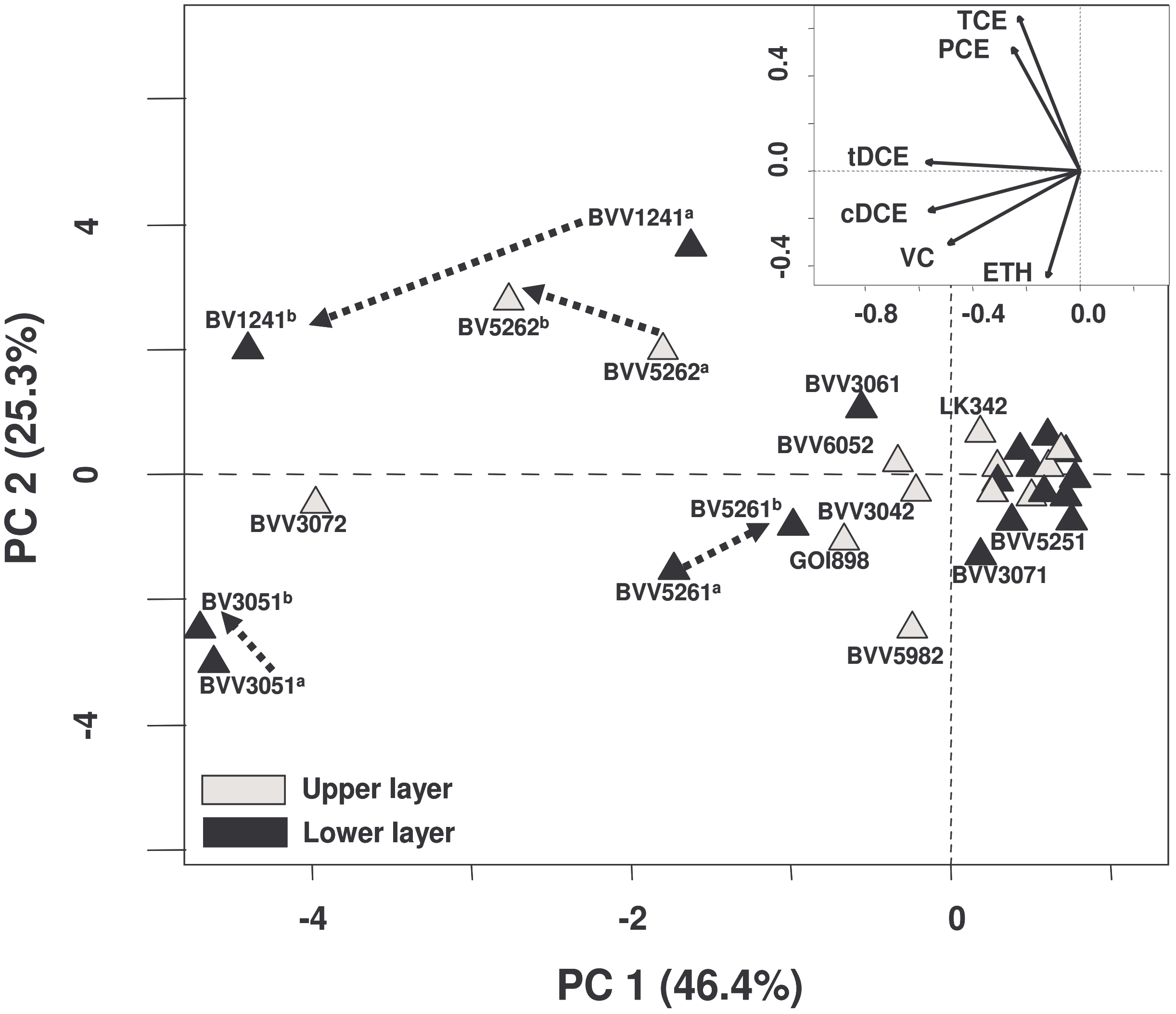

Fig. 3b: PCA ordination plot of the groundwater samples according to the amount of the chlorinated ethenes and ethene in the Tertiary aquifer. Description vectors correspond to: VC, vinyl chloride; tDCE, *trans*-DCE; cDCE, *cis*-DCE; PCE, tetrachloroethene; TCE, trichloroethene; ETH, ethene. Objects correspond to the wells. Values on the axes indicate % of total variation explanation by the corresponding axis (PC 1, principal component axis 1; PC 2, principal component axis 2). Arrows indicate the shift of the same object over time.

[a] Samples collected during the first sampling campaign (04/2005)

[b] Samples collected during the third sampling campaign (05/2006)

3.4. Investigation of the groundwater bacterial community using DGGE

DGGE provided an integrative overview on the composition of bacterial communities in the contaminated groundwater system (Fig. S3, Supplementary Material). Groundwater samples contained numerous bacterial types corresponding to the number of bands detected by DGGE for each sample (range 10–17). Quaternary samples tended to have fewer DGGE bands (10–13) than observed for the Tertiary samples (12–17) (Tables S4 and S5, Supplementary Material).

The Mantel correlation test revealed that no correlation could be observed between the assessed geochemical variables and the DGGE profiles (r Mantel, 0.067; P = 0.30). However, the chlorinated ethene concentrations were selected from among the environmental variables according to their high probability of correlation with the DGGE profiles (rMantel, 0.214; P = 0.06). Pairwise comparisons of bacterial species composition based on the Sørensen similarity index (S.I.) resulted in values between 0.17 and 0.67, but a large majority (77%) of the comparisons display a S.I.<0.4, indicating a relatively high b-diversity (data not shown). CCA analysis used for correlating the DGGE profiles of the groundwater samples with the contaminant composition revealed distinctive patterns (Fig. 4). Symbols in the plot lying close together are likely to display similar bacterial community patterns. The symbols are mostly grouped according to contaminant concentration, which indicates that the chlorinated ethene concentrations influence the bacterial community structure. It is likely that the ethene, trans-DCE and TCE concentrations had the strongest effect on the bacterial community structure (r^2>0.5, P<0.03). The chlorinated ethene concentrations explained 56.3% (P<0.005) of the variance in the DGGE profiles, whereas 43.7% could not be explained by the selected variables. The bacterial community from the Tertiary aquifer wells BVV5261 and BVV3051, where high ethene production and high DCE concentrations were observed, displayed a similar structure, but substantially differed from the other samples. The bacterial communities from the upper and lower Quaternary aquifers, evolving in an analogous contaminant and geochemical environment (Fig. 2), seem to be highly similar, with the exception of the community from the wells RT07 and RT071. The community profile of this last sample was found to be analogous to the community from the well BVV1241. In both wells, the concentrations of TCEs were substantially higher than in the other wells.

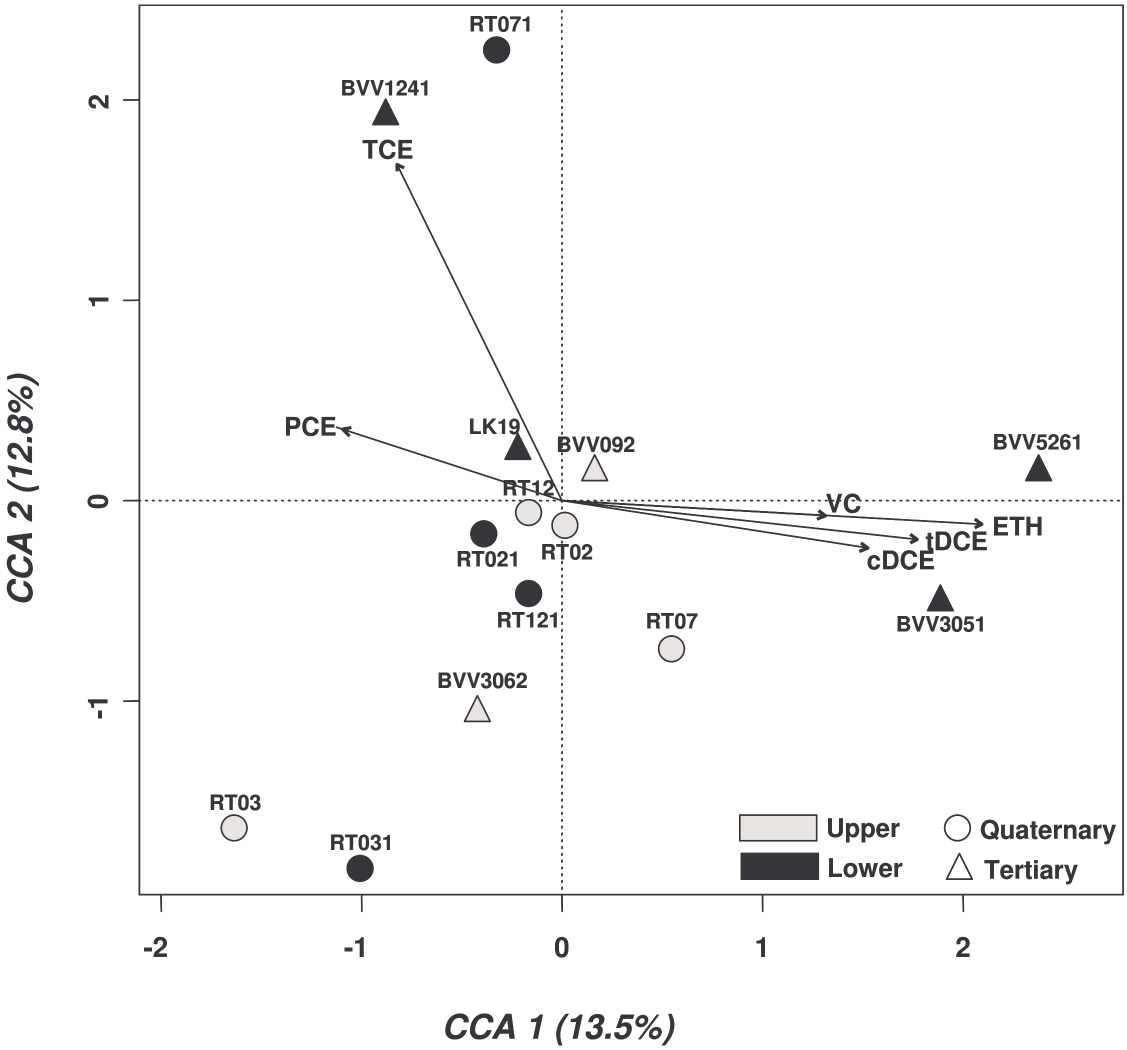

Fig. 4: Canonical correspondence analysis (CCA) of bacterial community patterns generated by 16S rRNA DGGE analysis for different chlorinated ethenes contaminated groundwater samples. Chlorinated ethenes concentrations are used as quantitative explanatory variables. The value (%) on the axes indicates the percentage of total variation which they explain. The variance decomposition analyses of the CCA on the bacterial community patterns showed that the chlorinated ethenes concentration explained 56.3% of the variance. Sum of all canonical eigenvalues, 0.922; total inertia, 1.636; Monte Carlo overall permutation test, *P*<0.005.

4. Discussion

4.1. Geochemistry and contaminant concentration analysis

We investigated multiple and converging lines of evidence to characterize natural attenuation of chlorinated ethenes in various geological units of a contaminated groundwater system. The temporal trend in contaminant concentration data was briefly illustrated by observing the evolution of the contaminant patterns over time in five key wells of the Tertiary aquifer (BVV1241, BVV3051, BVV5261, BVV5262 and BVV3062). In particular, a temporal change in the concentrations was observed in these wells evaluated at day 0 and day 395. Indeed, the flooding event of the Mulde River in August 2002 has affected the water levels, hydraulic gradients and overall contaminant shifts, explaining the observed temporal evolution of contaminant concentrations in this area (Wycisk et al., 2005). Overall, three main differences between the previous and the actual flow situations can be distinguished: (i) contaminants have spread to the east during the last few decades, but since August 2002 they have tended to the north-northeast direction; (ii) contaminants in the Tertiary aquifer are migrating more slowly than in the Quaternary aquifer; and (iii) the post-flooding development of the modelled path lines suggests a geochemical situation where organic compounds are partially desorbing from the aquifer matrix.

Therefore, the general effect of flooding is characterized by an increase in the concentrations of individual organic substances, particularly in the areas of higher hydraulic conductivity. The use of ordination methods for the analysis of the spatial and temporal development of the geochemical conditions in each aquifer revealed the predominant trends in the data sets. In particular, it showed a close relationship between the geochemistry of the upper and lower layers of each aquifer, and revealed uncommon geochemical and concentration patterns. For instance, the exploration of the concentration patterns in the Tertiary aquifer revealed that the majority of them were similar. The existence of some uncommon patterns reflected, however, the subsurface heterogeneity of contaminant transformation. These wells displaying uncommon contaminant concentrations and geochemistry footprints may be key components of the monitoring network to evaluate natural attenuation spatially and temporally. Moreover, the PCA allowed the identification of several processes putatively controlling the redox conditions in groundwater. For instance, the analysis revealed that biodegradation processes in the Tertiary aquifer were putatively associated with depressed levels of dissolved oxygen, nitrate and, to some extent, sulphate, and with higher concentration of ferrous iron (Fig. 3a). However, the PCA of the Quaternary aquifer emphasized a more confused redox status as several processes a priori co-exist, as described by the measured variables (Fig. 2). Estimating the redox status often remains arduous in shallow groundwater. This is generally due to both the difficulty to describe the predominant TEAPs considering exclusively electron acceptors and final products, as well as the possible occurrence of concurrent abiotic transformations (e.g. precipitation of S^{2-} as FeS and pyrite) (Chapelle et al., 1995). Therefore, the measurements of intermediate products (e.g. H_2) in groundwater could be particularly relevant to further support the existence of predominant TEAPs associated with biodegradation activities in dynamic, shallow aquifer systems (Ryu et al., 2004). Moreover, a relationship between variables does not systematically imply a causal biogeochemical relationship and existing correlations between descriptors in a PCA require cautious interpretation (Legendre and Legendre, 1998). Nevertheless, the PCA clearly separated the samples according to their geographical origin. The samples of the northeast zone of the investigated area were mainly characterized by high concentrations of PCE, TCE and the systematic detection of ethene, coinciding with relatively lower concentrations of the DCE and VC intermediates, relatively higher nitrite concentrations and lower redox potential values. The concentrations of nitrate and nitrite, as well as the redox potential value strongly suggest the occurrence of an anaerobic environment in this zone, allowing reductive dechlorination at a preliminary stage, as substantiated by the isotope composition analysis.

4.2. Isotope composition

The isotope analysis provided an indication of the ongoing in situ biodegradation and characterized the dehalogenation process spatially and over time in both the Quaternary and Tertiary aquifers. The interpretation of the chlorinated ethene transformation based on isotope analysis allowed distinguishing two characteristic stages in the degradation progress. The newly formed chlorinated ethene was relatively light compared with the educt, and the product became heavier in the course of the degradation, in accordance with previous observations (Nijenhuis et al., 2007). Particularly in the Quaternary aquifer, it is likely that a degradation has taken place since industrially produced PCE (δ[PCE] = -37.2‰ to -23.2‰) and TCE (δ[TCE] = -31.9‰ to -27.8‰) were generally more depleted (van Warmerdam et al., 1995). Moreover, the overall dominance of the cis-DCE isomer over the trans- in the Quaternary aquifer and in some wells of the Tertiary aquifer apparently indicates biological production, as industrial grade DCE usually consists of 60–70% trans-DCE (Ullmanns, 1975). In the Tertiary aquifer, the parallel observation of the ethene concentrations and its isotope composition over time showed that only small amounts of contaminant may have been converted and that dechlorination was in the preliminary phase, in concordance with the recent evolution of the concentration patterns.

