HELMHOLTZ
CENTRE FOR
ENVIRONMENTAL
RESEARCH – UFZ

PhD Dissertation 08/2010

Molecular biological, physiological and isotope chemical analyses of BTEX (bio)degradation processes within a contaminated aquifer

Steffi Herrmann

ISSN 1860-0387

Molecular biological, physiological and isotope chemical analyses of BTEX (bio)degradation processes within a contaminated aquifer

By the Faculty of Chemistry and Physics
of the Technische Universität Bergakademie Freiberg

approved

THESIS

to attain the academic degree of
Doctor rerum naturalium
Dr. rer. nat.

submitted

by Diploma Geoecologist Steffi Herrmann
born on the 16.06.1980 in Dresden

Assessors: Prof. Dr. Michael Schlömann, Freiberg
Prof. Dr. Lorenz Adrian, Berlin
Dr. Hans-Hermann Richnow, Leipzig

Date of the defence: 28.05.2010

Supervisors:

Dr. Carsten Vogt, UFZ, Leipzig
Dr. Hans-Hermann-Richnow, UFZ, Leipzig
Prof. Dr. Michael Schlömann, TU Freiberg

Abstract

The BTEX compounds comprise benzene, toluene, ethylbenzene, and xylene and are the most abundant organic groundwater pollutants. They are of major concern due to their mobility and toxicity. These aromatics are known to be degraded by microorganisms under aerobic and anaerobic conditions. However, little is known about the mechanisms of the anaerobic degradation of BTEX compounds, in particular the structure and function of microbial communities involved in the anaerobic degradation of benzene. The aim of this thesis was to investigate the anaerobic degradation of benzene and other BTEX compounds on a structural and functional (metabolic) basis.

One reason for the sparse knowledge regarding anaerobic benzene degradation is the lack of available cultures. Benzene-degrading microbial communities were directly enriched within a BTEX-contaminated sulphidogenic aquifer using *in situ* microcosms filled with different solids. These *in situ* microcosms were further incubated in the laboratory and stable growing benzene-degrading sulphate-reducing batch cultures developed depending on the presence of the solids sand or pumice.

Microbial key players involved in anaerobic benzene mineralisation were identified by means of DNA-stable isotope probing (SIP). ^{13}C-label incorporation was detected in DNA of two phylotypes within the consortium: a phylotype affiliated to the family *Peptococcaceae* (genus *Cryptanaerobacter/ Pelotomaculum*), the other belonging to the ε-*Proteobacteria*. The results strongly indicate that benzene is syntrophically mineralized by those two phylotypes and sulphate-reducing organisms, whereas the *Peptococcaceae* phylotype is assumed to carry out the first steps of benzene degradation.

The initial reaction of different benzene degradation pathways (ring activation under anoxic conditions or catalyzed by a monooxygenase or dioxygenase) was investigated in laboratory experiments using reference cultures. The respective biodegradation pathway was deciphered by using two-dimensional compound specific isotope analysis (2D-CSIA). Correlation of hydrogen and carbon isotope effects ($\Lambda = \Delta_H/\Delta_C \approx \varepsilon_H/\varepsilon_C$) yielded values that were unique for distinct initial benzene activation mechanisms. Specific Λ values were < 2 for dihydroxylation and between 3 and 11 for monohydroxylation as initial benzene-attacking mechanism. Under anaerobic conditions Λ values were about 16 for degradation under nitrate-reducing conditions, and > 22 for degradation under sulphate-reducing and methanogenic conditions.

The initial reaction for anaerobic alkylbenzene degradation catalysed by benzylsuccinate synthase (Bss) was investigated by 2D-CSIA. Different pure and mixed cultures able to degrade *m*-xylene, *o*-xylene, or *p*-xylene under sulphate- or nitrate-reducing conditions were analysed. Λ values differed significantly for the analysed cultures and ranged between 12 ± 4 and 29 ± 5, indicating slightly different reaction mechanisms for Bss catalysed reactions. A similar variation had been observed for Bss catalysed toluene activation with Λ values of 4 ± 3 for a phototrophic organism, 11 – 14 for nitrate-reducing organisms and 28 – 31 for sulphate-reducing organisms.

Isotope geochemical tools and *in situ* SIP techniques developed within the PhD thesis were applied to evaluate an air-sparging trail located at the source zone of a BTEX-contaminated aquifer. Within the BMBF founded project BEOQUE air-sparging was performed to study the enhancement of natural attenuation processes with regard to benzene removal, in particular processes of microbial degradation. Monitoring of removed benzene concentrations revealed that air-sparging is not sufficient under the given hydrogeochemical conditions of the aquifer. Several methods were used for monitoring biodegradation. MPN and isotope fractionation analyses gave evidence for microbial degradation. Experiments with Bactraps exposed to the aquifer by direct push technique indicated that 0.8 to 6% of the overall removed benzene was biodegraded.

Contents

1

Introduction

1.1 BTEX contamination

Hydrocarbons are the most common class of low molecular carbon compounds in nature. They comprise the most important energy sources for humans and serve as major resources for the chemical industry (crude oil, natural gas, coal, methane hydrates). They are synthesised either by geochemical processes of organic material or by biological activities. Hydrocarbons constitute a group of compounds with high structural diversity. They include (i) saturated compounds (aliphatic and alicyclic alkanes), (ii) compounds containing C – C double-bonds (alkenes), (iii) compounds with C – C triple-bonds (alkynes), and (iv) mono- and polycyclic aromatic hydrocarbons. Details regarding the chemistry of these compounds can be found in most organic chemistry textbooks (Streitwieser and Heathcock 1980; Morrison and Boyd 1986).

Since the beginning of the industrial revolution within the 18th century and the exploitation of petroleum hydrocarbons, huge amounts of those compounds entered environmental spheres due to careless handling during production, transportation and application, improper waste disposal, spills, and leakages.

In ecological terms, many hydrocarbons are precarious due to properties like persistence, toxicity and spreading with groundwater. Aromatic hydrocarbons, in particular BTEX compounds (benzene, toluene, ethylbenzene, and xylene, Figure 1.1) are of major concern. They are used as solvents and petroleum-constituents and are major educts for the chemical industry. Due to high production rates they are among to the most common environmental contaminants of soils and groundwater. BTEX compounds have relatively high water solubility. Hence, spills that enter aquifers and get in contact with groundwater can dissolve and form huge contamination plumes.

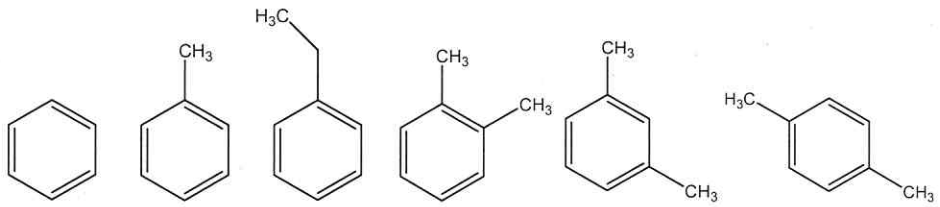


Figure 1.1: Structural formulas of BTEX compounds: benzene, toluene, ethylbenzene, *ortho*-, *meta*-, and *para*-xylene.

BTEX compounds are toxic substances. Toluene and xylenes can damage the central nervous system, kidneys, liver and fertility (toluene) or cause nausea and headaches (xylenes) (see MAC and TRGS values in Table 1.1). Additionally, toluene can enhance carcinogenesis by other compounds (Dean 1978). Benzene presents the highest risk to human health, as it is deemed to cause leukaemia and other mutagenic disorders (Angerer 1983; US-EPA 1998). These properties make it necessary to understand their behaviour within aquifers.

Aromatic compounds are ubiquitous in the environment. Next to the glycosyl moiety the aromatic ring is the most common structure in nature (Dagley 1981). With evolution, microorganisms could develop abilities to utilise BTEX compounds, although those compounds are chemically very stable.

Table 1.1: Properties of BTEX compounds (van Agteren *et al.* 1998).

name	molecular-weight [g/mol]	density [kg/l]	T_m [°C]	T_b [°C]	vapour-pressure [kPa]	water-solubility [mg/L]	Henry's law constant [Pa m^3/mol]	log $K_{o/w}$ (-)	MAC-value [mL/m^3]
benzene	78.1	0.878	5.5	80.1	10.13	1780	547	2.13	1*
toluene	92.1	0.867	-95	110.8	2.93	515	669	2.65	50
ethylbenzene	106.2	0.867	-95	136.2	0.93	152	588	3.20	n.s.
o-xylene	106.2	0.880	-25	144.4	0.67	175	496	2.95	100
m-xylene	106.2	0.864	-48	139.0	0.80	200	699	3.20	100
p-xylene	106.2	0.860	13	138.4	0.87	198	709	3.18	100

density, vapour pressure, water solubility are referred to 20°C; T_m melting point, T_b boiling point; $K_{o/w}$ distribution constant of a substance within octane and aqueous phase; MAC maximum concentrations at the workplace; *technical rules for hazardous substances (TRGS 1997); n.s. not specified

The first reports documenting the microbial degradation of aromatic compounds were published early in the last century (Söhngen 1913). Since the 1960s many investigations were conducted to elucidate biodegradation of BTEX compounds regarding physiology, biochemistry and molecular biology (Hopper 1978; Gibson and Subramanian 1984; Dagley 1986; Smith 1990). Prior to the 1980s, microbial hydrocarbon catabolism studies were all performed under aerobic conditions and the accepted paradigm at that time was that anaerobic hydrocarbon degradation by microorganisms was "negligible" (Atlas 1981).

This changed in 1985 with a publication of Kuhn *et al.* (1985). This study confirmed the existence of microorganisms able to degrade BTEX compounds (dimethylbenzenes) in the absence of oxygen. The organisms used nitrate as electron acceptor instead.

Within aquifers contaminated with organic compounds, aerobic degradation processes quickly deplete available oxygen, thus the majority of contaminants will need to be degraded anaerobically without the utilisation of oxygen. Hence, since the first publication there has been a significant increase in the number of studies investigating anaerobic degradation processes, but they are by far still not as well understood as aerobic processes.

1.2 Aerobic degradation of BTEX compounds

Since the 1960s, a large number of aerobic organisms (esp. *Pseudomonas* species) has been cultivated and isolated from environmental samples which are able to degrade BTEX compounds using them as electron and/or carbon source. Intensive studies have been performed with those isolates to investigate biochemical processes of aerobic degradation. The initial attack of benzene is catalysed by mono- and dioxygenases incorporating oxygen into the aromatic ring (Gibson and Subramanian 1984). Dioxygenases use both oxygen atoms of the molecule to hydroxylate the ring twice, thus forming *cis*-diols. Oxidised co-factors (NAD^+ or $NADP^+$) are used to cleave two hydrogen atoms from

those diols to form catechols (Figure 1.2D). Monooxygenases use only one oxygen atom and catalyse the formation of monohydroxylated aromatics (phenols) (Figure 1.2A).