4.3. Dehalogenating guild

Although many bacterial groups are involved in reductive dechlorination of chlorinated ethenes, *Dehalococcoides* is the only group known to completely dechlorinate PCE/TCE to ethene and is highly evolved to utilize dehalogenated organic compounds and hydrogen (Major et al., 2003; Seshadri et al.,2005). Hence, the detection of *Dehalococcoides* species may play a critical role in the evaluation of natural attenuation in anaerobic groundwater. The other groups of microorganisms capable of partial dechlorination of highly chlorinated ethenes to cis-DCE may also contribute to dechlorination in the contaminated system. Interestingly, the detection of *Dehalococcoides* sp. DNA in well BVV1241, displaying a pH value o5, coincided with high ethene production. Conversely, no detection was yielded at well LK19 (pH=3.6). These results are in accordance with Bowman et al. (2006), who detected *Dehalococcoides* sp. DNA in a moderately acidic (pH=5.1) anaerobic groundwater contaminated by chlorosolvent-containing DNAPL. However, most of the pH values recorded in the investigated wells were in the typical range from 6.3 to 7.2. Unexpectedly, *Dehalococcoides* sp. DNA was detected in oxygenated groundwater (C_{O2}>2mg L^{-1}), where concentrations of chlorinated ethene were mostly below the detection limits (wells BVV5881 and BVV589). At those wells, degradation activity may occur, but ethene concentrations remained below the detection limit. The detection of *Dehalococcoides* sp. DNA is most likely due to the presence of inactive bacteria or target DNA fragments transported with the groundwater flow. Conversely, in several wells, the correspondence between the presence of putative dehalorespiring organisms and ethene production was not systematic. In contaminated aquifers, both competition with H_2-consuming bacteria for the shared electron donor and the geochemical environment may influence the growth and colonization pattern of dehalorespiring bacteria. Nitrate, which was present in relatively high concentrations in the wells RT121, RT07 and RT03 of the Quaternary aquifer, may act as a competing terminal electron acceptor depleting H_2 or organic carbon concentrations, which may also hinder growth of *Dehalococcoides* bacteria (Fennell and Gossett, 1998; Yang and McCarty, 1998). Interestingly, the absence of dehalorespirers was associated with concentrations of Fe(II) 420 mg L^{-1} and sulphate 4800 mg L^{-1} in the Tertiary wells BVV1242, BVV1252, BVV538 and BVV6051.

The geochemical analysis of the Tertiary aquifer by PCA (Fig. 3a) revealed that these samples displayed uncommon geochemical patterns in terms of ferrous iron and sulphate as well as electric conductivity and temperature values. The combination of high concentrations of dissolved iron and sulphate may inhibit growth of *Dehalococcoides* and other dehalorespiring organisms, when iron and sulphate reduction compete as electron accepting processes. However, DNA of *Dehalococcoides* sp. was found to be associated with a maximal sulphate concentration of 1480 mg L^{-1} (well BVV6052), suggesting that sulphate was not used as a major electron acceptor at this well. Overall, these findings suggest (i) the present target DNA being under the detection limit, (ii) the involvement of other bacteria in the reductive dechlorination process and/or (iii) the presence of inhibitory compounds in the groundwater interfering with the PCR reactions. For instance, no organisms were detected in well BVV3071, which reaches the extensive natural lignite strata spread in the Bitterfeld aquifers. Leaching of humic compounds into the groundwater likely occurs and appears to have deleterious effects on PCR reactions (Wilson, 1997).

Biological reductive dechlorination requires a source of electrons, e.g. dissolved hydrogen or organic substrates. The putative release of DOC, mainly consisting in humic substances, from the natural lignite layers may ensure a sustainable source of electron donors for the bacterial community, which may in turn supply the reductively dechlorinating guild with specific electron donors. Interestingly, *Dehalobacter* and *Dehalococcoides* spp., known to be generally restricted to the use of H_2 as an electron donor (Holliger et al., 2004), were detected in several samples. Thus, processes governing the H_2 flow and distribution as well as high sulphate and ferrous iron concentrations may be critical and indicative of the extent and sustainability of natural attenuation in the investigated area.

4.4. Bacterial community structure

To further document the proposed approach, the spatial changes in the bacterial community composition during in situ biodegradation of chlorinated ethenes were investigated in the studied groundwater system. First, DGGE was used for making rapid qualitative comparison of community diversity over space. The levels of richness observed in this study were in the range of what has been observed for aquifers contaminated with other compounds (Feris et al., 1997, 2004; Wenderoth et al., 2003). Interestingly, in the wells showing relatively high specific richness levels, high ethene concentrations and depleted ethene carbon isotope composition supporting the occurrence of in situ biodegradation were simultaneously observed. This stays consistent with the feature of highly productive microbial communities, in which large and efficient energy fluxes would favour the specific richness (e.g. Zhou et al., 2002). Furthermore, the analysis of the relation between environmental factors and the bacterial community structure by DGGE patterns revealed that the groundwater community structure was substantially influenced by the concentrations of contaminants. Indeed, the chlorinated ethene concentration values explained a considerable part (56.3%) of the overall variance in the DGGE profiles. These community shifts together with contaminant exposure may result from acclimatization or an increased adaptation of a specialized mixed, relatively rich bacterial community to high contaminant concentrations. According to previous reports, the ability of strains or mixed cultures to dechlorinate PCE was not hindered at concentrations about three times higher than the highest contaminant concentration observed in the wells in Bitterfeld (Amos et al., 2007; Sleep et al., 2006). In particular, profiles of wells with high PCE and TCE (wells BVV1241 and RT071) or DCEs, VC and ethene (wells BVV3051 and BVV5261) exposure revealed a noticeable difference in the community composition. Interestingly, in these wells, isotope signature, concentrations analysis and taxon-specific detection strongly support the existence of dechlorinating activities. Though contaminant concentration seems to play a key role, it does not represent the sole functional relevant difference between the wells. Thus, though the explanation percentage of the variance by the tested factors was statistically significant, 43.7% of the variance in the bacterial community structure data remains unexplained. This means that a considerable part of the bacterial populations in the contaminated groundwater system was most probably affected by factors other than the chlorinated ethene concentrations. The analyses were carried out in field conditions, resulting in a greater heterogeneity between samples even if they originated from the same geological unit. Because aquifers are highly heterogeneous and discontinuous systems, other factors such as intra- and inter-species competition, presence or absence of specific microhabitat, nutrient flux (in particular, of potential electron donors) and transport properties at different scales have influenced the bacterial community structure (e.g. Zhao et al., 2005; Kao et al., 2001).

Further study in more controlled field conditions and the determination of key-aquifer material properties at sampling sites can help in the identification of some of these factors. Moreover, though contaminant concentrations were the most critical environmental characteristics showing strong coincidence with specific populations, other variables may also be important. However, these variables could not be detected given the characteristics of the site, the limits of the sampling methods and the measurement procedure applied in this study. It is likely that the inherent complexity and scale of aquifer systems will very often limit the ability to assess the relationship between groundwater bacterial community structure and environmental characteristics.

5. Conclusion

The following conclusions can be drawn from the present study:

1. The integrated approach provided evidence for natural attenuation of PCE and TCE to ethene, which is particularly critical in the highly hydraulic conductive layers of the Quaternary Bitterfeld aquifer. The presence of cis-DCE over large areas of the aquifer, the isotope data and the results of taxon-specific molecular analyses strongly support the occurrence of reductive dechlorination.

2. Molecular investigations showed a complex bacterial community potentially associated with in situ biodegradation of chlorinated ethenes. The geochemical habitat of the potentially dehalorespiring bacteria appears varied, but some key geochemical variables might influence their presence and distribution. They may indirectly govern the extent of in situ biodegradation along a vertical gradient in the geologically complex Bitterfeld system.

3. The relationship between environmental variables, in particular geochemistry and aquifer material characteristics, and the in situ activity of dehalorespiring microorganisms need to be further investigated. In particular, more detailed spatial and temporal geochemical analysis would be required to understand more accurately the relationship between the dynamics of the contaminant concentrations and the groundwater chemistry in the shallow Quaternary aquifer.

4. The canonical correspondence analysis indicated that the chlorinated ethene concentration patterns affect the bacterial community structure in the investigated system. However, the reciprocal relation is also conceivable in systems experiencing intense natural attenuation. Combining microbial fingerprinting techniques with a statistical approach may lead to the definition of new hypotheses and to new prospects in terms of spatial and temporal functioning of contaminated groundwater microbial systems.

5. Data reduction and spatial–temporal integration is a major cost component in MNA and represents a crucial step for both process and performance (long-term) monitoring to make data readily accessible to regulators and stakeholders (WSRC-STI-2006-00084, 2006). The approach followed in this study illustrates the possible benefit of ordination methods to analyse spatial and/or temporal variations of geochemical, contaminant and bacterial characteristics in the framework of natural attenuation process assessment. Future uses may include identification and analysis of the relationship between key-indicator parameters to distinguish changes in baseline conditions, as well as supporting detailed analysis of large data sets from the outcome of traditional and new monitoring methods to assess natural attenuation.

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Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.watres.2007.08.035.

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Section 3: Variability of bacterial community and in situ biodegradation of chlorinated ethenes along a vertical profile in a heterogeneous groundwater system

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Abstract
Variability of the bacterial community structure, hydrogeochemical indicators and the carbon stable isotope composition of chlorinated ethenes was assessed along a hydrogeologically heterogeneous, 50 m depth vertical profile. Substantial shifts in the hydrogeochemistry, bacterial community structures and the distribution of putative dehalorespirers (*Dehalobacter* spp., *Desulfitobacterium* spp., *Dehalococcoides* spp. or *Geobacter* spp.) were observed with depth according to the contaminant concentration. Sequences could be affiliated with at least 10 classes of the domain *Bacteria* and β- and γ-*Proteobacteria* accounted for > 18 % of 16S rRNA genes clone libraries. Biodegradation of chlorinated ethenes was assessed by compound-specific stable isotope analysis (CSIA). An isotope balance was applied in order to enhance the evaluation of vinyl chloride degradation while taking into account the complete reductive dechlorination sequence. Hydrogeochemical and microbiological indicators as well as the isotope balance, revealing isotopic shift > 8 ‰ between the sources and the plume fringe area, demonstrated the occurrence of reductive dechlorination of chlorinated ethenes at the investigated depths. Isotope balance values varied from -14.4 to -4.4 ‰ over the vertical profile, which suggested varying extent of chlorinated ethenes biodegradation activity. Overall, the study revealed a complex linkage between bacterial community structures, hydrogeochemical variables and hydrogeological conditions.

Running Head: Biogeochemical variability in contaminated aquifers

1. Introduction

Aquifers are dynamic ecosystems showing complex interactions between physical, chemical and biotic components (Haack *et al.*, 2004; Lehman, 2007). In these environments, hydraulic characteristics as well as contaminant bulk may display a high spatial variability and reflect the heterogeneity of the lithological composition as well as the grain size distribution (Boyd *et al.*, 2007). Therefore, aquifers can be considered as heterogeneous assemblages of discrete macro- and micro-scale habitats, providing various living conditions, which influence the distribution of the microbial communities and their functional activities (Goldscheider *et al.*, 2006). As a result of the biogeochemical heterogeneity, ground water flow segments may pass zones of different reaction conditions with respect to biodegradation processes, which complicates the evaluation of the fate of hazardous organic chemicals.
Interpretation of contaminant degradation processes controlling non-uniform contaminant plumes in heterogeneous aquifers is likely to be confounded with data collected in long-screened wells straddling zones of varying reactivity. Alternatively, fine-resolution groundwater analysis over a vertical scale may permit to characterize in detail microbial and hydrogeochemical processes at presumably critical zones of a contaminant plume (Wilson *et al.*, 2004). For instance, biodegradation processes occurring at contaminated-pristine water interfaces characterizing the plume fringe may be critical for limiting the transport of toxic reductive dechlorination intermediates, such as dichloroethenes (DCE) and carcinogenic vinyl chloride (VC) (Bouwer, 1994; McCarty & Semperini, 1994). Under highly reducing conditions generally achieved at central zones of a plume, these lower chlorinated ethenes are believed to mostly originate from sequential biotic dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) and tend to accumulate (McCarty & Semperini, 1994; Vogel, 1994; MaymoGatell *et al.*, 1997). At the fringe area, degradation processes are assumed to differ from those occurring at the core because dispersive mixing processes between hydrogeochemically different types of water generally occur (van Breukelen & Griffioen, 2004). However, knowledge about such interfaces with respect to biogeochemical processes associated with chlorinated ethenes biodegradation is so far limited.

Evaluating biodegradation processes in groundwater systems generally requires a comprehensive approach relying on both hydrogeochemical measurements of contaminant transformation and information on associated microorganisms (Weiss & Cozzarelli, 2008). Hydrogeochemical methods may allow tracking on a spatial and/or temporal scale the changes of metabolites production and redox conditions at complex field sites. This information can be advantageously coupled with an evaluation of isotope fractionation of reactants or products occurring during biotransformation by the means of compound-specific isotope analysis (CSIA) (Meckenstock *et al.*, 2004). CSIA relies on the occurrence of isotope fractionation processes consisting of the discrimination of the light (e.g. ^{12}C, ^{1}H) and the heavy (e.g. ^{13}C, ^{2}D) isotopes during biodegradation reactions. This concept has been proven useful to analyze *in situ* biodegradation of chlorinated ethenes in groundwater systems (Hunkeler *et al.*, 1999; Sherwood Lollar *et al.*, 2001; Nijenhuis *et al.*, 2007; Imfeld *et al.*, 2008). However, interpretation of the CSIA may be hampered when degradation reactions are sequential, hereby involving several intermediates. For instance, during reductive dechlorination of higher chlorinated ethenes, the fate of degradation intermediates, such as TCE, DCE and VC is simultaneously controlled by their production (associated with depletion in ^{13}C) and further degradation (leading to enrichment in ^{13}C). This would lead to an opposite fractionation effect affecting the isotope signature of individual intermediates (Hunkeler *et al.*, 1999; Morrill *et al.*, 2006). In such case, a comparative interpretation of the relationship between contaminant depletion and target-degradation process based on the concentration and isotope data would require taking into account the complete reaction chain in a balance calculation.

More insight into bioremediation processes can be offered by nucleic acid based investigations. Potential degrading microorganisms can be targeted at various taxonomic levels. For instance, members of the genera *Dehalobacter, Desulfitobacterium,* *Dehalococcoides* or *Geobacter* have been reported to use chloroethenes as terminal electron acceptors and can then be used as indicators of potential dechlorinating activity (Loffler *et al.*, 2000; Hendrickson *et al.*, 2002; Duhamel & Edwards, 2006; Imfeld *et al.*, 2008). Furthermore, the microbial community structure and composition associated with *in situ* bioremediation processes can be characterized using microbial community analysis techniques, such as molecular fingerprinting or cloning-sequencing (Eyers *et al.*, 2004; Feris *et al.*, 2004). Subsurface microbial community diversity, structure and degradation activity are likely to change as a function of hydrogeological, geochemical or contaminant mass transfer variations. Therefore, more consistent insight into contaminant attenuation potential can be gained by correlating the obtained community structure with the hydrogeochemical variability across ecological scales of interest. In this respect, multivariate analyses help addressing the relationship between microbial structures and environmental variables (Ramette, 2007).

In this study, we assessed the variability of biogeochemical processes associated with chlorinated ethenes transformation along a vertical profile across a heterogeneous groundwater system. The primary objective was to evaluate the bacterial community structure and diversity associated with ongoing degradation activity at discrete-depth intervals. For this purpose, the variability of the bacterial community structures, hydrogeochemical indicators and the carbon stable isotope composition of chlorinated ethenes was assessed over time in a multilevel monitoring well located at the fringe of a chlorinated ethenes' plume. To evaluate the *in situ* biodegradation of chlorinated ethenes, an isotope balance based on contaminant concentrations and isotopic composition patterns was calculated. The variability of the bacterial community structures was assessed by molecular fingerprinting techniques and detailed analysis of two clone libraries. Putative members of the dehalorespiring guild were detected at discrete-depths using classical 16S rRNA gene biomarkers. Multivariate analyses were applied to evaluate the relationship between selected hydrogeochemical variables, including the contaminants isotope compositions, and the bacterial community structures, hence providing information about the functional linkage between microbial community structures, geochemical and hydrogeological conditions, and contaminant degradation.

2. Materials and methods

2.1. Site and vertical profile description

The investigated well is located in a research area of about 4 km^2 of the industrial Bitterfeld/Wolfen contaminated mega-site, about 130 km south of Berlin, Germany. *Cis*-1,2-dichloroethene (*cis*-DCE) represents a dominant contaminant, which accumulates as a result of dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE). Previous studies have suggested the occurrence of *in situ* dehalogenation activities, which implies that Monitored Natural Attenuation may be considered as a remediation option (Nijenhuis *et al.*, 2007; Imfeld *et al.*, 2008). As a consequence of the flooding event of the open pit mine by the Mulde River in August 2002, the hydraulic gradient is progressively turning towards the northeast (Wycisk *et al.*, 2003). The multilevel sampler permitting the acquisition of depth-discrete groundwater samples is located at the north-east fringe of the DCE plume (Fig. S1) and intercepts the re-directed contaminant flow along 50 m depth across the groundwater system. The multilevel samplers consist of 100 mm diameter high-density polyethylene (HDPE) screened tubes. The 10 adjacent sampling points were separated from 2 to 4 m and were numbered starting with 1 from the top towards the bottom for identification. The detailed position of the filter screens and the physical characteristics of the corresponding sediment layers are provided in Table S1. The overall geological setting and the hydrogeological situation of the investigation area has been described previously (Wycisk *et al.*, 2003; Nijenhuis *et al.*, 2007). Briefly, the sampling points reach two porous aquifers displaying different hydrogeological characteristics. Sampling points 1 to 4 (2 to 21 meters below the surface) are located in the upper aquifer consisting of Quaternary sands and gravels (K_f = 0.5 to 4 x 10^{-3} m s^{-1}), partially underlayed by Upper Oligocene lignite seam. Sampling points 5 to 10 (25.5 to 49.5 meter below the surface) are located in the Tertiary aquifer consisting of

Upper Oligocene micaneaous sands of different hydraulic conductivity ($K_f = 0.5\text{-}2 \times 10^{-5}$ m s^{-1}) in its upper and lower parts.

2.2. Groundwater collection

Two groundwater samplings were conducted, the first in 05/2006 and the second in 07/2007, at the multilevel sampler. In 07/2007, samples were also taken from selected surrounding wells (Fig. S1) by the IFUA Umweltberatung und Gutachten GmbH (Germany). Groundwater samples were collected using a submersible peristaltic pump (Grundfos, Bjerringbro, Denmark). Dissolved oxygen, pH, conductivity, redox potential and temperature were directly measured in the field. To ensure representative sampling, the sampling points were purged to replace at least one tube volume and until field parameters were constant. During each sampling campaign, aqueous samples retrieved from each depth were systematically dispensed into 100 ml volatile organic compounds (VOCs) vials for concentration analysis (headspace free), 20 ml high-density polyethylene bottles for geochemical analysis, and 2 x 1 L cleaned and autoclaved glass bottles for molecular investigations. For isotope composition analysis of chlorinated ethenes, additional samples were collected and dispensed in separate 250 ml glass vials (~30 ml headspace) containing NaCl at saturation point to inhibit further microbial activity. In parallel, 100 ml full glass vials conserved with 1 ml of 12N HCl (pH of the final solution < 1) were taken for isotope composition analysis of chlorinated ethenes by the purge-and-trap procedure (see next section). The water samples were sealed with Teflon-coated septa, stored on ice and directly transported to the laboratories for chemical analysis.

2.3. Hydrogeochemical and isotopic analysis

Details of the hydrogeochemical and isotopic analysis are provided in supplementary information (SI), section 1.2. VOCs were quantified according to the EN ISO 10301. Geochemical parameters (Fe^{2+}, Fe, Cl^-, SO_4^{2-}, NO_3^-, NO_2^-, PO_4^{3-}, F^-, NH^{4+}, Mn^{2+} and H_2S) were determined by using DIN and DIN EN ISO standards and laboratory procedures. Stable carbon isotope compositions of the chlorinated ethenes were measured using a gas chromatography-combustion-isotope ratio mass spectrometry system (GC-C-IRMS) as described earlier (Nijenhuis *et al.*, 2007). Samples with cumulated chlorinated ethenes concentrations below 1 µM typically found in the Quaternary aquifer were analysed using a purge-and-trap procedure prior injection in the GC-C-IRMS (adapted from EPA method 524.2; EPA, 1995).

2.4. Definitions and isotope balance

The carbon isotope ratio for an individual compound is reported in δ-notation [‰] relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna) (Coplen *et al.*, 2006). The analytical error was ≤ 1 δ unit and incorporated both the accuracy of the measurement and the reproducibility on triplicate measurements of the sample.

The isotope signature of the total chlorinated ethenes ($\delta^{13}C_{CE}$) was calculated by multiplying the molar concentration of each compound (C_i) with its respective carbon isotope signature ($\delta^{13}C_i$), adding all contributions and dividing by the total molar concentration of all chlorinated ethenes (C_{CE}) (Eq. 1).

$$\delta^{13}C_{CE}[‰] = \frac{\sum (C_i * \delta^{13}C_i)}{C_{CE}} \qquad (1)$$

The *isotope balance* refers to the weighted cumulative isotope signatures of the chlorinated ethenes ($\delta^{13}C_{CE}$, isotope balance) and is distinct from the directly measured isotope signature ($\delta^{13}C_i$) of the single species of chlorinated ethenes. The uncertainty associated with the isotope

balance was calculated based on the error propagation using the standard deviation (Eq. 2).

$$\Delta_{tot}\delta_{stdev} = \frac{\sqrt{\sum\left(c_i \Delta\delta_i\right)^2}}{\sum c_i} \qquad (2)$$

For all samples the standard deviation for the values of the isotope balance was ≤ 1 ‰.

2.5. Molecular analysis

DNA extraction. Preparation of the samples for DNA extraction from the water samples was carried out within 5 hours after water collection. Groundwater samples filtered through a sterile 0.2 µm membrane (MoBio Water DNA kit, Carlsbad, CA, USA) and stored at -20°C until extraction. DNA from cells was extracted using a FastDNA spin kit (BIO101, La Jolla, CA, USA) according to the manufacturer's instructions and eluted in nuclease-free water (50 µl).

16S rRNA gene-targeted PCR. PCR was used to partly amplify 16S rRNA genes from *Bacteria*. PCR mix per reaction contained 1X PCR + $MgCl_2$ buffer (Qiagen, Hilden, Germany), 0.2 mM (each) deoxynucleoside triphosphate (Qiagen), 0.5 µM (each) forward and reverse primer (Invitrogen), 1.5 U of HotStar*Taq* DNA polymerase (Qiagen), 1:10 dilution of DNA template and molecular grade water (Promega). Eubacterial primers 27f (Lane, 1991) and 1378r (Heuer *et al.*, 1997) were used to amplify almost the complete 16S rRNA gene using the following program: 95°C (15 min), followed by 30 cycles of 95°C (30 s), 51°C (30 s), and 72°C (50 s), completed with an additional 30 min at 72°C. The second round of PCR for DGGE analysis employed universal primers GC968f (Nubel *et al.*, 1996) and 1378r. The conditions for PCR amplifications were as follows: 95 °C (15 min) followed by 30 cycles of 95 °C (30 s), 60 °C (1 min), 60 °C to 55 °C (0.5 °C min^{-1}), 72 °C (1 min), and a final extension for 30 min at 72 °C. In parallel, to test the presence of the genera *Dehalobacter* (Schlotelburg *et al.*, 2002), *Desulfuromonas* (Loffler *et al.*, 2000) and *Geobacter* (Duhamel & Edwards, 2006), a second round of PCR with specific primers was performed using the PCR products from the universal 16S rRNA gene amplification as template, as previously described (Imfeld *et al.*, 2008). A Dehalococcoides-specific amplification protocol was used for detecting *Dehalococcoides*-affiliated bacteria as described previously (Hendrickson *et al.*, 2002).

Molecular fingerprinting analysis of microbial communities

Denaturing gradient gel electrophoresis (DGGE). DGGE was carried out using the DCode Universal Mutation Detection System (BioRad, Munich, Germany), as previously described (Imfeld *et al.*, 2008). The DGGE fingerprints were normalized according to the reference patterns and compared using the GelComparII software (Applied Maths, Kortrijk, Belgium). DGGE banding patterns were then converted to a binary numerical matrix based on presence or absence of bands and subsequently used for statistical analysis.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP). Terminal-restriction fragment length polymorphism was carried out on an ABI 3130xl DNA capillary sequencer equipped with 50 cm long capillaries and POP 7 electrophoresis matrix. The detailed protocol is provided in the SI, section 1.3.

Cloning-sequencing*.* Two groundwater samples representative for the Quaternary and Tertiary aquifers (depths 4 and 7, at 20 and 35.5 meters below surface, respectively) were selected for setting up 16S rRNA gene libraries. The detailed cloning-sequencing protocol is provided in the SI, section 1.3. Following chimera detection, the examination of phylogenetic relatedness and taxonomic assignments (confidence threshold of 80%) was performed using the naïve Bayesian rRNA Classifier and the Sequence Match tools of the RDP-II (release 9.61, http://rdp.cme.msu.edu/), respectively. Nucleotide sequence data reported are available in the EMBL database under the accession number from FM206116 to FM206292.

2.6. Data analysis

All statistical analyses were carried out using the R software (R Development Core Team, 2008; Version 2.6.2). The detailed analysis procedure is provided in SI, section 1.4.

Principal component analysis (PCA). PCA based on the *z*-scores was used to assess the gradients in the hydrogeochemical data of the sampling campaigns 2006 and 2007 and to gain insight into the relationships between the hydrogeochemical variables. Contaminants displaying concentration values above the detection limit (> 5 µg L^{-1}) at one depth at least were selected. The hydrogeochemical data set eventually consisted of 9 geochemical and 10 contaminant variables. Additionally, PCA based on the covariance matrix and using the Hellinger transformation was used to analyze the DGGE data obtained in 2006 and 2007. The relationship between the community profiles and the corresponding hydrogeochemical variables was interpreted by fitting *a posteriori* the environmental vectors onto the respective PCA as a result of their calculated correlations. The significance of the fitted vectors was assessed using a permutation test (1000 permutations).

Multiple factor analysis (MFA). Biogeochemical processes in 2007 were holistically analyzed by integrating the DGGE, T-RFLP and the complete hydrogeochemical data (i.e. including chlorinated ethenes isotope composition values) in a single analysis; the three blocks of variables were simultaneously analyzed and compared in a global MFA (Escofier & Pages, 1994; Becue-Bertaut & Pages, 2008). The goal of this analysis was first to obtain an integrative picture of the bacterial structure and hydrogeochemical variability along the profile and second to evaluate the level of agreement between the hydogeochemical, the DGGE, and the T-RFLP profiles in 2007. The importance of each data set was weighted by the inverse of their corresponding highest eigenvalue ($1/\lambda$) and the respective data blocks were concatenated in a global matrix. The matrix was then analyzed by PCA to reveal the common structure by projecting the respective data sets onto the final ordination plot.

3. Results

3.1. Hydrogeochemical variability

The contaminant profiles dramatically changed in the Tertiary aquifer between 2006 and 2007 (Tables S2 and S3). In particular, *trans*- and *cis*-1,2-DCE concentration values were 2 to 8 fold higher in 2007 and reached 87 and 407 µg L^{-1} from 12 and 59 µg L^{-1} in 2006, respectively, at depth 9. In the Tertiary aquifer, concentrations of VC and ethene were below 10 µg L^{-1} in 2006 and increased up to 74 and 57 µg L^{-1} in 2007, respectively. This suggested reductive dechlorination activity. However, VC and ethene concentrations remained under the detection limit in the Quaternary aquifer (< 5 µg L^{-1}). PCE concentration values also remained stable under the detection limit in both aquifers. The concentration values of some redox-sensitive constituents also varied over time. While dissolved oxygen concentrations remained below 0.8 mg L^{-1} and redox potential values suggested predominant and stable anoxic conditions in both aquifers, concentration values for ferrous iron, ammonia, and manganese generally increased over time in both aquifers with values up to 3, 2 and 1 order of magnitude higher in 2007 than in 2006, respectively. In parallel, the pH values slightly decreased (6.3 ± 0.1 and 5.7 ± 0.1 in 2006 and 2007, respectively). Nitrate and nitrite concentrations were systematically below the limit of detection, suggesting insignificance of nitrate as an electron acceptor or immediate nitrate reduction reaction. Ferrous iron represented about 2 and 90 % of total iron in 2006 and 2007, respectively. Along with the slightly positive redox potential values over the whole profile (+24 ± 17 mV), it suggested in our case the occurrence of iron-reducing processes in 2007. However, transport of ferrous iron from adjacent zones of the aquifer where other redox reactions prevailed may have occurred equally. While the background concentration of sulphate in the groundwater ranged from 800 to 1000 mg L^{-1}, only traces of sulphide were detected along the whole profile in 2006 and 2007.

PCA carried out on the hydrogeochemical data obtained at discrete-depth intervals illustrated the temporal and spatial variability occurring in the system during the investigation period (Fig. 1A). Profiles from 2006 scored negatively, whereas profiles from 2007 scored positively along the *x*-axis (PC1), expressing 55 % of the variance in the data set. On the variables plot, scores of PC1 correlated positively to redox-sensitive constituents and to contaminants (Fig. 1B). Hence, both the geochemistry and contaminant load contributed the hydrogeochemical variation between 2006 and 2007. Moreover, hydrogeochemical profiles could also be distinguished along the *y*-axis (PC2) on the basis of their hydrogeological origin in 2007 and to a lesser extent in 2006 (Fig. 1A). Profiles from the Quaternary and the Tertiary aquifers clearly formed separated clusters in 2007, which emphasized differences of the hydrogeochemical conditions between the two superimposed aquifers. Contaminant concentrations in the Tertiary samples were relatively higher, whereas ferrous iron, sulphate, ammonia and manganese concentration values were higher in the Quaternary. Overall, the analysis revealed a dominant temporal change of the hydrogeochemistry over variations at discrete-depth intervals along the vertical profile. However, the magnitude of the change strongly varied along the vertical profile and mirrored the hydrogeological setting.

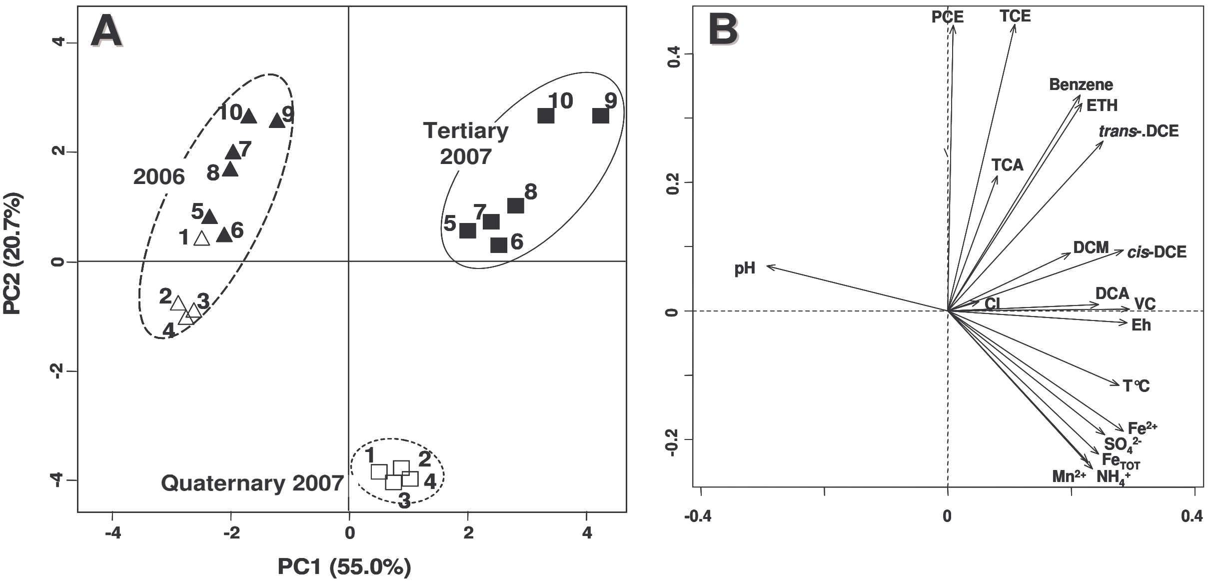

Figure 1: PCA ordination plot of hydrogeochemical characteristics of porewater samples (A) and of hydrogeochemical variables (B) collected at discrete-depth intervals from the multilevel well in 2006 and 2007. (A) Values on the axes indicate % of total variation explanation by the corresponding axis (PC 1, principal component axis 1; PC 2, principal component axis 2). The first and second principal component accounted for > 70% of the variance in the data set. Objects are labeled according to the sampling year and their originating aquifer (Δ and ▲: 2006, □ and ■: 2007, Δ and □: Quaternary aquifer and ▲and ■: Tertiary aquifer) and numbered starting with 1 from the top according to increasing depth. (B) Description vectors correspond to: Fe^{2+}, ferrous iron; ΣFe, Total iron; Cl^-, chloride; NH_4^+, ammonium; Eh, redox potential; SO_4^{2-}, sulphate; Mn^{2+}, manganese; SO_4^{2-}, sulphate; Eh, redox potential; T℃, temperature; ETH, ethene; *cis*-DCE, 1,2-*cis*-dichloroethene; *trans*-DCE, *trans*-1,2-dichloroethene; VC, vinyl chloride; DCM, dichloromethane; DCA, 1,2-dichloroethane; TCA, trichloroethane; TCE, trichloroethene; PCE, tetrachloroethene.