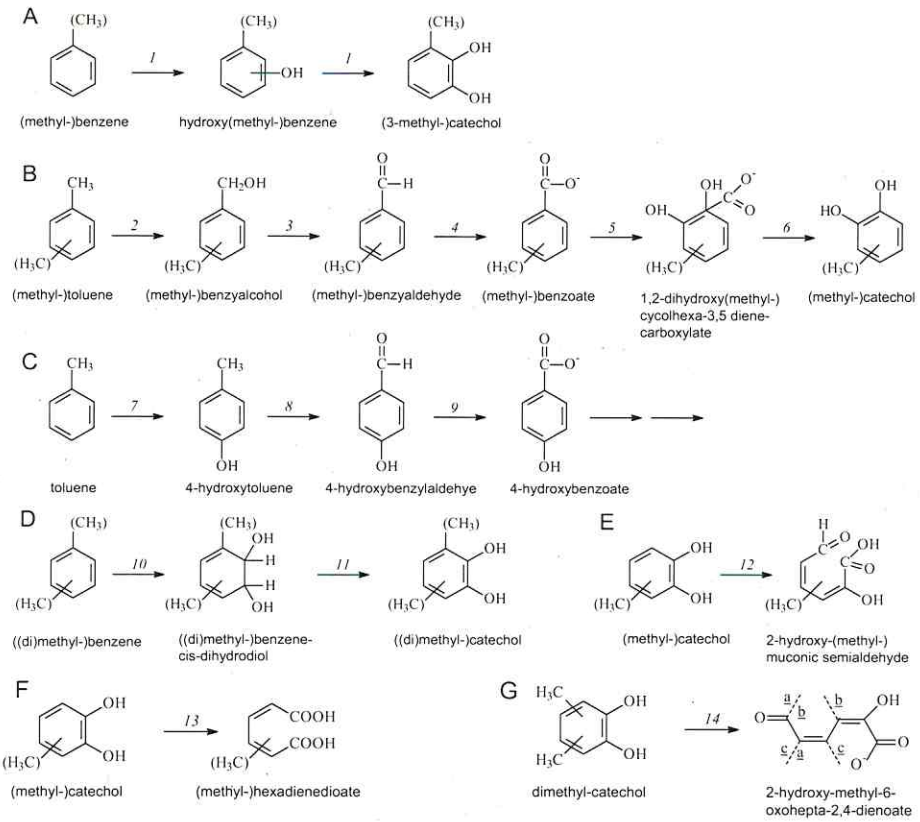


Figure 1.2: A-D) Initial enzymatic (*1, 2, 7, 10*) steps of aerobic degradation of BTEX compounds by A) ring-monooxygenases (*1* = *e.g.* T2MO, T3MO) in *e.g. Ralstonia pickettii* PK01, *Burkholderia cepacia* G4; B) side-chain-monooxygenases (*2* = *e.g.* xylene-monooxygenase, toluene-side-chain-monooxygenases) with subsequent oxidation steps catalysed by benzylalcohol dehydrogenases (*3*), benzylaldehyde dehydrogenases (*4*), benzoate-1,2/toluate dioxygenases (*5*), and 1,2 dihydroxy-(methyl-)cyclohexa-3,5 diene-carboxylate dehydrogenase (*6*) in *e.g. Pseudomonas putida* mt-2, *Pseudomonas* Pxy, *Sphingomonas yanoikuyae* B1; C) ring-monooxygenases (*7* = *e.g.* T4MO) with subsequent oxidation steps catalysed by 4-cresol dehydrogenases (*8*), and 4-hydroxybenzaldehyde dehydrogenases (*9*) in *P. mendocina* KR1; D) ring-dioxygenases (*10*) and subsequent catalysis by a dihydrodiol dehydrogenase (*11*) in *e.g. P. putida* F1, *P. fluorescens* CFS215, *B.* sp. strain JS150. E-G) illustrates ring-cleavage steps of key intermediates, catechols, of aerobic degradation by E) catechol-2,3-dioxygenases (*12*, *meta*-cleavage), F) catechol-1,2-dioxygenase (*13*, *ortho*-cleavage), G) catechol-2,3-dioxygenases (*14*) resulting in products with different locations of methyl-groups, detected during a and c) *o*-xylene and b) *p*-xylene degradation by *Rhodococcus* sp. strain Yu6. (Davey and Gibson 1974; Worsey and Williams 1975; Gibson *et al.* 1990; Shields *et al.* 1991; Whited and Gibson 1991; Haigler *et al.* 1992; Mikesell *et al.* 1993; Olsen *et al.* 1994; Jørgensen *et al.* 1995; Zylstra and Kim 1997; Jang *et al.* 2005).

Two possible reaction mechanisms for the initial attack are proposed incorporating activated oxygen either between a C – H-bond, or between a C – C-bond by formation of epoxides and subsequent reorientation. Catechols are formed afterwards by the same enzymes. These key intermediates of aerobic aromatics degradation are subsequently cleaved by ring-dioxygenases either between hydroxylated carbons (1, 2-dioxygenases, intradiol- or *ortho*-cleavage) or at a bond between one hydroxylated and one non-hydroxylated carbon (2, 3-dioxygenases, extradiol- or *meta*-cleavage) (Figure 1.2E-F). During the *ortho*-cleavage pathway acetyl-CoA and succinic acid are formed via *cis, cis*-muconic acid. Both subsequently enter the tricarboxylic acid cycle. The *meta*-cleavage pathway leads via 2-hydroxymuconononic semialdehyde to pyruvate and acetaldehyde which are also intermediates of the tricarboxylic acid cycle.

Aerobic degradation of toluene, ethylbenzene, and xylenes can analogously be initialised by ring-hydroxylating mono- and dioxygenases (Figure 1.2A, D). Additionally, those compounds can be attacked at the side chain (Figure 1.2B, C) by successive oxidation of the alkyl-aromatics to the corresponding alcohols, aldehydes and carbonic acids ('upper' pathway). During the 'lower' (or *meta*) pathway the carbonic acids are further transformed to catechols, which are subsequently degraded to pyruvate and acetaldehyde (Ramos *et al.* 1987; Burlage *et al.* 1989; Smith 1990).

1.3 Anaerobic degradation of BTEX compounds

Since the first published study in 1985 by Kuhn *et al.*, numerous investigations have been performed focusing on anaerobic degradation of BTEX compounds (Schink *et al.* 1992; Heider *et al.* 1999; Spormann and Widdel 2000; Phelps and Young 2001; Widdel and Rabus 2001; Chakraborty and Coates 2004; Heider 2007; Foght 2008; Fuchs 2008). Under anaerobic conditions nitrate, iron, manganese, sulphate and carbon dioxide can serve as terminal electron acceptors. Most extensively studied is the anaerobic degradation of toluene among the BTEX compounds. A large number of organisms have been isolated which are able to mineralise toluene under anoxic conditions, *e.g. Thauera*- and *Azoarcus*-species as nitrate-reducers (Dolfing *et al.* 1990; Evans *et al.* 1991a), *Geobacter metallireducens* as iron (III)-, manganese(IV)- and nitrate-reducers (Lovley *et al.* 1989; Lovley and Lonergan 1990; Coates and Lovley 2003), and species of δ-*Proteobacteria* as well as Gram-positives belonging to the genus *Desulfotomaculum* as sulphate reducers (Rabus *et al.* 1993; Beller *et al.* 1996; Harms *et al.* 1999b; Morasch *et al.* 2004b). Liu *et al.* (2004) showed that a Gram-positive organism, 97% similar to *Desulfosporosinus meridiei*, is able to link toluene degradation to arsenate reduction. Also, several mixed cultures were able to degrade toluene under methanogenic conditions (Grbic-Galic and Vogel 1987; Edwards and Grbic-Galic 1994; Washer and Edwards 2007). See Table 1.2 for organisms that have been isolated to date able to degrade BTEX compounds under anoxic conditions and the respective electron acceptors.

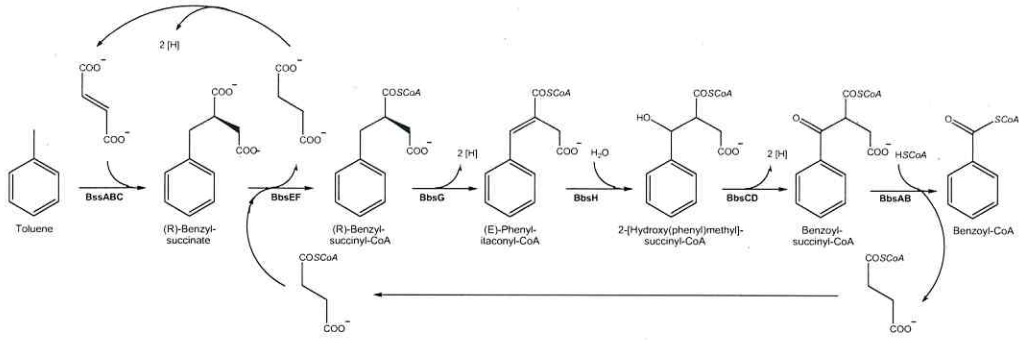


Figure 1.3: Pathway of anaerobic toluene degradation within the denitrifying bacterium strain EbN1 according to Kube *et al.* (2004). BssABC, benzylsuccinate synthase; BbsEF, succinyl-CoA:(R)-benzylsuccinate CoA-transferase, BbsG, (R)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase; BbsAB, benzoyl-succinyl-CoA thiolase.