3.2. Variability of stable carbon isotopic composition

Due to the overall contaminant concentration increase in 2007, compound-specific isotope analysis of chlorinated ethenes was performed to gain insights into the significance of biodegradation and to evaluate the variability of degradation reactions along the vertical profile (Table S5). The isotopic signatures over the profile ranged from -33.6 to -32.6, -22.2 to -15.0, -18.3 to -2.1 and -17.2 to -8.0 ‰, for PCE, TCE, *cis*- and *trans*-DCE, respectively. PCE, TCE and *cis*-DCE were more enriched in ^{13}C at the multilevel well than at the identified apparent source area locations (Nijenhuis *et al.*, 2007; Imfeld *et al.*, 2008) and at the adjoining wells (encoded BVV, Fig. S1 and Table S5). This supports the occurrence of biodegradation in both aquifers. Furthermore, the DCE isomers were generally enriched in ^{13}C compared to the respective discrete-depth isotopic signatures of VC and ethene ($\delta^{13}C$ = -25.3 to -10.2 and -35.0 to -27.1 ‰, respectively). The formed ethene was lighter than VC and likely becomes heavier in the course of VC degradation, indicating varying degrees of microbial conversion. Similar trends were observed in culture experiments completely degrading PCE to ethene (Bloom *et al.*, 2000; Cichocka *et al.*, 2007).

Comparison of the isotopic signatures between depths is complicated because simultaneous production and dehalogenation of TCE, DCE and VC may generate opposite isotope fractionation patterns for these species. Therefore, an isotope balance was calculated to overcome the hindrance associated with the interpretation of isotope data using the Rayleigh concept obtained at discrete-depth intervals along the profile and to evaluate the occurrence of VC degradation. Figure 2 shows the change of cumulated concentration values and balanced isotopic signatures of chlorinated ethenes ($\delta^{13}C_{CE}$) over depth. In the Quaternary aquifer, $\delta^{13}C_{CE}$ values ranged from -11.6 ± 0.8 to -10.8 ± 0.7 ‰ and did not vary significantly. In contrast, the range of variation was higher in the Tertiary aquifer and values ranged from -14.4 ± 0.6 to -4.4 ± 0.6 ‰. The highest $\delta^{13}C_{CE}$ values of the vertical profile were observed at depths 7 and 8, suggesting relatively larger VC degradation at those depths. The lowest value was observed at depth 9, which coincided with the highest cumulated contaminant concentrations. This suggested varying extent of VC degradation between aquifers but also between depths in the Tertiary aquifer. To attest the occurrence of VC biodegradation in this area of the plume fringe, spatial distribution of concentration and $\delta^{13}C_{CE}$ values within the plume area were also considered (Fig. S2). The concentration values at the apparent plume sources in both the Tertiary and the Quaternary aquifers were 1 to 3 orders of magnitude larger than the values retrieved at the multilevel well. Significantly lower $\delta^{13}C_{CE}$ values were found at the apparent plume source areas in both aquifers (Tertiary: $\delta^{13}C_{CE}$[BVV1241] = -30.4 ± 0.2, $\delta^{13}C_{CE}$[BVV3051] = -23.9 ± 0.3, Quaternary: $\delta^{13}C_{CE}$[BVV3063] = -35.2 ± 0.3 ‰) and in other parts of the plume (Fig. S2). Thus, the decrease of chlorinated ethenes concentration from the source to the investigated area of the fringe zone was associated with a significant enrichment in ^{13}C, resulting in balanced isotopic shift ranging from + 8 to + 18 ‰. This indicates that vinyl chloride, initially produced *via* reductive dechlorination of higher chlorinated ethenes, further was subjected to *in situ* biodegradation in both aquifers.

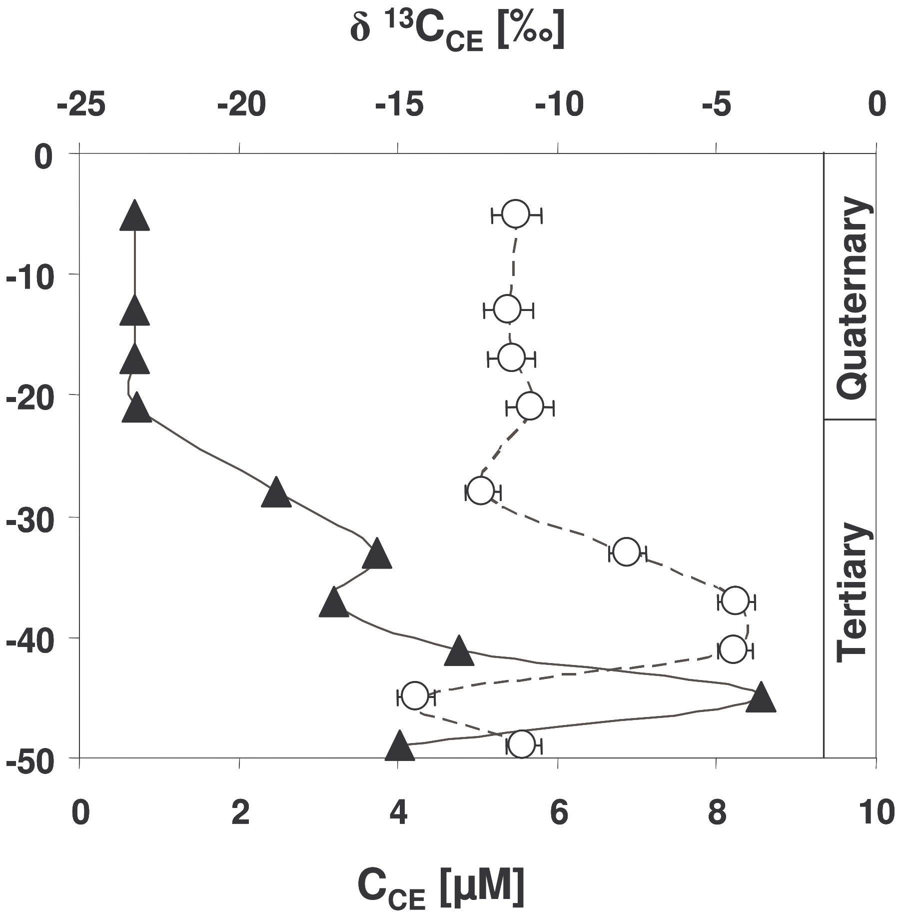

Figure 2: Depth profiles of concentration (▲) and isotopic balance (○) of the total chlorinated ethenes (C_{CE}) along the vertical profile in 2007. Lower $\delta^{13}C_{CE}$ values were found at the apparent plume source areas in both aquifers (Tertiary: $\delta^{13}C_{CE}$[BVV1241] = -30.4 ± 0.2, $\delta^{13}C_{CE}$[BVV3051] = -23.9 ± 0.3, Quaternary: $\delta^{13}C_{CE}$[BVV3063] = -35.2 ± 0.3 ‰).

3.3. DGGE analysis of groundwater bacterial community structure in 2006 and 2007

DGGE patterns of PCR-amplified partial 16S rRNA genes obtained from groundwater samples collected along the vertical profile in 2006 and 2007 provided complex and heterogeneous microbial fingerprints (Fig. S3). The variability of the bacterial community structures was investigated by PCA performed on Hellinger transformed data (Fig 3). Despite several attempts of PCR optimization, the amplification of sample 9 failed, probably due to the presence of inhibiting humic substances. Therefore, this sample was excluded from further analysis. Most samples of the Quaternary aquifer scored negatively along the *x*-axis (PC1) and were separated from the samples of the Tertiary, scoring positively, regardless of the year. Hence, the ordering of the samples partly mirrored the hydrogeological structure of both superimposed aquifers, reflecting in turn the contaminants distribution. PCA also revealed that bacterial community structures of the Tertiary aquifer corresponding to the depths 5, 6, 9 and 10 substantially changed over time. However, these samples were found together in 2007. Conversely, bacterial community structures found at depths 7 and 8 were similar and did not display significant change over time. Hence, while the whole Tertiary aquifer was subjected to similar changes in contaminant concentrations over time, the extent of the change in bacterial community structures varied at discrete-depth intervals.

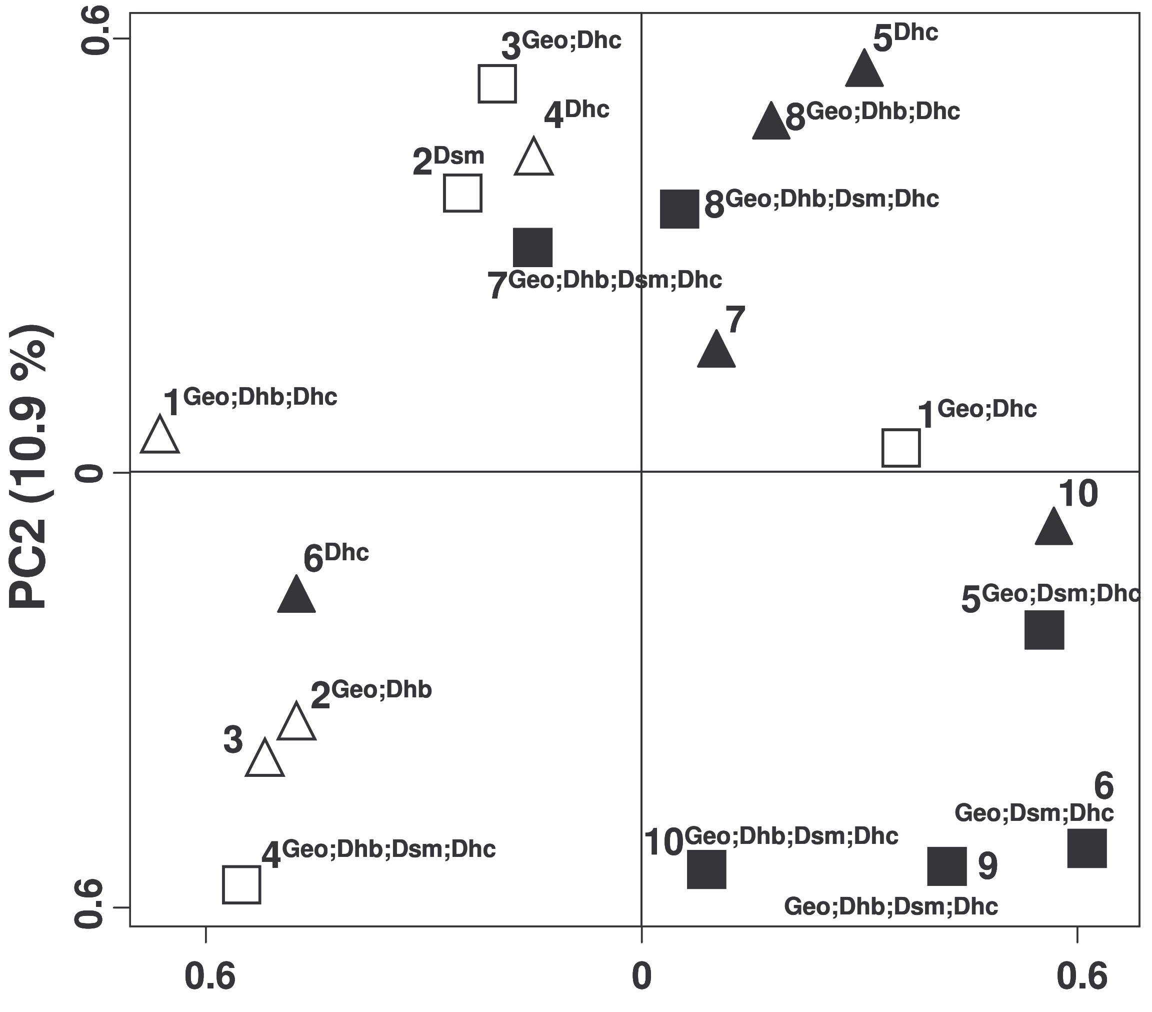

Figure 3: Principal component analysis of DGGE bacterial community patterns generated with PCR-products obtained from contaminated groundwater for the years 2006 and 2007 (λ = 0.712). Samples are labeled according to the sampling year and their originating aquifer (Δ and ▲: 2006, □ and ■: 2007, Δ and □: Quaternary aquifer and ▲and ■: Tertiary aquifer). Samples are numbered starting with 1 from the top according to increasing depth. The values on the axes indicate the percentage of the total variation, which they explain. The presences of putative dehalorespiring bacteria obtained from the taxon-specific detection assay are given for each sample as follows: Geo = *Geobacter* sp., Dhb = *Dehalobacter* sp., Dsm = *Desulfuromonas* sp., Dhc = *Dehalococcoides* sp.

The hydrogeochemical variables explaining the largest amount of variation in the bacterial community structures were correlated *a posteriori* onto the PCA results obtained with DGGE patterns. Among all hydrogeochemical variables, contaminants were found to be predominantly associated with the observed changes. These were, from the most to the least significant explanatory variable, benzene ($p<0.010$) > *trans*-DCE ($p<0.015$) > *cis*-DCE ($p<0.025$) > ethene ($p<0.033$) > 1,2-dichloroethane ($p<0.037$) > TCE ($p<0.040$) > dichloromethane ($p<0.043$) > trichloromethane ($p<0.044$) > 1,2,2-trichloroethane ($p<0.046$) > 1,2-trichloroethane ($p<0.049$).

3.4. Global ordination of bacterial fingerprints and hydrogeochemistry in 2007

Due to the overall contaminant concentration increase, the isotopic signature of chlorinated ethenes was measured in 2007. Therefore, both T-RFLP and DGGE as well as the complete hydrogeochemical data sets of the year 2007 were jointly explored by multiple factor analysis (MFA) to gain detailed insights onto the variability of bacterial community structures and the hydrogeochemistry along the vertical profile. No T-RFLP fingerprint could be generated for depth 1; this sample was excluded from the analysis.

A global correlation between the different sets of variables was assessed by the RV coefficient of relationship. The DGGE and T-RFLP weighted data sets were relatively close (rv = 0.73), whereas they were similarly distant from the hydrogeochemical data set (rv = 0.63 and 0.58). This emphasized a fair similarity between the DGGE and T-RFLP fingerprints and a moderate relationship between the bacterial community structures and the hydrogeochemical measured variables, respectively. The MFA scores plots (Fig. 4A) showed the distribution of the centroïds corresponding to the sampled depths. The three associated partial points corresponded to the object position (obtained by the PCAs performed separately on the three analyzed data sets). In a general sense, the hydrogeochemistry mostly influenced the position of the centroïds and was often decreasing the distances obtained between the samples issued from a same aquifer. This suggested lesser variability in the hydrogeochemical conditions found in each aquifer when compared to the higher variability of the corresponding bacterial community structures. Samples 2, 3 and 4 of the Quaternary aquifer showed negative scores along the *x*-axis (Dim 1) and were separated from samples of the Tertiary aquifer, scoring positively. In the loading plot of the hydrogeochemical variables (Fig. 4B), PC1 showed strong positive loadings (> 0.8) on ethene, VC, TCE, benzene and toluene, indicating that samples displaying higher scores along the Dim 1 axis were associated with a higher contamination load. Conversely, negative loadings (< -0.9) on hydraulic conductivity, sulphate, ammonia and manganese also suggested a ranking of the samples with respect to these variables. Depths 2, 7 and 8 scored negatively along the *y*-axis (Dim 2) and were clearly separated from depths 9 and 10, which scored positively. Dim 2 axis also expressed a relatively high part of the data variance (20.4 %) and mainly showed strong negative correlation on the balanced isotopic signature values of chlorinated ethenes (Fig. 4B). Thus, samples scoring low along this last axis were associated with higher $\delta^{13}C_{CE}$ values.