The biochemical pathway of toluene degradation has been basically elucidated by studies examining toluene-degrading *Thauera* and *Azoarcus* species (see Figure 1.3). Benzylsuccinate synthase (BssABC) catalyses the initial addition of fumarate to the methyl group forming the intermediate benzylsuccinate (for an overview see Heider 2007). More precisely, BssABC contains a stable glycyl radical which is proposed to initially abstract a hydrogen atom from a neighbouring cysteine residue to form a reactive thiyl radical (Leuthner *et al.* 1998; Krieger *et al.* 2001). The thiyl radical abstracts subsequently a hydrogen atom from the methyl moiety, leading to a benzyl radical intermediate which attacks the double bond of fumarate, forming a benzylsuccinyl radical (Beller and Spormann 1998). Finally, the benzylsuccinyl radical obtains a hydrogen atom from the cysteine yielding the end product benzylsuccinate, which is (via several intermediate steps) further oxidised to benzoyl-coenzyme A, the central intermediate of anaerobic degradation of aromatics (Leuthner *et al.* 1998).

The *bss* operon (siehe Figure 1.4) contains the genes *bssA*, *bssB*, *bssC*, encoding respective subunits of the enzyme, and *bssD*, encoding an enzyme which activates BssABC. The function of *bssE* is so far not fully understood (Hermuth *et al.* 2002). The gene encoding the alpha-subunit of the enzyme, *bssA*, was detected within the genome of all so far isolated organisms able to degrade toluene anaerobically (Winderl *et al.* 2007).

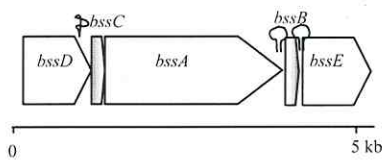


Figure 1.4: The *bss* (benzylsuccinate synthase) operon within *Thauera aromatica* K172 (modified according to Hermuth *et al.* (2002)).

The anaerobic degradation of xylenes was observed under nitrate- and sulphate-reducing as well as methanogenic conditions (Edwards and Grbic-Galic 1994). So far, only organisms able to mineralise xylenes with nitrate and sulphate as electron acceptor could be isolated (Rabus and Widdel 1995; Hess *et al.* 1997; Harms *et al.* 1999b; Morasch *et al.* 2004b; Chakraborty *et al.* 2005). According to Krieger *et al.* (1999), the degradation pathway is analogous to the one of toluene by an initial addition of fumarate to one methyl group and the formation of methyl-benzylsuccinic acid.

Until now, only two strains able to degrade ethylbenzene with nitrate as electron acceptor have been isolated. In the case of those strains the compound is activated by an initial dehydrogenation (ethylbenzene dehydrogenase - EDB) of the methyl group of the ethyl side chain to form 1-phenolethanol, which is further oxidised to the aromatic ketone acetophenone (Rabus and Widdel 1995; Ball *et al.* 1996; Rabus and Heider 1998). Kniemeyer *et al.* (2003) isolated another strain (EbS7) which is able to mineralise ethylbenzene using sulphate as electron acceptor and adding fumarate to the methyl group of the compound to form 1-phenylethyl succinic acid (analogue to the anaerobic degradation of toluene and xylene).

As a non-substituted aromatic compound, benzene has a very stable structure with a high topological resonance energy (Aihara 1992). Therefore, the activation energy is relatively high and an enzymatic attack difficult (Widdel and Rabus 2001). For those reasons benzene was considered to be not degradable by anaerobic organisms. However, anaerobic degradation of benzene was observed with the electron acceptors nitrate (Major *et al.* 1998; Burland and Edwards 1999), iron(III) (Lovley *et al.* 1996), sulphate (Lovley *et al.* 1995; Coates *et al.* 1996) and carbon dioxide (Grbic-Galic and Vogel 1987; Weiner and Lovley 1998b). Two organisms of the genus *Dechloromonas* (RCB and JJ) have been isolated which are able to degrade benzene under nitrate-reducing conditions (Coates *et al.* 2001b). Chakraborty *et al.* (2005) demonstrated that *Dechloromonas aromatica* RCB is also able to use the other BTEX compounds as carbon sources. Furthermore, perchlorate, chlorate, and oxygen may serve as electron acceptors for those organisms. Recently, the genome of RCB was fully sequenced (NC_007298). It contains no genes for any of the key enzymes of anaerobic metabolism of hydrocarbons, phenol or other aromatic compounds, such as benzylsuccinate synthase, phenylphosphate synthetase and carboxylase, or benzoyl-CoA reductase (Heider 2007).

Beside the *Dechloromonas*-strains, two further organisms (strain DN11 und AN9) were recently isolated able to metabolise benzene. They showed a 99% similarity to *Azoarcus evansii* and *Azoarcus* sp. ToN1, respectively. Both strains use nitrate as electron acceptor (Kasai *et al.* 2006).

Table 1.2: Survey of all isolated strains able to mineralise benzene, toluene, ethylbenzene or/and xylene under anaerobic conditions. Possible electron acceptors are mentioned within the table (modified from Weelink (2008)).

strain	name or next relative (with % similarity)	Growth on: benzene	toluene	ethylbenzene	xylene	references
strain T	*Azoarcus* sp. strain T		NO_3^-		NO_3^- (m)	Dolfing *et al.* (1990)
GS-15	*Geobacter metallireducens*		Fe^{3+} [(b)]			Lovley and Lonergan (1990)
K172	*Thauera aromatica*		NO_3^-			Schocher *et al.* (1991)
T1	*Thauera aromatica* T1		NO_3^-			Evans *et al.* (1991a)
Tol-2	*Desulfobacula toluolica*		SO_4^{2-}			Rabus *et al.* (1993)
8 strains	*Azoarcus tolulyticus* Tol4, *Azoarcus tolulyticus* Td15, *Azoarcus toluvorans* Td21		NO_3^-		NO_3^- (m)	Fries *et al.* (1994)
4 strains	*Azoarcus* sp. EbN1, ToN1, PbN1, mXyN1		NO_3^-	NO_3^-		Rabus and Widdel (1995)
EB1	*Azoarcus* sp. strain EB1			NO_3^-		Ball *et al.* (1996)
PRTOL 1	*Desulforhabdus amnigenus* (96%)		SO_4^{2-}			Beller *et al.*(1996)
63 strains	*Azoarcus toluclasticus*		NO_3^-			Fries *et al.* (1997)
14 strains	*Azoarcus tolulyticus* (97-98%)		NO_3^-		NO_3^- (m)	Hess *et al.* (1997)
TRM1			SO_4^{2-}			Meckenstock (1999)
oXyS1	*Desulfosarcina ovata*[c]		SO_4^{2-}		SO_4^{2-} (o)	Harms *et al.* (1999b)
mXyS1	*Desulfococcus multivorans* (86.9%)		SO_4^{2-}		SO_4^{2-} (m)	
pCyN1	*Azoarcus* sp. EbN1 (100%)		NO_3^-			Harms *et al.* (1999a)
TACP	*Geobacter grbiciae* TACP		Fe^{3+}			Coates *et al.* (2001a)
RCB, JJ	*Dechloromonas aromatica* RCB	NO_3^- [(a)]	NO_3^-	NO_3^-	NO_3^- (m,o,p)	Chakraborty *et al.* (2005)
	Dechloromonas sp. JJ	NO_3^-	NO_3^-			Coates *et al.* (2001b)
S2	*Thauera aminoaromatica* S2		NO_3^-			Mechichi *et al.* (2002)
EbS7	strain mXyS1 (96%)			SO_4^{2-}		Kniemeyer *et al.* (2003)
OX39	*Desulfotomaculum* strain R-acetonA179 (96%)		SO_4^{2-}		SO_4^{2-} (m,o)	Morasch *et al.* (2004b)
Y5	*Desulfosporosinus meridiei* (97%)		AsO_4^{3-}			Liu *et al.* (2004)
DNT-1	*Thauera aminoaromatica* (99%)		NO_3^-			Shinoda *et al.* (2004)
4 strains	*Magnetospirillum magneticum* AMB-1 (99-100%)		NO_3^-			Shinoda *et al.* (2005)
DN11, AN9	*Azoarcus evansii* (99%), *Azoarcus* sp. ToN1 (99%)	NO_3^-				Kasai *et al.* (2006)
H3	*Desulfotignum toluenicum*		SO_4^{2-}			Ommedal and Torsvik (2007)

'm' stands for growth on *m*-xylene, 'o' for *o*-xylene and 'p' for *p*-xylene ; [a] *Dechloromonas aromatica* RCB can additionally use perchlorate and chlorate and [b] *Geobacter metallireducens* uses additionally nitrate and manganese(IV) as electron acceptors; [c] formerly *Desulfosarcina variabilis* renamed by Kuever *et al.* (2006)

The pathway of anaerobic benzene degradation is so far not elucidated. The following possible initial reactions to activate the aromatic ring are under discussion (see Figure 1.5): hydroxylation to form phenol (Vogel and Grbic-Galic 1986a; Caldwell and Suflita 2000; Chakraborty and Coates 2005; Ulrich *et al.* 2005), methylation to form toluene (Coates *et al.* 2002; Ulrich *et al.* 2005), and carboxylation to form benzoic acid (Caldwell and Suflita 2000; Phelps *et al.* 2001; Kunapuli *et al.* 2008), with subsequent transformation to benzoyl-coenzyme A and further ring cleavage, respectively (Coates *et al.* 2002). Based on compound-specific isotope analysis Mancini *et al.* (2008) postulated different initial reaction mechanisms for degradation under nitrate- and sulphate-reducing/methanogenic conditions, respectively.

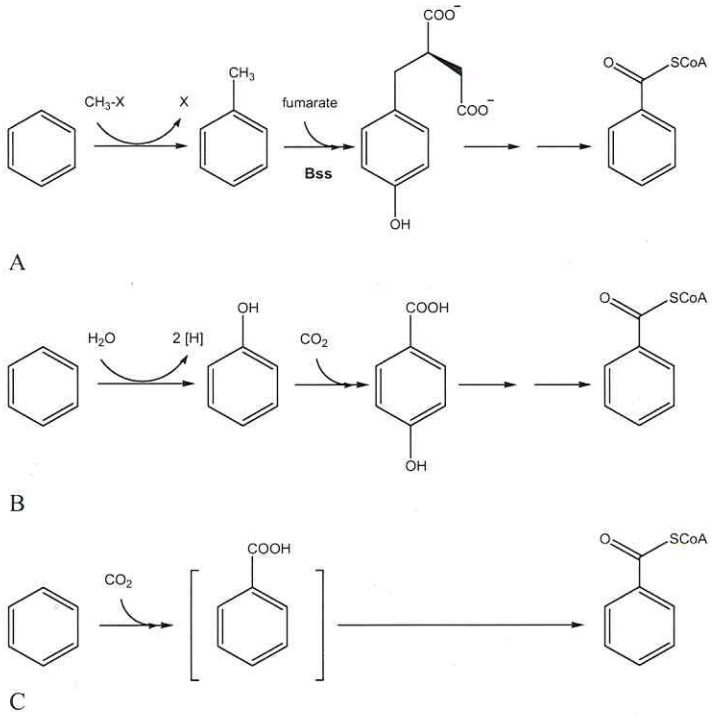


Figure 1.5: Postulated pathways of benzene degradation: activation of benzene ring by A) methylation to form toluene whose degradation is catalysed by the enzyme benzylsuccinate synthase (Bss); B) hydroxylation to form phenol which further leads to hydroxyl benzoic acid; C) carboxylation to form benzoic acid. All intermediates are further degraded via benzoyl-CoA.

BTEX compounds show great differences regarding their respective biological half-life period. Anaerobic toluene and *ortho*-xylene degradation is the most rapid, followed by *meta*-xylene, ethylbenzene, and *para*-xylene degradation (Wiedemeier *et al.* 1999). Benzene is considered to be persistent in the presence of other BTEX compounds (Krumholz *et al.* 1996; Cunningham *et al.* 2001; Da Silva and Alvarez 2004). Anaerobic benzene degradation seems to take place only if no other carbon source is available.