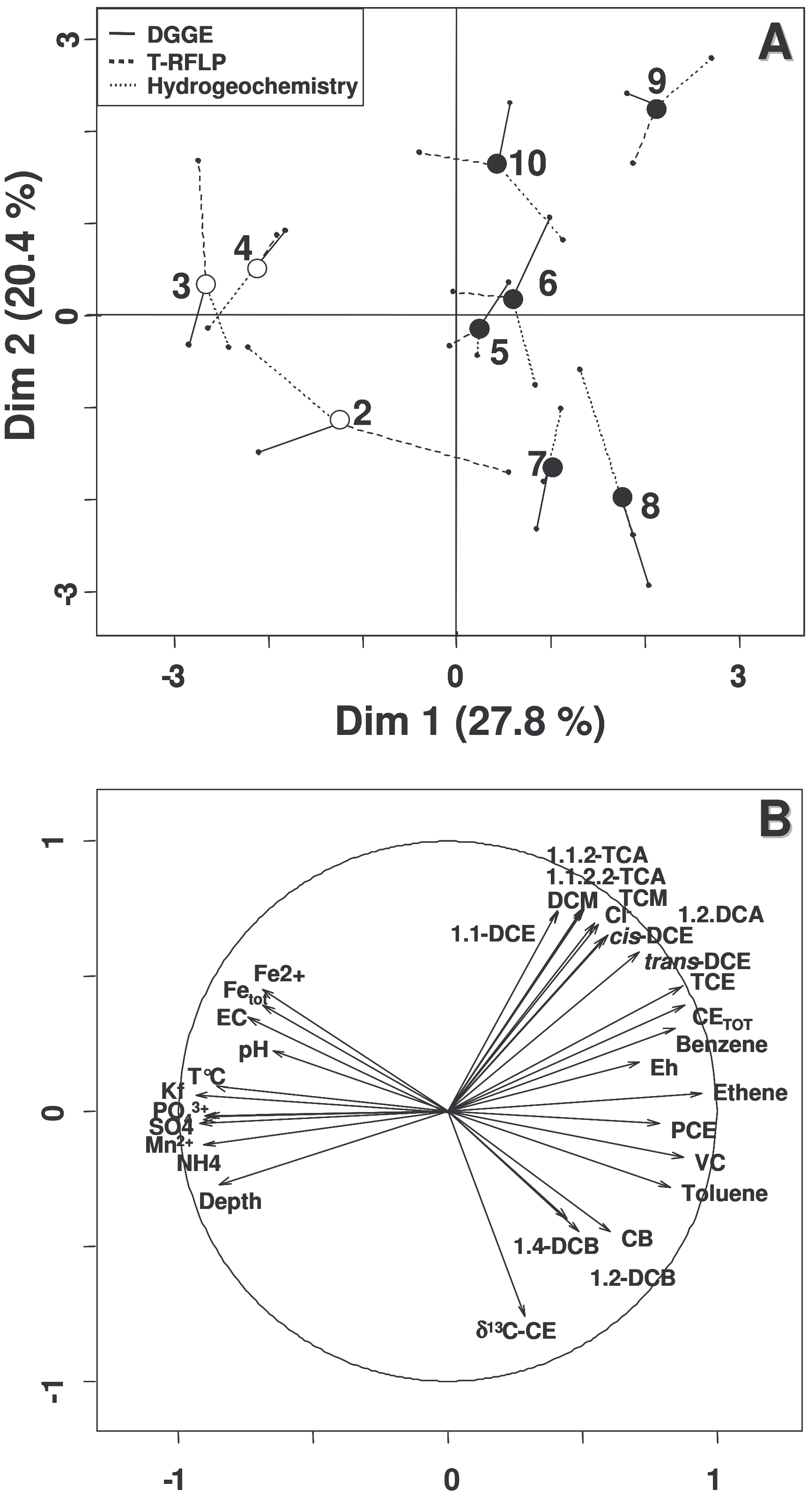

Figure 4: Global analysis of the T-RFLP, DGGE and hydrogeochemistry data sets of 2007 by multiple factor analysis. (A) The superimposed representation shows one global point for each investigated depth (○: Quaternary, ●: Tertiary aquifer) and the three associated partial points corresponding to the three data sets (DGGE, T-RFLP

and hydrogeochemistry). A line segment links the position of the bacterial (DGGE or T-RFLP) or hydrogeochemical patterns for each depth to the global position. Samples are numbered starting with 1 from the top according to increasing depth. The values on the axes indicate the percentage of the total variation, which they explain. (B) MFA ordination plot representing the hydrogeochemical variables corresponding to groundwater samples 2007. Description vectors correspond to: Fe^{2+}, ferrous iron; ΣFe, Total iron; EC, electric conductivity, T°C, temperature; K_f, hydraulic conductivity; PO_4^{3-}, phosphorus; SO_4^{2-}, sulphate; Mn^{2+}, manganese; NH4, ammonium; Cl^-, chloride; Eh, redox potential; 1.1-DCE, 1,1-dichloroethene, DCM, dichloromethane; 1.1.2.TCA, 1,1,2-trichloroethane; 1.1.2.2.TCA, 1.1.2.2-tetrachloroethane; TCM, trichloromethane; 1.2.DCA, 1,2-dichloroethane; *cis*-DCE, 1,2-*cis*-dichloroethene; *trans*-DCE, *trans*-1,2-dichloroethene; TCE, trichloroethene; CE_{TOT}, total chlorinated ethenes concentration (sum of respective concentrations); PCE, Tetrachloroethene; VC, vinyl chloride; CB, monochlorobenzene; 1.2-DCB, 1,2-dichlorobenzene; 1.4-DCB, 1,4-dichlorobenzene; δ^{13}C-CE, isotopic balance.

3.5. Distribution of potential members of the dehalorespiring guild

The presence of *Dehalococcoides*, *Dehalobacter*, *Desulfuromonas* and *Geobacter* DNA was tested at discrete-depth intervals along the vertical profile in the years 2006 and 2007 using taxon-specific assays. In 2006, the presence of at least one of the targeted genera could be proven at six depths (Fig. 3). *Dehalococcoides*-affiliated bacteria could be detected at depths 1, 4, 5, 6 and 8. *Dehalococcoides* spp., *Geobacter* spp. and *Dehalobacter* spp. DNA were concomitantly detected at depths 1 and 8. In 2007, putative dehalogenators could be detected at all investigated depths along the whole profile (Fig. 3). *Dehalococcoides* spp. DNA was found at all depths, except at depth 2. In particular, at least three of the targeted genera could be concomitantly detected at seven of the ten investigated depths. DNA of the four targeted genera could be simultaneously detected at the lowest depths (7, 8, 9 and 10), where high contaminant concentrations occurred. Overall, the taxon-specific assays suggested substantial changes in the distribution of potential members of the dehalogenating guild along the vertical profile between the years 2006 and 2007.

3.6. Bacterial community composition

Two 16S rRNA clone libraries were constructed to gain more insight in the bacterial composition of the communities found in both aquifers. A total of 150 and 130 clones were obtained from depth 4 (Quaternary aquifer) and depth 7 (Tertiary aquifer), respectively. Restriction pattern analysis further refined these sequences, with a resulting 93 and 85 OTUs for depths 4 and 7, respectively. Although the rarefaction curves indicated a tendency of saturation, discovery of additional sequences is expected by increasing the number of investigated clones (Fig. 5).

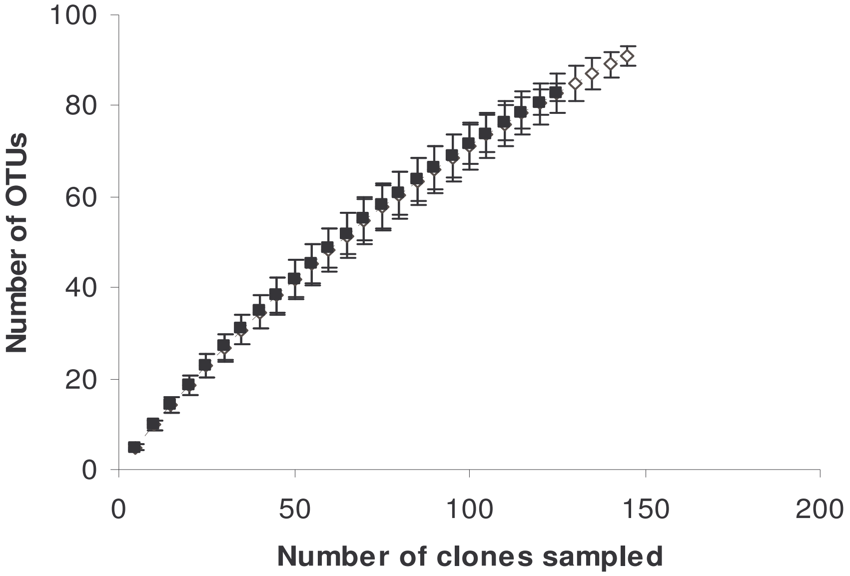

Figure 5: Rarefaction curve of bacterial 16S rRNA gene clones recovered from groundwater at depth 4 (19.5 meters below surface) and depth 7 (36.5 meters below surface) from the multilevel well. The studied depths are represented as follows: black squares, depth 4; white diamonds, depth 7. Error bars show the upper and lower 95% confidence limits.

Clone sequences could be affiliated with at least 10 classes of the domain *Bacteria* (Tables 1, S6 and S7). The majority of the clones (>50%) were not assigned to any phyla at the confidence threshold of 80%. Both clone libraries were dominated by the phylum *Proteobacteria* (approximately 25 %). Sequences affiliated to the *β* and *δ-Proteobacteria* were retrieved in higher numbers than the other groups in both libraries. However, the relative frequency of *δ- Proteobacteria* sequences was higher at depth 4, whereas the relative frequency of *β-Proteobacteria* affiliated sequences was higher at depth 7. At depth 7, some sequences within the *β*-subgroup were found similar to *Denitratisoma* sp. (Seqmatch score = 0.84) and *Thiobacillus* sp. (Seqmatch score = 0.72) clones retrieved from a mixed-waste groundwater contamination (unpublished) and lake sediment (Qu *et al.*, 2005), respectively. Other sequences detected at depth 4 were affiliated to *Acidobacteria* (8.0 %), *Chloroflexi* (4.7 %), and *γ-Proteobacteria* (4.0 %), whereas these groups represented respectively less than 1 % at depth 7. *γ-Proteobacteria* clone sequences grouped into two families, including *Syntrophaceae*, retrieved from both depths, and *Desulfobacteraceae*, exclusively at depth 4. Some clones shared close homology with sulphate-reducing *Desulfomonile*, *Desulfobacca* and *Desulfitobacterium* genera affiliated sequences. Clone sequences affiliated to *Desulfomonile* sp. displayed a high level of similarity with a clone retrieved from sulphate-reducing, toluene oxidizing cultures (Beller *et al.*, 1992), itself closely related to an anaerobic dehalogenating bacterium from marine sediments (Sun *et al.*, 2001). Conversely, a number of sequences affiliated to *Verrumicrobia* (1.5 %) and *Lentisphaerae* (0.8 %) were exclusively found at depth 7. Clones belonging to the less-known candidate divisions OD1 and TM7 could be retrieved from both depths. *Acidobacteria* clone sequences grouped with clones detected in flooded, anaerobic environment (e.g. rice paddies) and one sequence shared homology with a clone detected in nitric acid, uranium bearing wastes, fuel and chlorinated hydrocarbons contaminated sediments (Abulencia *et al.*, 2006). Within the phylum *Chloroflexi*, clone sequences were affiliated to the *Anaerolineae* class. Noticeably, one clone from depth 4 was closely related to an uncultured bacterium sequence associated with PCE dechlorination and recently retrieved from anoxic river sediment by RNA-based stable isotope probing (Table S6, clone 150, Seqmatch score = 0.82) (Kittelmann & Friedrich, 2008).

Table 1: Relative clone frequencies in major phylogenetic groups of the clone libraries from depth 4 (Quaternary aquifer, 19.5 meters below surface) and depth 7 (Tertiary aquifer, 36.5 meters below surface) of the multilevel well.

Group	Relative frequency [%]	
	Depth 4	Depth 7
Proteobacteria	**24.7**	**26.9**
α-Proteobacteria	1.3	1.5
β-Proteobacteria	10.7	19.2
δ-Proteobacteria	8.7	4.6
ε-Proteobacteria	0	0.8
γ-Proteobacteria	4.0	0
Unclassifed-*Proteobacteria*	0	0.8
Acidobacteria	8.0	0.8
Bacteroidetes	6.0	6.2
Chloroflexi	4.7	0.8
OD1 Division	2.0	2.3
OP10 Division	0.7	0
TM7 Division	0.7	1.5
Verrucomicrobia	0	1.5
Lentisphaerae	0	0.8
Firmicutes	1	0.8
Unclassifed Bacteria	52.7	58.5
Number of clones	**150**	**130**
Number of OTUs	**93**	**85**

4. Discussion

4.1. Relationship between microbial communities and environmental variables

One of the major challenges in microbial ecology is to understand how microbial communities are patterned with spatial and temporal heterogeneities in the environment (Horner-Devine & Bohannan, 2006). In this study, a correlation between bacterial community structures, hydrogeochemical and hydrogeological variables could be observed. Apparent strong contrasts in spatial hydrogeological properties, coupled with heterogeneous distributions of physical and chemical properties between the two superimposed aquifers, were clearly defining diverse habitats in which complex bacterial community structures were maintaining reductive dechlorination activities. However, this does not imply that the bacterial communities were stably maintained in terms of structure and diversity at this ecosystem scale. Although the PCA analysis of DGGE patterns of the years 2006 and 2007 did not result in clearly distinct clusters of samples, the dissimilarity between samples from the Tertiary aquifer in 2007 and the other samples indicated that part of the microbial community changed with the development of the contaminant plume. The analysis also raised statistically significant relationships between the variation of the community structure and the contaminant concentrations. Similarly, the comprehensive multiple factor analysis of the year 2007 also showed that variations of the bacterial community structures could be related to the hydrogeology, which indirectly reflected the distribution of contaminant concentrations over the vertical profile. In this analysis, samples from the less-contaminated Quaternary aquifer could be distinguished from samples of the Tertiary aquifer when coupling the two bacterial fingerprinting analyses and the hydrogeochemical profiles into one single analysis. DGGE and T-RFLP profiling methods were similar and displayed about 3/4 of homologous information. While DGGE may be useful to routinely screen structural changes of low complexity groundwater community in response to contaminant perturbation, it may not reflect minor species variation. In contrast, T-RFLP displays the high-resolution and reproducibility of automated sequencing technologies (Nocker *et al.*, 2007; Smalla *et al.*, 2007). Nevertheless, DGGE and T-RFLP belong to different fingerprinting families and entail different drawbacks and limitations (Nocker *et al.*, 2007). Therefore, the reliability of the interpretation is likely strengthened when the outputs of the different bacterial profiling methods are coupled, while benefiting of their comparative advantages. This study showed that it can be efficiently achieved by multiple factor analysis (MFA).

The distinction between “non-contaminated” and “contaminated” samples with respect to both bacterial and archaeal community structures was previously reported in BTEX contaminated aquifers (Roling *et al.*, 2001; Mouser *et al.*, 2005). Hence, distinction between plume and pristine microbial profiles previously reported at the plume scale seemed to apply also on a temporal scale at the plume fringe or on a vertical scale across the heterogeneous aquifers. A contamination stress due to the plume development may have shaped the initial microbial community structures at discrete-depth and may lead to acclimatization of organisms capable of utilizing or surviving the contaminant. This phenomenon may have generated similar bacterial community structure at depths 5, 6, 9 and 10 of the more heavily contaminated Tertiary aquifer in the year 2007. It also resulted in relatively rich microbial communities and did not reduce the diversity, as suggested by a similar range of DGGE bands in the patterns from 2006 and 2007 (Fig. S3). Conversely, a convergence in the community structuring could not be observed in the less-contaminated Quaternary aquifer, and the bacterial profiles substantially differed between depths. Furthermore, the observed hydrogeochemical and microbial community changes also partly mirrored the physical heterogeneity of the vertical profile, mainly characterized by two aquifers of heterogeneous hydrogeological nature. The spatial variation of the solid phase may have partly influenced the microbial diversity at discrete-depth intervals by controlling the distribution, fluxes and bioavailability of electron donors and acceptors as well as nutrients and exposure to contaminants. By controlling mass transfer of reactive species, pore velocity may indirectly select microbial populations adapted to a particular flow regime and flux rate of contaminant (Cunningham & Fadel, 2007). Hence, the variability of hydrogeological characteristics may also influence the degree of heterogeneity of the microbial structure and composition.

The analysis of the clone libraries revealed that the majority of the recovered sequences could be affiliated with obligatory, anaerobic bacteria, which is in agreement with the prevailing hydrogeochemical characteristics of the aquifers. Similar types of organisms have been widely described in hydrocarbons- and chlorinated solvents contaminated anoxic aquifers (e.g. Dojka *et al.*, 1998). Moreover, sulfate-reducing bacteria were likely an important component of the microbial community, in accordance with previous report (Kleikemper *et al.*, 2002). However, high background concentration of sulphate in groundwater hindered a concentration-based sensitive analysis of sulphate reduction and no direct geochemical indications could be inferred. The formation of iron sulfide minerals in the presence of ferrous iron may have lead to underestimation of total concentration of sulphide. Interestingly, some sequences were found related to potential contaminant degraders. In particular, several sequences in the Quaternary aquifer were affiliated to the *Anaerolineae* classes, suggesting that non-Dehalococcoides *Chloroflexi* populations, which might also be linked to reductive dehalogenation, are present (Watts *et al.*, 2005). This also emphasizes the relevance of detailed sampling of the microbial community for better delineating the microbial diversity potentially associated with *in situ* transformation of chlorinated ethenes. However, the majority of bacterial sequences detected at both depths could not be taxonomically assigned at fair confidence level with previously reported sequences (Table S6 and S7).