1.4 Natural attenuation and enhanced natural attenuation of BTEX-contaminated aquifers

In the last decades 'natural attenuation' (NA) or the reduction of contaminant concentration and contaminant mass by relying on the assimilative capacity of a groundwater system developed to a valuable remediation strategy since it is nonintrusive, cost-effective and does not require the transfer of the contamination from the aquifer. Natural attenuation results from the integration of several subsurface mechanisms, both destructive and nondestructive. Processes that result only in the reduction of a contaminant's concentration but not of the total contaminant mass in the system are termed nondestructive and include hydrodynamic dispersion, sorption, volatilisation, and dilution (Wiedemeier *et al.* 1999). More important in ecological terms are processes that result in a reduction in the total mass of contaminant in the system. These are referred to as destructive. The dominant destructive attenuation mechanism acting on the BTEX compounds is biodegradation.

In many cases, before a contaminant plume reaches potential receptor exposure points, natural attenuation will reduce dissolved contamination concentrations below regulatory standards such as maximum contamination levels (MCLs) or other remediation goals (Wiedemeier *et al.* 1999). 85 to 90% of petroleum contamination plumes are stable or are even shrinking (Rice *et al.* 1995; Mace *et al.* 1997). Numerous reports have been published documenting natural attenuation of BTEX compounds in aquifers, groundwater and sediments; the reader is directed to the review of Widdel and Rabus (2001)

However, in some cases NA is insufficient and needs some supporting measures. 'Enhanced natural attenuation' (ENA) comprises measures to stimulate or accelerate physical-chemical and biological processes of natural groundwater purification. These processes may either cause an increase of the sorption capacity (immobilisation, (bio-) precipitation) or may provide electron acceptors/donors, nutrients (biodegradation/biostimulation) and/or microorganisms (bioaugmentation) (www.euwelcome.nl).

Due to relatively high water solubility and volatility (see Table 1.1) it is difficult to bind BTEX compounds irreversibly to adsorbents. Therefore, the usage of materials such as activated carbon requires sufficient amounts which is cost-intensive and needs immense effort to be installed at contaminated sites with large extents.

More often considered are processes that stimulate or enhance the microbial activity. During microbial degradation of BTEX compounds, the contaminants are used as carbon and/or energy source. To enable growth, microorganisms also need macro nutrients (nitrogen and phosphor), micro nutrients (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, S^{2-}, co-factors like heavy metals), electron acceptors (oxygen as electron acceptor within aerobic metabolism; iron(III), nitrate, sulphate, manganese(IV) and CO_2 within anaerobic processes) and optimal environmental conditions (temperature, pH, salinity, presence of inhibitors and nutrients) (Fiorenza and Ward 1997; Holliger *et al.* 1997; Lovley and Coates 1997; Mandelbaum *et al.* 1997; Salanitro *et al.* 1997; Field 2002; Lin *et al.* 2002; Van Hamme *et al.* 2003;

Villatoro-Monzon *et al.* 2003; Chakraborty and Coates 2004; Schulze and Tiehm 2004; Jahn *et al.* 2005; Dou *et al.* 2008). The degradation potential is strongly dependent on the availability of those metabolism relevant compounds which may be supplied by the groundwater flow in the aquifer. Thereby, a determining factor is the groundwater velocity. Intrinsic biodegradation processes may be limited by the rate groundwater provides the required substances and removes inhibiting by-products, respectively (NRC 1993).

The supply of electron acceptors has been widely studied within the last decades (Olsen *et al.* 1995; Kao and Borden 1997; Cunningham *et al.* 2001; Atteia and Franceschi 2002; Schreiber and Bahr 2002; Maurer and Rittmann 2004; Reinhard *et al.* 2005). In this regard, the supply of oxygen in order to stimulate aerobic degradation is a common bioremediation technique (Lovley and Lloyd 2000). To increase oxygen concentration of groundwater, as main limiting factor of intrinsic aerobic degradation within contaminated aquifers, numerous methods were developed, *e.g.* air-sparging, injection of oxygen releasing compounds (*e.g.* hydrogen peroxide or magnesium peroxide), and trapped gas phases (Fry *et al.* 1996; Fiorenza and Ward 1997; Waduge *et al.* 2004; Yang *et al.* 2005). An enhancement of microbial degradation of mono-aromatic compounds or fuels by air-sparging (injection of air-oxygen beneath the groundwater table) was observed within studies of *e.g.* Johnston *et al.* (1998; 2002), Zucchi *et al.* (2003), Cavalca *et al.* (2004), Waduge *et al.* (2004), Connon *et al.* (Waduge *et al.* 2004; 2005), and Yang *et al.* (2005). Applications of oxygen releasing compounds also increased the concentration of dissolved oxygen and improved the degradation potential, but were connected with disadvantages like toxicity and microbial inhibition whilst usage of high concentrations of H_2O_2 (Morgan and Watkinson 1992; Morgan *et al.* 1993). However, the usage of oxygen as stimulant is linked to some difficulties. Generally, laminar flow conditions of the groundwater prevent effective mixing of O_2 and any other oxidant in the groundwater flow and the dosing of stimulants is a very challenging problem in any ENA measure. The low water solubility of oxygen (8-10 mg L^{-1}) additionally complicates mixing into the groundwater. Hence, several studies focused on the optimisation of air-sparging programs concerning injection intensity and rate (constant vs. pulsed injection) or addition of tensides to lower the water surface tension (Hall *et al.* 2000; Kim *et al.* 2004; Yang *et al.* 2005; Kim and Annable 2006). But quite often studies only consider the physical effect of stripping of volatile substances while injecting air to the groundwater disregarding microbial processes (Johnson *et al.* 1993; Johnston *et al.* 1998; Murray *et al.* 2000; Peterson *et al.* 2000). Another disadvantage of oxygen is that any reduced compound (*e.g.* sulphur, sulphides, Fe(II), organic compounds, ammonia, FeS species) may lead to the consumption of oxygen. Moreover, formed precipitates, like iron oxides, may block pore volume and decrease the permeability of the aquifer causing a decreased supply with nutrients by a decelerated groundwater velocity.

Hence, studies focused on microbial stimulation by supply with other electron acceptors. Enhanced biological activity on BTEX degradation was demonstrated for nitrate (Ball and Reinhard 1996; Schreiber and Bahr 2002), sulphate (Anderson and Lovley 2000) and iron-chelates supply

(Lovley *et al.* 1996). But also the application of those substrates is linked with both advantages and disadvantages. Compared to oxygen, sulphate and nitrate have a high water solubility. Thus, they can easily be dosed to groundwater and can be transported with groundwater flow in widespread areas, respectively. Moreover, sulphate can accept twice as many electrons ($8e^-$ equivalents per mol) than oxygen; therefore required concentrations are lower. However, the oxidation potential ($E_h^0(w)$ = -0,22V $SO_4^{2-} \rightarrow HS^-$) is much lower compared to all in this content mentioned electron acceptors.

Iron(III)-ions have a high oxidation potential ($E_h^0(w)$ = +0,36 V $FeOOH \rightarrow FeCO_3$), but accept only one electron equivalent per mol and would have to be added in very large amounts in hypothetical ENA measures. Iron(III)-ions (1 mg L^{-1}) are even less water soluble than oxygen (8-10 mg L^{-1}) making an effective dosing nearly impossible. Lovley *et al.* (1994) demonstrated an increase of water solubility and a better bioavailability of iron-ions bound in chelates like EDTA. However, EDTA may act as competitive carbon source and inhibits microorganism growth dosed in high concentrations (Haas and DiChristina 2002), respectively.

Of all electron acceptors for anaerobic processes nitrate has the highest oxidation potential ($E_h^0(w)$ = +0,42 V $No_3^- \rightarrow N_2$). It effectively stimulated microbial *in situ* growth on alkyl-benzenes (Hutchins 1991; Barbaro *et al.* 1992; Reinhard 1993; Sweed *et al.* 1996; Wiesner *et al.* 1996). But, nitrate is potentially toxic, and thus can not be added at high levels (< 100 mg L^{-1}). Cunningham *et al.* (2001) avoided toxicity effects of electron acceptors by using smaller amounts of sulphate and nitrate at the same time. The authors demonstrated that this combination effected a general acceleration of pollutant degradation (in this case of BTEX). Furthermore, they showed a preferential consumption of nitrate within degradation of toluene and ethylbenzene, but sulphate within xylene degradation, thus increased the diversity of degraded compounds and the pool of involved organisms, respectively. Degradation of benzene occurred not before 15 months of incubation and probably not until all other BTEX compounds were degraded, which supported former studies considering benzene recalcitrant under anaerobic conditions as long as other carbon sources are present (Kuhn *et al.* 1988; Evans *et al.* 1991b; Hutchins 1991; Acton and Barker 1992; Barbaro *et al.* 1992; Edwards and Grbic-Galic 1992; Hutchins 1992; Hutchins 1993; Alvarez and Vogel 1995; Thierrin *et al.* 1995; Ball and Reinhard 1996; Langenhoff *et al.* 1996). Except for one study, stimulation of benzene degradation in the presence of other carbon sources was never shown under anaerobic and *in situ* conditions, respectively (Anderson and Lovley 2000). Hence, in the case of benzene-contaminated aquifers oxygen is preferably added to stimulate microbial degradation, though its disadvantages.

Stimulating effects by adding nutrients to BTEX-contaminated aquifers has been poorly investigated. However, at least two examples of successful treatment exist for a stimulation of degradation within anaerobic, diesel fuel contaminated areas poorly equipped with nutrients: Cross *et al.* (2006) observed an improved microbial degradation under nitrate-reducing conditions by addition of ammonium, nitrate, and phosphate to groundwater microcosms. Powell *et al.* (2006) showed a

stimulating effect on denitrifying hydrocarbon metabolisers within nutrient-poor Antarctic soil after supply with nitrate, ammonium, calcium, sulphate and phosphate.

Also biostimulation by bioaugmentation and thus introduction of organisms capable of degrading organic contaminants under field conditions has hardly been observed by any study, so far. Da Silva and Alvarez (2004) inoculated percolated columns filled with aquifer material with enrichment cultures and demonstrated increased benzene degradation under methanogenic conditions, contrarily to columns without injection. But long acclimation periods were required and the observation could not be verified in the field. Possibly, a versatile organism like *D. aromatica* RCB, able to mineralise BTEX compounds under aerobic, nitrate-, perchlorate, and chlorate-reducing conditions, could function in bioaugmentation approaches. However, survival rates of added microbes in competition with indigenous microorganisms adapted to *in situ* conditions of the aquifer are questionable (Foght 2008).

1.5 Verification of *in situ* biodegradation

To verify whether microbial degradation of a pollutant at a contaminated site takes place, under which conditions and to which extent different qualitative and quantitative methods are used. They can be roughly divided into biological and geochemical methods. The next paragraphs introduce some of those methods, their advantages and limitations, and they provide information on literature that complement the topics or discuss certain methods in more detail.

Geochemical methods:

Electron donor and acceptor measurements: Many scientist use geochemical methods, targeting inorganic and organic species in the aqueous, gaseous and solid phase in the subsurface, to assess microbial activity (see the review of Cozzarelli and Weiss (2007)). These methods include studies on mass loss of contaminants (Barker *et al.* 1986; Eganhouse *et al.* 2001) and changes in redox sensitive species (Christensen *et al.* 2000). A combination of both via electron donor and acceptor balances helps to characterise the microbial community and their physiological properties (Wiedemeier *et al.* 1999). For instance, the amount of used oxygen and the simultaneous decrease of pollution concentration can be used to characterise the extent of aerobic microbial activity. Analogously, concentration changes of nitrate, sulphate, iron(III), manganese(IV) and carbon dioxide can be used as indicators of anaerobic degradation processes. Unfortunately, the consumption of electron acceptors corresponds to the sum of all degradation reactions within the aquifer, thus not exclusively to the amount of pollutant. Moreover, the degradation of single contaminants can not be detected in most cases by calculating electron donor and acceptor balances. Nevertheless, measurement and observation of electron acceptor concentrations provide useful information on how long a certain electron acceptor will be available for pollutant degrading processes.

Detection of metabolites: Geochemical methods also include the detection of metabolites which are formed during the degradation process (Beller *et al.* 1995). Ideally, the formation of those metabolites is specific to the degradation process of the parent contaminant. Thus, the metabolites should not be formed during degradation of other compounds or abiotically. They should be chemically relatively stable, thus they can be detected on the one hand; on the other hand they have to be biologically labile enough, so one can conclude a recent degradation by their presence (Phelps and Young 2002). (Alkyl-) Benzylsuccinates (as the product of fumarate addition) fulfil those criteria and therefore can be used as specific indicator metabolites for the anaerobic degradation of TEX-compounds (Beller *et al.* 1995; Elshahed *et al.* 2001b; Reusser and Field 2002; Reusser *et al.* 2002; McKelvie *et al.* 2005). In contrast, (alkyl-) benzoates (intermediates of the lower degradation pathway) are not specific enough, as they may also be formed under aerobic conditions and during degradation of various aromatic compounds (Meckenstock *et al.* 2004b). Metabolites often occur in very small amounts (normally orders of magnitude less than the parent hydrocarbons). Thus, the detection is often difficult due to the low concentration (Caldwell and Suflita 2000; Griebler *et al.* 2004b), the heterogeneity (Kazumi *et al.* 1997) and the ephemeral character as a result of degradation of different members of the consortium (Beller 2000).