The description of the bacterial composition was further enhanced by the more sensitive, double step PCR-based taxon-specific assays targeting the guild of reductive dehalogenators. While these groups may not represent an abundant part of the microbial community, their activity and function is assumed to be critical at the plume fringe. Their putative dechlorination capacity was very likely not altered at the observed concentration levels (Sleep *et al.*, 2006). The distribution of the potential members of the dehalogenating guild over the vertical profile partly mirrored the temporal hydrogeochemical variation. For instance, *Geobacter* sp. and *Desulfuromonas* sp. were widely distributed over the profile in 2007 but not in 2006. These iron-reducing and metabolically versatile microorganisms are obligate anaerobes capable of reductive dechlorination. They may play a critical role in the dechlorination of chlorinated ethenes at the plume fringe, as iron reduction may inhibit reductive dechlorination by other species (Sung *et al.*, 2003; Sung *et al.*, 2006). Furthermore, the taxon-specific assays showed the presence of *Dehalococcoides*-affiliated organisms at almost all investigated depth in 2007. This also emphasized the sensitivities of this specific-PCR protocol, ranging from 10 to 1000 copies of the gene per reaction mixture, over the cloning-sequencing procedure (Hendrickson *et al.*, 2002). Although members of the genera *Geobacter*, *Dehalobacter* and *Desulfuromonas* are capable of partial dechlorination of PCE and TCE to *cis*-DCE, *Dehalococcoides* is the only group known today to completely dechlorinate PCE/TCE to ethene and is highly evolved to utilize halogenated organic compounds and hydrogen (MaymoGatell *et al.*, 1997; Seshadri *et al.*, 2005). Thus, the wide distribution over the profile of putative dehalogenators in association with evidences of dechlorinating processes suggested favorable conditions for chlorinated ethenes biodegradation in this zone of the plume fringe. Moreover, the correlation between the nearly ubiquity of Dehalococcoides-like bacteria along the vertical profile and the occurrence of complete reductive dechlorination support the involvement of these group of bacteria in the observed degradation process.

4.2. Variability of in situ biodegradation processes and isotope balance

Changes in both the bacterial community structures and the distribution of members of the potential dechlorinating guild over the vertical profile also coincided with varying extents of isotopic shifts during contaminant degradation at discrete-depth intervals (Fig. 2). At the investigated monitoring well, significantly higher enrichment in ^{13}C in the residual fractions of chlorinated ethenes species was observed compared to the previously reported source areas. Isotopically lighter VC and ethene with respect to their parent-compounds indicated ongoing *in situ* biodegradation at discrete-depth intervals over the whole vertical profile (Table S5). Indeed, non-destructive processes, which may occur in these aquifers, are not expected to cause significant isotope fractionation (Huang *et al.*, 1999; Poulson & Drever, 1999; Slater *et al.*, 2000; Schüth *et al.*, 2003). Furthermore, while PCE and TCE abiotic degradation have been reported, this process is unlikely to occur in the investigated groundwater system, since it appears to be pH-dependent, with significant degradation rate at pH values > 8, and half-life value have been reported to be one order to magnitude lower than biotic transformation (Butler & Hayes, 1999; Jeong *et al.*, 2007). pH values over the vertical profiles were systematically < 7 (Tables S2 and S3).

However, the isotopic signature of VC may reflect in our case both its production from DCEs and its degradation to ethene. This represents limits in the application of the Rayleigh concept for interpreting the isotope shifts of single contaminant species. Therefore, the evaluation of VC degradation over the profile was upgraded by calculating an isotope balance ($\delta^{13}C_{CE}$). It included both concentration and isotopic composition values of PCE, TCE, *cis*- and *trans*-DCE (DCEs) and VC, separately calculated for each investigated depths. In this study, significant enrichment of $\delta^{13}C_{CE}$ was interpreted as indicative of VC biodegradation. This interpretation is based upon assumptions discussed for two main cases. A closed isotope balance involves all the chlorinated ethenes species and implies that PCE is reductively dechlorinated to TCE, TCE to DCEs, and DCEs to VC. This dechlorination sequence is then the sole process affecting the concentration and/or the isotope composition of PCE, TCE and DCEs within the balanced system. In this case, the isotope fractionation, which may occur during the various reductive dechlorination steps from the higher chlorinated ethenes to VC (i.e. PCE to TCE, TCE to DCEs and DCEs to VC), does not affect the cumulative $\delta^{13}C_{CE}$ value, regardless the extent of isotope fractionation at each step. However, the occurrence of an isotope fractionation during further degradation of VC should generate a significant ^{13}C enrichment of $\delta^{13}C_{CE}$ and represent a prerequisite to indicate *in situ* degradation of VC. In the case of a not closed isotope balance, an alternative degradation pathway co-exists with the dechlorination sequence. This pathway is associated with a loss of carbon from the pool of PCE and/or TCE and DCEs. This loss affects then the concentration and/or the isotopic composition of the involved chlorinated ethenes species. In this case, the reductive sequence from PCE to VC is no more balanced with respect to concentration and/or isotopic values, and the relative enrichment of individual species reflects its degradation. The degree at which the isotope balance will be debased depends on the relative contribution of the alternative pathway(s) with respect to reductive dechlorination but a significant ^{13}C enrichment of $\delta^{13}C_{CE}$ will always indicate biodegradation of VC. Moreover, a significant positive shift of the isotope signatures of the less-chlorinated ethenes between the source and the fringe of the plume will always indicate *in situ* degradation. Though anaerobic microbial oxidation of DCE and VC may theoretically represent an alternative pathway (Bradley & Chapelle, 1996; Bradley & Chapelle, 1998), hydrogeochemical, isotopic and microbiological indicators strongly suggested that reductive dechlorination represented a dominant degradation mechanism over the vertical profile.

While the isotope measurements of single species suggested conversion of *cis*-DCE and VC at all depths, the isotope balance revealed important variations both between aquifers and between depths. The physical and geochemical uniformity of the Quaternary aquifer may have lead to spatially homogenous degradation reactions, as suggested by similar $\delta^{13}C_{CE}$ values across the segment. The high hydraulic conductivity and putative groundwater mixing process may have reduced the mass transfer limitation of both contaminant and electron donors. The release of reduced organic compounds from the lignite layer underlining this aquifer might have ensured a sustainable source of electron donors for the present dehalorespirers (Imfeld *et al.*, 2008). In contrast, the range of variability of VC conversion was larger in the Tertiary aquifer, characterized by more heterogeneous sediments and contaminated load. The variability of $\delta^{13}C_{CE}$ values may also reflect different characteristics of the microbial communities, whose members may respond differently according to discrete-depth conditions and influence the different stages of the sequential reductive dechlorination reaction. For instance, $\delta^{13}C_{CE}$ values found at depths 7 and 8 were significantly higher, coinciding with the presence of a more complex dehalogenating guild. While bacterial community structures of depths 7 and 8 were similar, they differed from structures found at other depths of the Tertiary aquifer. Furthermore, structures of these communities did not substantially vary with the arrival of the contaminant plume. This may reflect the maintenance of key inter-populations interactions and functions of the community in the presence of the contaminant load and with respects to contaminant degradation.

The progress of reductive dechlorination activity at discrete depth intervals is likely controlled by 1) the local hydrological and geochemical conditions, 2) the fermentation of reduced organic compounds released from the lignite layer, which may generate available of electron donor species, such as H_2, and 3) the activity of an appropriate guild of dehalogenating microorganisms. In this study, the taxon-specific assays showed the varying distribution of several putatively active dehalorespirers along a heterogeneous vertical profile. Though the variation of local conditions with respect to geochemistry and/or availability of specific electron donor species may explain the observed variation in the complexity of the dehaloresping guild between depths, the ongoing degradation activity putatively performed by the members of the guild could be inferred based on the isotope data analysis and the detection of ethene. Molecular tools for detecting the presence of putatively actively degrading microorganisms coupled to isotope composition and metabolite concentrations analyses allowed to efficiently document *in situ* biodegradation of chlorinated ethenes while investigating local reaction conditions in the heterogeneous aquifers. However, numerous factors, including the diversity and abundance of the active dehalogenating microorganisms and their specific interactions with the ambient environment, may explain the substantial variability of VC degradation reaction between depths, as revealed by the isotope analysis. In combination with local hydrological and geochemical conditions, this may lead to a segmentation of the reaction rates over the vertical profile. If the various flow segments reach a common point and unite (e.g., in an integrative monitoring well), the interpretation of contaminant degradation based on isotope data, applying the Rayleigh concept, may be limited (Kopinke *et al.*, 2005). Nevertheless, the presence of ethene as well as the parallel analysis of the isotope balance and the isotopic patterns of single chlorinated ethenes species indicated variability of VC dechlorination over the profile. It also suggested a relationship between heterogeneity of discrete-depth conditions and degradation processes. A better understanding of how reactive zones with respect to chlorinated ethenes degradation are delineated in heterogeneous groundwater systems is required to improve the monitoring of natural attenuation processes. This study shows that this issue can be tackled by an integrative approach based on hydrogeochemical indicators, microbial community characterization and an isotopic analysis.

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4

Summary, Conclusions and outlook

1. Summary and Conclusions

This thesis aimed at gaining knowledge about biogeochemical processes in chlorinated solvents contaminated model wetlands and aquifers, and is particularly focused on the *in situ* biodegradation processes. The different studies that constitute this work primarily addressed the destructive removal of toxic and priority reductive dechlorination intermediates, such as dichloroethenes, vinyl chloride or chlorobenzene in the environment. These investigations are to be placed in the context of assessing Natural Attenuation at complex contaminated environmental systems and specifically addressed a scientific gap, namely the biogeochemical processes associated with natural attenuation of chlorinated solvents at anoxic aquifers and groundwater-surface water interfaces. In particular, the occurrence of *in situ* biodegradation of lower chlorinated solvents over both spatial and temporal scales requires evidences. However, microbial and chemical processes interact to control the chemical evolution of groundwater. This in turn may affect both the extent and the nature of biodegradation processes, which often limits a conclusive interpretation of ongoing *in situ* processes. Therefore, a thorough biogeochemical understanding of systems contaminated with organic chemicals potentially subjected to various degradation pathways necessarily requires concurrently taking into account the relationships between ambient hydrological and hydrochemical conditions and indigenous microbial community.

For this purpose, several complementary techniques were coupled in order to gain robust evidences of *in situ* degradation, while simultaneously investigating hydrochemical conditions associated with the target microbial processes (Chapter 1, section 1 and 2). This approach was found relevant when investigating spatially plumes of chlorinated solvents (Chapter 3, section 1 and 2), interfaces or transitional zones, such as fringes of contaminant plumes (Chapter 3, section 3), as well as model systems for groundwater-surface water transition zones (Chapter 2), where ambient conditions widely varied over both spatial and temporal scales. The state of the art of hydrogeochemical methods, CSIA and microbial analyses used in this thesis as well as possible approaches for characterizing *in situ* degradation of organic chemicals in saturated, complex systems have been previously summarized in the form of a review paper (Chapter 1, section 2). Therefore, this conclusion focuses on the limits and potential of linking the outcome of the various groups of methods to constitute an integrative approach enabling a thorough characterization of key biogeochemical processes in systems contaminated with chlorinated solvents (section 1.1). This is tackled in the following sections with respect to i) *in situ* biodegradation by means of isotope tools (section 1.2.), ii) the elucidation of prevailing degradation pathway (section 1.3.), and iii) the relationship between environmental factors and *in situ* microbial communities (section 1.4.) and dehalogenating guilds (section 1.5.). Finally, prospective experimental and conceptual developments with respect to the assessment of natural attenuation of chlorinated solvents in aquifers and groundwater-surface water zones are proposed (section 3.).

1.1. Relevance of an integrative approach

An appropriate monitoring strategy would ideally enable i) assessing the status and contribution of the different contaminant removal processes, and ii) evaluating the long-term maintenance of the organic chemicals mobilization and/or transformation function of a system. However, degradation of chlorinated solvents may occur within biogeochemically heterogeneous systems *via* sequential reductive dechlorination and/or alternative pathways that mostly cannot be appropriately characterised by one single method. The different studies of the present thesis underscored the importance of integrative experimental designs based on both *in situ* hydrogeochemical and microbiological indicators to create a stronger basis for evaluating *in situ* biogeochemical processes at contaminated sites and thus improve the quality and reliability of the interpretation.

Microbial transformation processes associated with changes of chlorinated solvents concentrations were documented by hydrogeochemical analyses that included mass loss of contaminant, production of degradation metabolites, as well as changes in redox-sensitive species. The analysis of target contaminant(s) behavior was partly achieved by combining an evaluation of footprints of biogeochemical reactions along with concentration variations of both the targeted contaminant and its degradation metabolites. Chemical and geochemical signatures resulting from reductive dechlorination of highly chlorinated solvents were typically characterized by the presence of less-chlorinated metabolites, detected under various conditions prevailing in the milieu, ranging from iron- to methanogenic reducing conditions (Chapters 2 and 3).

However, degradation of dehalogenated metabolites as DCE or VC by alternative pathways, such as aerobic and anaerobic oxidation, may have co-occurred at the investigated groundwater systems and model wetlands, or even predominated at certain periods or zones (refer to e.g. Chapters 2, section 2). In this case, the detection of degradation metabolites appeared difficult due to their ubiquitous presence and/or transient occurrence. Furthermore, steep gradients where several degradation mechanisms may co-occur over both spatial and temporal scales can not be appropriately characterized and the interpretation can be misleading with respect to the real contribution of the ongoing degradation processes (Chapter 2, section 2). Moreover, at strictly anoxic plumes where reductive dechlorination of the chlorinated solvent dominated, the direct interpolation of decreasing contaminant concentrations along the plume centreline for further estimation of the biodegradation was delicate, due to possible co-occurrence of contaminant attenuation due dilution as well as the complexity of the geology limiting a fair knowledge of the flow path (Chapter 3, section 1 and 2).

Though some geochemical variables may strengthen evidences for the progress of *in situ* degradation of chlorinated solvents at the investigated systems scale, they rarely conclusively attest it. Nevertheless, the hydrogeochemical analysis permitted to gain a snapshot of the prevailing redox conditions while investigating anoxic aquifers (Chapter 3), or information on the spatiotemporal evolution of the hydrochemical framework while investigating model wetland systems (Chapter 2). In particular, information on contaminant concentrations and redox-sensitive species constituted a necessary basis upon which microbial and compound-specific isotope analyses could be successfully implemented to investigate more specific aspects of the *in situ* degradation of chlorinated solvents. In particular, this step allowed gathering knowledge on hydrological and hydrochemical conditions of the investigated system, which is a prerequisite for using CSIA, and thus to further characterize contaminant transformation.

Another important target was to gain knowledge about the distribution and potential activity of microorganisms directly or indirectly involved in the degradation of chlorinated solvents in aquifers and model wetlands over both spatial and temporal scales. This can be tackled by a reductionist and/or by a holistic approach. Obtaining a “representative” pure culture of an organism that carries out relevant biogeochemical process represent a preliminary -often necessary- step to relate a microorganism to a specific environmental function and thus to gain key physiological information related to contaminant degradation. For instance, this approach has been used to investigate the variability of the carbon isotope fractionation upon PCE and TCE reductive dechlorination reaction (refer to ANNEX A and B). Complementarily, because *in situ* degradation potential and activity inherently depend on the composition and function of indigenous microorganisms, molecular techniques allowed investigating the structures and composition of the indigenous microbial community in a more holistic way. The concomitant hydrochemical and microbial characterization also gave the opportunity to investigate what environmental factors are associated with the *in situ* distribution and potential activity of microorganisms (Chapter 2, section 3; Chapter 3, sections 2 and 3). For this purpose, the complex sets of data were further separately treated by exploratory statistical analysis in order to gain additional insight into the development of observed biogeochemical processes (e.g. PCA, refer to Chapter 2, section 1 or Chapter 3, section 2). Additionally, interpretative statistical treatments coupling both hydrochemical and microbial data sets were also employed to enhance the interpretation of existing relationships between the microbial community structures and the hydrochemical framework (e.g. CCA or MFA, refer to Chapter 3, sections 2 and 3, respectively). In a general sense, the combination between microbial and hydrochemical information enhanced the mechanistic understanding of the relationship between microbial species diversity and environmental factors, such as contaminant concentrations, prevailing TEAPs or even hydrogeological variables (Chapter 2, section 3 and Chapter 3, section 2 and 3). However, molecular techniques have inherent methodological biases that may cause incorrect estimation or even no detection of targeted microorganisms, as discussed in the different studies. Though qualitative information on spatial or temporal changes of the microbial communities can be provided by DNA fingerprinting methods, tracking changes in both species composition and degrading function at contaminated sites remains challenging as well as time and cost-demanding. Moreover, chlorinated solvents degradation can be performed under various ambient conditions and by several degradation pathways that involve different, only partly known, microbial guilds and degradation mechanisms. This uncertainty largely contributes to complicate the interpretation.

Further coupling of hydrogeochemical and molecular techniques to compound-specific isotope analysis (CSIA) is thus relevant for improving qualitative and quantitative aspects of *in situ* biodegradation of chlorinated solvents. In particular, as the extent of isotope fractionation mainly depends on the biochemical reaction mechanism involved, this technique may particularly help documenting and characterizing biodegradation and corresponding pathways during spatiotemporal surveys in complex environmental systems.

1.2. Assessing i*n situ* biodegradation of chlorinated solvents by isotope tools

In this thesis, CSIA was primarily used to provide a qualitative evidence of chlorinated solvents degradation. The obtained isotopic data then were used as a variable in further statistical treatment (Chapter 2, section 2; Chapter 3). For the investigation of CB degradation, CSIA was combined with tracer experiments employing ^{13}C-labelled CB (Chapter 2, section 1; Chapter 3, section 1). Additionally, an isotope balance was calculated at two different field sites to support the interpretation of VC and CB degradation, while taking into account the complexity due to the sequential chlorinated solvents degradation (Chapter 3, sections 1 and 3).

In a general sense, these different investigations showed the potential and reliability of CSIA to assess the degradation of intermediate and accumulating compounds originating from the sequential degradation of higher chlorinated compounds under various hydrological and hydrochemical conditions. The interpretation of isotopic data was primarily performed by comparing i) the isotopic signature of intermediate compounds with the signature of their respective precursor and product, and ii) the isotopic signatures at local point within the contaminant plume with the isotopic signature at the appearant source area(s). In a second step, the isotopic data were put together with other hydrogeochemical variables in a multivariate statistical treatment to investigate possible correlations between them (Chapter 2, section 2 and Chapter 3). At the investigated field site, CSIA showed that concentration decreases observed between the *a priori* contaminant sources and the plume fringes were associated with degradation of VC or CB. Other, non-destructive natural attenuation processes were not expected to result in significant changes of the isotopic signatures, as discussed case by case in the different studies. In the model wetlands, depletion of CB (Chapter 2, section 1) or DCE (Chapter 2, section 2) concentration values over the flow path generally coincided with significant enrichment of heavy isotopes within the non-degraded contaminant fraction. These studies represent the first contributions successfully using CSIA to monitor degradation processes of organic chemicals in engineered wetland systems. The isotope data also demonstrated DCE transformation, with concomitant VC accumulation and further degradation to non-toxic ethene varying over spatial and temporal scales both at the Bitterfeld site (chapter 3, section 1 and 2) and in the model wetland (chapter 2, section 2). In the model wetland experiment, CSIA allowed an integrative assessment of the *in situ* biodegradation activity over the flow path by coupling the concentrations and isotopic signatures of DCE using a Rayleigh model. This approach permitted to obtain an integrative, "mean enrichment factor" that then was compared to reference factors (Chapter 2, section 2). In parallel, the microbial diversity and the composition of the putative dechlorinating guild of bacteria was investigated at discrete interval over the flow path and punctually during the investigation period (Chapter 2, section 3). The presence of putative dehalogenators in association with evidences of dechlorinating processes indicated favorable conditions for DCE reductive dechlorination within the wetland.

Additionally, CSIA was applied to demonstrate CB degradation both in a constructed wetland (chapter 2, section 1) and in a contaminated field site (chapter 3, section 1). In parallel to CSIA, i*n situ* microcosm systems loaded with ^{13}C-labelled CB were used in wetland sediment and in groundwater in order to investigate in more details the biotransformation of CB. In both studies, incorporation of ^{13}C-labelled carbon derived from the CB into bacterial fatty acids substantiated *in situ* degradation of CB, whereas the detection of ^{13}C-labelled benzene indicated reductive dehalogenation of CB. However, the use of such tracer experiment implies that the contaminant is metabolized as a carbon source by the potential degraders, resulting in an incorporation of ^{13}C-labelled carbon into the microbial biomass. Therefore, degradation of halogenated compounds *via* reductive dehalogenation can not be documented by this method, as in this case, the contaminant molecules are used as electron acceptors. In this thesis, stable isotope tracer (^{13}C) experiments provided evidence for anaerobic CB degradation in the two types of systems, although only very small portions of the contaminant (nmol) were mineralized. This approach can be combined with laboratory microcosms and was revealed as valuable to analyze the slow degradation of recalcitrant organic chemicals. The application of modern analytical techniques for isotope analysis enables stable isotope probing of cell components, such as fatty acids that permit the sensitive elucidation of carbon fluxes on a molecular level. Additionally, *in situ* microcosms were also used as pre-enrichments to improve cultivation efficiency of the microbial community involved in the degradation processes (Nijenhuis *et al.*, 2007), thus efficiently linking field and laboratory investigations. Prospectively, this approach can also be applied to detect the biodegradation and to investigate the persistence of a variety of recalcitrant environmental pollutants using ^{13}C or ^{15}N stable isotope tracers used for the synthesis of cell components.

Though CSIA is generally employed to characterize the degradation of a single contaminant, the isotope signature of an intermediate compound formed during a reductive dechlorination sequence reflects simultaneously its production from its direct higher-chlorinated precursor and its degradation. Therefore, the study of chlorinated compounds *in situ* degradation implies taking into account both reductive dechlorination sequences and possible parallel pathways. In a dechlorination sequence, a Rayleigh model can only be applied to the initial dechlorination step, because the isotope ratio of the residual reactant is solely controlled by degradation of the reactant. In contrast, the isotope ratios of the intermediate degradation products are affected simultaneouly by both production and degradation of the compound and a Rayleigh model cannot be directly applied. In a closed system, the isotope balance is expected to remain at the initial value of the initial, precusor compounds, and the final δ^{13}C value of accumulated product is expected to equal the δ^{13}C value of the precursor compound if the mass balance covers all carbon compounds (Hunkeler et al., 1999; Bloom et al., 2000). In this thesis, an isotope balance was calculated with the goal to overcome the limit in the application of the Rayleigh concept posed by the simultaneous enrichment and depletion in ^{13}C of reductive dechlorination intermediates during *in situ* degradation of chlorinated benzenes (Chapter 3, section 1) or ethenes (Chapter 3, section 3). For the evaluation of VC or CB degradation an isotope balance ($\delta^{13}C_{\text{Chlorinated ethenes or benzenes}}$) including both concentration and isotopic composition values of the single chlorinated compounds was used. The enrichment in ^{13}C in VC or CB and an increase of the isotope balance above that of the appeарent source areas allowed demonstrating VC and CB biodegradation as well as the relative variation of the degradation reaction along a vertical profile (Chapter 3, section 3) and horizontally across a contaminant plume (Chapter 3, section 1), respectively.

This approach is particularly promising in the case of a closed isotope balance, reasonably occurring when reductive dechlorination represents the predominant degradation pathway in a closed system. The degree at which the isotope balance may be debased depends on the relative contribution of the alternative pathway(s) with respect to reductive dechlorination. Nevertheless, a significant ^{13}C enrichment of $\delta^{13}C_{\text{Chlorinated ethenes or benzenes}}$ will always indicate biodegradation of VC or CB. In this thesis, the calculation of an isotope balance was particularly found useful to assess qualitatively *in situ* degradation of CB and VC over a spatial scale at complex sites where reductive dechlorination occurred. It also allowed revealing spatial variations in the VC degradation process while taking into account the whole degradation sequence (Chapter 3, section 3).

1.3. Characterizing degradation pathways by CSIA

It is very often necessary to determine the prevalent degradation pathway at contaminated sites or in engineered treatment systems, including constructed wetlands. Though the presence and concentrations of dehalogenated intermediates is highly informative for ascertaining the occurrence of reductive dechlorination, the measurement of degradation products can in many cases be labour intensive and rarely conclusive for distinguishing pathways. The mechanism of a chemical reaction results in a characteristic isotope effect. Therefore, the use of CSIA to retrieve field-specific isotopic enrichment factors that can be later compared with reference factors entails potentials to distinguish different biodegradation pathways over space or time.

In this thesis, DCE was used as a model tracer compound in a model wetland investigation (Chapter 2, section 2). DCE can be degraded *via* both oxidation and reductive dechlorination. However, each degradation pathway implies a different mechanism and occurs under a specific range of reducing conditions. Isotopic and concentration data were then used to retrieve enrichment factors from the model wetland in order to monitor the prevailing biodegradation pathway over both spatial and temporal scales. Hence, isotope fractionation analysis was used to identify biodegradation pathways based on existing reference enrichment factors determined for cometabolic aerobic DCE degradation that significantly differs from those determined for anaerobic degradation *via* reductive dechlorination. The extent of apparent fractionation varied and mirrored both changes in the predominant degradation mechanism and in the hydrogeochemistry. Thus, the parallel investigation of prevailing hydrochemical and TEA processes was highly relevant, as their developments was associated with changes in both microbial and degradation mechanisms.

This approach could be extended to track in complex environmental systems such as wetlands other compounds that can be degraded *via* various pathways, mainly depending on the dominant hydrochemical conditions. For instance, benzene, toluene or MTBE are common environmental contaminants and hence represent interesting candidates for further investigations. Indeed, the oxidation and reduction of these compounds occur as a function of the ambient conditions and involve different enzymatic transformation mechanism that could be distinguished in complex environmental site on the basis of CSIA. Moreover, studying this compound presents the advantage that the degradation metabolites are partly characterized and can be used in a mass balance in order to provide a supplementary evidence for the ongoing pathway(s). However, a fundamental prerequisite for applying this approach is a fair knowledge of the system background and functioning. In particular, the flow path should be identified and parallel processes that may contribute to contaminant mass removal over the investigation period should be identifiable and quantifiable.

1.4. Factors influencing microbial community structures in heterogeneous systems contaminated with chlorinated solvents

A fundamental question when investigating contaminated systems is how contaminant, hydrochemistry and hydrology influence the biocenose over space and time, in particular with regard to its *in situ* biodegradation potential and function. Studies presented in this thesis specifically focused on microbial processes and biodegradation potential at groundwater-surface water interfaces in a model system (Chapter 2, section 3), along a contamination plume (Chapter 3, section 2) and at a plume fringe (Chapter 3, section 3). Distribution of contaminant and redox sensitive species concentrations was considered as a critical factor that may influence *in situ* degrading microbial activity. To address this issue, microbial fingerprints were interpreted along with the hydrogeochemical data sets and possible relationship between hydrogeochemical variables and community structures were highlighted by means of multivariate analysis.

Two different studies were performed at the Bitterfeld/Wolfen contaminated megasite, based on samples collected at different locations along the contaminant plume and at different sampling-resolutions (Chapter 3, sections 2 and 3). Interestingly, both studies revealed a statistically significant linkage between contaminant concentrations and microbial community structures over space. Though chlorinated ethenes dominated within the investigated groundwater system, the complex history of the Bitterfeld/Wolfen megasite resulted in a mixture of contaminants, whose quantitative and qualitative distribution may have influenced the biocenoses, and possibly degradation activities. On the one hand, contamination may decrease the specific richness due to a negative toxicity effect. On the other hand, an input of readily biodegradable matter delivers substrate for energy and growth and thus stimulates bacterial activity with a concomitant increase of the microbial diversity. At higher contaminated zones, xenobiotic compounds contributed significantly to the dissolved organic carbon in the plume. Therefore, as suggested in this thesis, a relationship between microbial community structures and degradation is likely to occur and thus microorganisms metabolizing available contaminants would make an important contribution to the total microbial community. Conversely, at less-contaminated zones such as the fringes of a plume, some bacterial species might simply not be detectable in a sample due to lower population densities, although they might be present in the aquifer. Hence, the apparent number of species observed with the employed molecular techniques and procedure followed might be underestimated, as suggested by the rarefaction analysis of the 16S rRNA genes clone libraries (Chapter 3, section 3). Despite existing biases due to sampling and the employed analytical procedures, the fingerprints of the microbial community (i.e. DGGE or T-RFLP) provided meaningful information on the presence and relative abundance of the species over both spatial and temporal scales and reflected biodiversity, which may in turn mirror environmental quality at chlorinated solvents contaminated sites. A thorough exploration of how different groups of variables forming complex data sets, including microbial fingerprinting, hydrogeochemical and hydrological variables, interact in heterogeneous system requires mathematical tool. For instance, an evaluation of the correlation and the respective contribution of the data sets to the overall variation was efficiently carried by means of multivariate statistical methods, namely a multiple factor analysis (MFA) (Chapter 3, section 3) (Escofier & Pages, 1994; Legendre & Legendre, 1998). However, it is likely that the inherent complexity and scale of aquifer systems will often limit the ability to precisely assess and predict the relationship between groundwater bacterial community structure and environmental characteristics.

Though contaminant concentrations were the most critical environmental characteristics influencing microbial community structures at the Bitterfeld/Wolfen site, other environmental variables may play a critical role. These variables may not have been directly revealed as particularly explanative given the characteristics of the site, the limits of the sampling methods and the measurement procedure applied in the studies. However, physical heterogeneity has a major impact on the pattern of transport and distribution of contaminants and other reactants, which in turn may affected the biocenoses. This phenomenon was observed across a vertical profile in the groundwater system, where the spatial variability of the hydrochemical and hydrological properties reflected the contaminant concentrations distribution and partly correlated with the variation of the microbial community structures (Chapter 3, section 2). However, the respective impact of contaminant *versus* geochemical or hydrological factors can hardly be separated under field conditions and is likely to change from site to site and over time. In the model wetland, nMDS analysis of bacterial community structures revealed that structural changes over time coincided with major changes of the hydrogeochemical conditions and TEAPs, although the DCE degrading function was maintained over time (Chapter 2, section 3). This study also revealed that substantial changes of the microbial community structures coincided with the detection of putative members of the dechlorinating guild. Changes at the biocenose level were in turn associated with a progressive change of the DCE degradation mechanism, that evolved towards prevaling reductive dechlorination at the end of the investigation period (Chaper 2, section 2 and 3).

Hence, a detectable response of the “total” bacterial community to specific environmental factors also emphasizes that spatial and temporal distribution of contaminant concentrations or other reactants should be cautiously considered when assessing *in situ* biodegradation in aquifers. Indeed, changes of microbial community structures may also mirror variations at the guild or population levels, which may in turn significantly influence degradation of chlorinated solvents, as shown in the model wetland (Chapter 2, section 3). At the investigated site, contaminant concentrations and other factors may affect the entire biocenose or specific organisms only. As groundwater organisms share complex ecological interactions, a direct impact on one species or guild may then induce indirect effect on other part of the microbial community. However, some numerically minor guilds of microorganisms can be essential for degradation processes. In this case, multivariate analysis of the relationship between overall bacterial DGGE profiles and hydrogeochemical factors and isotope signatures had limited value for identifying more specific microbial communities changes in system contaminated with chlorinated solvents.

1.5. Dehalogenating guilds in heterogeneous systems contaminated with chlorinated ethenes

In strictly anaerobic zones of the investigated aquifers and wetland, chlorinated ethenes degradation was assumed to be performed *via* reductive dechlorination by a specific guild of dehalogenating bacteria, and/or by less-known anaerobic metabolic or co-metabolic oxidising microorganisms. Therefore, taxon-specific assays targeting putative dechlorinating bacteria were systematically carried out.

As discussed in the section 3 of chapter 3, factors such as intra- and inter-species competition, presence or absence of specific microhabitat, nutrient flux and transport properties at different scales may have concomitantly influenced both the bacterial community structures and the dechlorinating guild. These factors may substantially affect the diversity, distribution and activity of members of the dechlorinating guild (Chapter 2, section 3; Chapter 3, section 3). Though changes in bacterial community structures occurred in the investigated groundwater system without loss of functionality with respect to contaminant degradation, hydrochemical and hydrogeological factors could explain the inter-dependent variability of both the spatial distribution of dechlorinating microorganisms and chlorinated ethenes degradation reactions (Chapter 3, section 3). In particular, important changes in the distribution of the putative dehalogenating bacteria could be observed along a vertical profile and could be linked to the movement of the contaminant plume (Chapter 3, section 3). Furthermore, studying the distribution of dehalogenating bacteria at the plume scale suggested that processes governing the flow and distribution of H_2 may be critical and indicative of the extent and sustainability of *in situ* biodegradation at the investigated area, whereas high sulphate and ferrous iron concentrations may hinder the constitution of a stable dehalogenating bacterial guild (Chapter 3, section 2). In this respect, the systematic measurements of H_2 and/or fermentable substrates such as lactate, propionate and butyrate for monitoring biodegradation of chlorinated solvents may give better insight into the *in situ* ecology of dehalogenating bacteria. If ambient conditions remain relatively stable, a complex dehalorespiring guild may then develop at certain location or time periods into robust consortia. This has been observed at the model wetland treating DCE where an anoxic milieu was progressively established, coinciding with both the detection of putative dehalogenators and evidences of reductive dechlorination activity at the system scale over 200 days (Chapter 2, section 3). In a stable dechlorinating guild, changes of conditions may hinder one member of the guild, but this loss may be compensated by another one to fill its role and keep the overall community functioning (Duhamel & Edwards, 2006).

Understanding of factors influencing the efficiency of dechlorinating consortia, and thus controlling the accumulation of certain chlorinated solvents at contaminated systems would require further knowledge about i) ecological factors influencing the degrading guild composition and distribution, ii) the environmental -probably largely underestimated- diversity of dehalorespiring microorganisms (Kittelmann & Friedrich, 2008), and iii) associated non-dechlorinating organisms. This thesis mainly tackles the first point, but also underscores the potential relevance of not known dechlorinating bacteria, in particular non-*Dehalococcoides Chloroflexi* that may be involved in the observed degrading activities (Chapter 2, section 3 and Chapter 3, section 3). The involvement and interaction of multiple populations in complex microbial communities appears essential for dechlorination under field conditions but also complicate the identification of key dechlorinating microorganisms (Richardson *et al*., 2002). Therefore, understanding the functioning of a dehalogenating guild would require information on the parallel *in situ* activity of organisms that may also control the extent of reductive dechlorination by transforming complex organic molecules to simpler ones or to H_2, or by providing specific trace nutrients to dehalorespirers. Laboratory mixed culture set-ups including various levels of complexity with respect to the number of dechlorinating strains and/or nondechlorinating organisms of interest, such as fermenters, acetogens, and methanogens, are thus of interest for gaining insights into the interaction between organisms and how these interactions influence degradation parameters such as rate or apparent isotopic fractionation.

2. Implications

The development of a thorough assessment strategy relying on different but complementary methods has been motivated by the need of developing robust, integrative and holistic concepts encompassing multiple lines of evidence for natural attenuation of chlorinated solvents at contaminated complex subsurface environments (US-EPA, 1999; McKelvie *et al*., 2007). This appears particularly relevant in the case of chlorinated solvents contaminated sites, as the intermediate dechlorination metabolites are usually more toxic than their parent compounds and can be degraded under various environmental conditions. However, the use of natural attenuation as part of a possible remediation approach depends on the defined remediation goals that differ from one country to another. In Germany, if compliance criteria are exceeded, remediation measure must be undertaken, as groundwater and soil itself are regarded as the direct receptor of the contamination (Rügner *et al*., 2006). Several concepts were applied in this thesis, namely the elucidation of DCE degradation pathways in complex systems, the isotope balance for the evaluation of reductive sequences and the use of tracer experiments with further microbial cultivation, and can be further used for investigating of the environmental fate of various recalcitrant organic chemicals. However, quantitative estimation of *in situ* biodegradation of chlorinated solvents and relative contribution of co-occurring degradation pathways require further fundamental investigations.

Data reduction and spatiotemporal integration represents a crucial step for both process interpretation and long-term performance monitoring to make data readily accessible to regulators and stakeholders (WSRC-STI-2006-00084, 2006). The approach followed in this thesis illustrated the benefit of ordination methods and multivariate statistics to analyze spatial and/or temporal changes of the hydrogeochemical and microbial components when assessing natural attenuation processes. These different developments, taken separately or in combination, may then provide valuable support and enlarge the available tools for evaluating chemicals for regulatory framework such as Registration, Evaluation and Authorisation of Chemicals (REACH, EC Regulation n°(1907/2006, 2006) recently developed in Europe.

Moreover, the approach may also be of interest to monitor the implementation of active remediation measures at chlorinated contaminated sites and systems (e.g. biostimulation, bioremediation or chemical treatments). The results of this thesis particularly underscore that concepts and methods currently used when assessing Natural Attenuation to provide quantitative and/or qualitative information about the reactive transport processes of contaminants in groundwater systems can be conveniently transferred and implemented in natural or engineered wetland studies and monitoring. The use of an integrative approach can complement the traditionnal assessment based on mass balance and budget calculations between the influent and the effluent water, and thus improve knowledge about the behaviour of organic chemicals in natural and engineered wetland systems.

From a research perspective, the use of an integrative approach to investigate hydrochemical and hydrological dynamic groundwater-surface water systems may permit to better address the linkage between environmental factors and *in situ* biodegradation of chlorinated solvents intermediates. Groundwater-surface water interfaces are relevant settings for evaluating the response of the biocenose as a function of a large spectrum of ambient conditions. The approach can be refined and adapted in the future to benefit from the developments of the respective fields from which its different components derive.

3. Outlook

Isotope analysis. The studies demonstrated that CSIA is a relevant component in integrative assessment approach targeting *in situ* biodegradation activity at site contaminated with chlorinated ethenes and benzenes. Further characterization of the prevailing degradation pathway in aquifers or wetlands can include the isotope signatures of more than one element to retrieve correlation patterns and identify degradation processes (Rosell *et al.*, 2007; Fischer *et al.*, 2007). For instance, the parallel measurements of the isotope composition of carbon and chlorine may permit to derive more accurate information on specific reaction mechanisms, and thus decrease the uncertainty related to both pathway identification and estimation of the relative contribution of ongoing pathways in hydrological characterized wetlands or aquifers. Recent progress in the measurements of the isotope signature of chlorine in chlorinated ethenes (Shouakar-Stash *et al.*, 2006; Sakaguchi-Soder *et al.*, 2007) permits to already foresee the routine use of a dual, carbon-chlorine approach to investigate the environmental fate of chlorinated solvents. Additionally, a better documentation of oxidative degradation pathways of dehalogenated compounds such as DCE, VC and CB at oxic-anoxic interface areas by means of CSIA requires reference experiments to i) clarify the specific degradation mechanism involved in these types of reactions, and ii) investigate the associated isotope fractionation patterns. This also underlines the interest of isolating microbial strains oxidizing chlorinated solvents in order to gather eco-physiological and genetic knowledge about such process. Furthermore, other processes than degradation, such as volatilization in wetlands or hydrophobic sorption in sediment organic matter rich aquifers, may contribute to significant contaminant removal. Though few studies have showed that non-destructive processes would not significantly affect the isotope composition of chlorinated solvents, a closer look on the influence of such processes on the isotope fractionation patterns of chlorinated ethenes and benzenes is required to develop further the concept of isotope fractionation of organic contaminants during biodegradation to monitoring *in situ* biodegradation in both wetlands and aquifers.

Microbial analysis. In parallel to the assessment of *in situ* degradation activity and pathways by means of CSIA, thorough microbial culture-independent investigations performed at particular discrete-intervals or time periods provided meaningful information. Recent development of 16S rRNA-targeted techniques may allow more rapid and reliable proof of the presence of the target sequence, while providing the pattern code for further taxonomic classification of detected sequence of specific dechlorinating microorganisms. For instance, multiplex hybridization and primer extension assays with several primers is promising

(Nikolausz et al., 2007; ANNEX C), and can be routinely applied to characterize *Dehalococcoides* sp. populations in contaminated aquifers or wetland samples in the framework of an integrative characterization. In parallel, functional genes can be concomitantly targeted for investigating relevant metabolic capabilities. For instance alkene monooxygenase genes, that are apparently well conserved, can be targeted in attempts to detect vinyl chloride-degraders at anoxic groundwater-oxic surface water interfaces (Mattes *et al.*, 2005) and investigate their ecology. Furthermore, assessment of chlorinated ethene-impacted sites often relies on the quantification of *Dehalococcoides* DNA or RNA biomarkers (i.e., gene transcripts) based on PCR-based tools targeting reductive dehalogenase genes implicated in chlorinated ethenes reductive dechlorination (i.e., *tceA*, *bvcA*, and *vcrA*). However, those tools are inherently limited in their capacity to identify novel dehalorespiring microorganisms as they rely on target gene sequences of known cultivated species. Therefore, the use of stable isotope probing techniques (DNA- or RNA-SIP) (Kreuzer-Martin, 2007; Neufeld *et al.*, 2007) can be relevant to study microbial community members involved in the metabolic aerobic or anaerobic oxidation of chlorinated solvents, or in reductive dechlorination using ^{13}C-labelled electron donors in experiment settings based on wetland or aquifer pore water or sediment samples. Microbial analyses achieved in this thesis were based on liquid phase samples alone, which might have provided an incomplete view of the systems microbiology (Lehman, 2007). Indeed, the liquid phase is generally less heterogeneously distributed than the solid material, and specific populations or activities may be asymmetrically distributed between the pore water and the sediment phases (Lehman & O'Connell, 2002). Although in routine monitoring sediments samples can not be systematically collected, partitionning of dechlorinating guild members and their activity between suspended and sediment is worth to be investigated.

Conceptual outlook. The concept of biogeochemical hot spot and hot moment (McClain *et al.*, 2003) can be applied to address the relationships between the development of biogeochemical processes and microbial biocenose activity within contaminated terrestrial-aquatic environments across multiple scales. Biogeochemical hot spots are patches that show disproportionably high reaction rate relative to the surrounding matrix, whereas hot moments are defined as short period of time that exhibit disproportionably high reaction rates relative to longer intervening time periods. In this thesis, a hot moment with respect to DCE degradation was observed in the model wetland at the beginning of the investigation, when the highest contaminant removal performance occurred (Chapter 2, section 2). Hot spots were encountered, for example, at some discrete intervals within the model wetland during the oxidative phase (Chapter 2, section 2), or at depth 8 in the multilevel monitoring well (Chapter 3, section 2) that displayed particularly higher degradation activity compared to other points of the investigated system in terms of contaminant mass removal, production of degradation metabolite and/or relative shift in the isotope signature. A better mechanistic understanding of such reactive spatial or temporal points with respect to transport and/or availability of limiting reactants may shed light on factors controlling the feasibility and extent of *in situ* chlorinated solvents degradation events. The biogeochemical development of such points can be followed over different scales by means of an integrative approach. The relationships between aquifer or wetland biological information (i.e. diversity patterns) and biogeochemical sets of data can be explored and jointly interpreted by numerical analysis (i.e. bioinformatics, biostatistics, modeling). Biogeochemical hot spots commonly occur at the boundary between two structural features (i.e. ecotone), at a broad spectrum of structural patterns, ranging from the micro-scale to the landscape. Hence, wetland systems engineered for the treatment of groundwater organic chemicals can be considered as artificial biogeochemical hot spots, in which reactants should be combined under ideal thermodynamic conditions for the purpose of water treatment over time. These systems also represent models to investigate natural processes and to predict the spatiotemporal distribution of hot events within natural wetlands. Reciprocally, the delineation and functional understanding of natural hot spots and hot moments in natural system at micro-, meso- and macro-scales may also permit optimizing treatment conditions within artificial systems. This brings-up, however, challenging issues related to the uncertainty associated with the up- or down scaling of the information gathered at one scale and to the comparison of methodologies employed at different scales to assess characteristics of hot biogeochemical spots and moments.

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**Corresponding author*

Conference proceedings

Oral presentations

Imfeld G., Estop C.A., Zeiger S., Richnow H.H. Assessment of in situ biodegradation of chlorinated ethenes using an integrative approach –Application to a model constructed wetland. AXIOM-VIBE-eTRAP Workshop *"Electron transfer processes at biogeochemical gradients"* from 4-7 March 2008 in Leipzig, Germany.

Imfeld G., Zeiger S., Estop C.A. , von Eckstädt C.V., Weber S., Richnow. Assessment of in situ biodegradation of *trans*- and *cis*-dichloroethene in groundwater treated in a laboratory-scale constructed wetland. 2nd International Symposium on Wetland Pollutant Dynamics and Control, WETPOL 2007. September 16-20 2007, University of Tartu, Tartu, Estonia.

Imfeld G., Nijenhuis I., Nikolausz M., Weber S., Richnow H.. Assessment of in situ degradation of chlorinated ethenes and bacterial community structure in a complex contaminated groundwater system. International Workshop on „Biogeochemical Processes in Groundwater Systems. September 10-11, 2007, GSF-National Research Center for Environment and Health, Munich, Germany.

Imfeld G., Nijenhuis I., Nikolausz M., Cichocka D., Köth A., Zeiger S., Weber S., Richnow H.H. Stable isotope fractionation indicates *in situ* biodegradation of chlorinated ethenes in an anaerobic aquifer (Bitterfeld, Germany). 29th Annual Meeting of the GASIR – German Association for Stable Isotope Research. October 4 – 6, 2006, Freiberg, Germany.

Brackevelt M., Rokadia H., **Imfeld G.**, Stelzer N., Weber S., Kuschk P., Richnow HH. (2006) Assessment of *in situ* biodegradation of monochlorobenzene in contaminated groundwater treated in a soil filter. 10th International Conference on Wetland System for Water Pollution Control. September 23-29, 2006, Lisbon, Portugal.

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Posters

Pieper H., Richnow H.H., Paschke H., **Imfeld G.**. Variations of *in situ* biodegradation of chlorinated ethenes and bacterial community dynamics along a complex vertical profile. AXIOM-VIBE-e-TRAP-HIGRADE Workshop: Electron Transfer at Biogeochemical Gradients. March 4th-6th, 2008, Leipzig, Germany.

Estop C.A., Zeiger S., Weber S., Richnow, **Imfeld G**. Assessment of *in situ* biodegradation of *trans*- and *cis*-dichloroethene in groundwater treated in a laboratory-scale constructed wetland. Summer School Chemicals in Soil: Interactions, Availability and Residue Formation. September 24-27, 2007, Leipzig, Germany. ***Public Best Poster Award.***

Imfeld G., Nijenhuis I., Nikolausz M., Weber S., Zeiger S., Richnow H. (2007). An integrated approach to assess in situ degradation of chlorinated ethenes in several geological units of a groundwater system. European Geosciences Union (EGU) General Assembly 2007. 15 – 20 April 2007, Vienna, Austria.
Biogeoscience Young Scientists' Outstanding Poster Paper (YSOPP) Award 2007.

Imfeld G., Nikolausz M., Nijenhuis I., Zeiger S., Köth A., Richnow H.H., Weber S. (2006). Assessment of the natural attenuation of chlorinated ethenes in an anaerobic contaminated aquifer. International Symposium on Environmental Biotechnology. July 9-13, 2006, Leipzig, Germany.

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Kabelitz N., Fischer J., **Imfeld G.**, Heipieper H.J. (2007). Monitoring bioremediation processes by using lipids as biomarkers. Annual Conference of the Association for the General and Applied Microbiology. April 1-4, 2007, Osnabrück, Germany.

Kabelitz N., Fischer J., **Imfeld G.**, Heipieper H.J. (2006). New tools to monitor bioremediation in soil – Application of membrane lipids as biomarkers. 2nd FEMS Congress of European Microbiologists – Integrating Microbial Knowledge into Human Life. July 4-8, 2006, Madrid, Spain.

Curriculum vitae

Gwenaël IMFELD
Born: May 6, 1979 in Geneva
Single
Swiss and French nationality

Educational background

2005-2008	**Ph.D. thesis in Isotope Biogeochemistry** at the Department of Isotope Biogeochemistry of the Hemholtz Centre for Environmental Research – UFZ, Leipzig, Germany. Framework : **EU** Marie Curie Host fellowship for Early Stage Research Training
2003-2005	**Master of advanced studies in Hydrology** and Water Resources Management at the Swiss Federal Institutes of Technology (EPFL), (ETHZ) and the Centre of Hydrogeology of the University of Neuchâtel (CHYN). Framework : Collaboration **EPFL** and **Indian Institute of Technology** (IIT)-Madras
1998-2003	**Master in Biology** at the University of Neuchâtel (Switzerland). Framework : **Indo-Swiss** collaboration in **biotechnology** (ISCB)

Professional experience

Since 10/2008	Post-doc	Laboratory of Hydrology and Geochemistry - Strasbourg (LHyGeS), UMR 7517, Ecole National du Génie de l'Eau et de l'Environnement Strasbourg (ENGEES)
2004-2007	Fund raiser and administrator	*Helvetas* (Swiss NGO for development and cooperation): project "Promotion of Organic Farming and Marketing in Lao PDR" (Laos); (300´000 Euro)
01/2005-04/2005	Scientific collaborator	Laboratory of Hydrology and Planning (HYDRAM), Federal Polytechnical School (EPFL), Lausanne
2002-2003	Instructor	Practical courses in microbiology at the University of Neuchâtel
09/2000-09/2001	President of a student association	Section of biology, University of Neuchâtel
07-09/1998-2000	Hospital helper	Department of psychiatry, University hospital, Geneva

Extra-curricular activities

Sport (trekking, running, badminton), botany, vice-presidency of a nature protection association (le Môle, Haute-Savoie, France), history

Annexes

ANNEX A: Factors controlling the carbon isotope fractionation

ANNEX B: Variability of carbon isotope fractionation

ANNEX C: Single-nucleotide primer extension assay

ANNEX D: Biodegradation of chlorobenzene in a constructed wetland