Compound specific isotope analysis (CSIA): A relatively new approach to detect biodegradation is the determination of isotope composition of individual contaminants, known as compound specific isotope analyses (CSIA) or stable isotope fractionation analysis (SIFA). This method can be used to prove microbial activity, to quantify biodegradation, and to elucidate degradation pathways. The principles of this method are described in section 1.6.1.

Laboratory vs. field based experiments: These methods can be applied *in situ* or *in vitro*, *i.e.* within laboratory microcosm approaches (often referred to as a bioassay or batch experiment). Laboratory approaches can be especially valuable in distinguishing biotic from abiotic chemical reactions, looking at toxicity effects, and evaluating the impact of changing electron acceptor or donor availability on biodegradation rates. However, laboratory approaches are linked with some disadvantages: *e.g.* collected samples may not be representative of the aquifer, microbial communities may be disturbed during sample collection or may change over time within microcosms. Heterogeneity of aquifers as complex systems concerning geochemistry, hydrogeology as well as microbiology also makes the execution of reproducible laboratory studies difficult. For those reasons some investigators prefer field-based (*in situ*) approaches like tracer tests and push-pull tests. Thereby, an experimental solution is injected into the aquifer, and changes in chemistry and microbiology are measured over time and/or space. These tests are well suited to calculate rates of terminal electron acceptor processes like nitrate reduction (Schroth *et al.* 1998), sulphate reduction (Kleikemper *et al.* 2002), or methanogenesis (Kleikemper *et al.* 2005). Tracer tests were also applied to examine the spatial variability of *in situ* microbial activity (Sandrin *et al.* 2004) as well as to quantify biodegradation of contaminants of interest (Fischer *et al.* 2006). Push-pull tests were used to analyse

the presence and activity of certain groups of organisms (Istok *et al.* 1997) and the overall community composition (Kleikemper *et al.* 2002). Furthermore, to determine biodegradation potentials *in situ* microcosms are used either by encasing a certain volume within an aquifer loaded with groundwater and spiked with a certain contaminant (Gillham *et al.* 1990; Mandelbaum *et al.* 1997) or by incubating a certain substrate within the groundwater of the aquifer via monitoring wells to investigate the *in situ* microbial community (Bennett *et al.* 2000; Reardon *et al.* 2004; Geyer *et al.* 2005; Kästner *et al.* 2006). Phylogenetic characterisation and identification of those microbial communities rely on microbiological methods which are described within the next paragraphs.

Microbiological methods:

Geochemical methods can provide information on fate and transformation rates of contaminants, but only microbiological methods give information about microbial community members and possible growth limitation factors. Microbiological methods can be divided into two broad categories of techniques, those which base on culture-dependent methods and those which are culture-independent Figure 1.6 gives a schematic overview on those methods and the respective information they provide.

Culture-dependent methods: With culture-dependent methods specific organisms, obtained from environmental samples, are cultivated under specific conditions (Madsen 2005). To cultivate aerobic bacteria, samples are commonly plated on general media like Bitek agar (de Lipthay *et al.* 2003), tryptic soy broth agar (Pickup *et al.* 2001), or R2A agar (de Lipthay *et al.* 2004). Concerning pollution degradation, bacteria can be grown more selectively with certain carbon sources either by plating on minimal media agar or by using the most probable number (MPN) technique (Christensen *et al.* 2000; Vogt *et al.* 2004). The latter one is also more suitable for anaerobic bacteria by providing certain electron acceptors within the liquid anoxic media and because anaerobic organisms often do not grow on agar plates. Besides, the AGAR-shake technique proved to be applicable for growing and even isolating of anaerobes (Cole *et al.* 1994). An important limitation of those methods is the inability of many groups of environmental bacteria to grow on standard media. Certainly, applications of (*in situ*) microcosms and percolated column experiments with sediment material and/or groundwater of contaminated aquifers improve the cultivability to a certain extent. However, in most cases the exact biogeochemical conditions of the aquifer cannot be simulated on the laboratory scale, thus cultivation of indigenous contaminant degrading organisms is often not possible. According to Amann *et al.* (1995) only 1 to 10% of microorganisms are cultivable. However, culture-based methods play an essential role in enumerating and isolating organisms and studying factors that might control their *in situ* activity (Figure 1.6).

Culture-independent methods: To avoid the limitations of culture-dependent microbiological methods during characterisation of microbial communities, more and more culture-independent techniques have been developed. These include methods that simply measure the total microbial abundance like microscopy and flow cytometry (see Figure 1.6), which also provide information on

diversity and morphology of community members (Bhupathiraju *et al.* 1999; Collier and Campbell 1999). However, these methods allow no conclusions concerning the phylogenetics of organisms. Therefore, moleculargenetic methods are used which base on comparative sequence analysis of ribosomal RNA-genes; mainly 16S rRNA-genes. These genes with a length of approximately 1500 bp carry next to evolutionary conservative also highly variable regions which allow a reliable reconstruction of phylogenetic relations (Woese and Fox 1977; Pace *et al.* 1986; Hugenholtz and Pace 1996). Almost the whole 16S rDNA molecule of each organism within the microbial community, either of field samples or of laboratory enrichments, can be amplified with universal primers by polymerase chain reaction (PCR) and sequenced. However, this requires on the one hand preceding cloning steps to separate gene fragments by means of fragment ligation into cloning vectors and vector transformation into competent cells (*e.g. Escherichia coli*) and on the other hand subsequent assembly of single sequence sections to total genomic sequences by means of bioinformatics. To get a quick overview on the diversity and relative abundance of the microbial community, fingerprinting-methods like 'terminal restriction fragment length polymorphism' (T-RFLP) (Liu *et al.* 1997), 'single-strand conformation polymorphism' (SSCP) (Schwieger and Tebbe 1998), 'denaturing gradient gel electrophoresis' (DGGE) (Muyzer *et al.* 1993), 'temperature gradient gel electrophoresis' (TGGE) (Muyzer and Smalla 1998), 'amplified ribosomal DNA restriction analysis' (ARDRA) (Massol-Deya *et al.* 1995) are used. Thereby, 16S rRNA genes are also amplified by PCR from a DNA-mixture and subsequently separated based on sequence specificity. SSCP separates enzymatically formed single-stranded DNA fragments of the same length due to a sequence specific formation of secondary structures which migrate distinct distances within a non-denaturising gel. Ideally, one pattern band represents one sequence. Comparison of patterns of different samples allows similarity suggestions of microbial communities. By subsequent addition of partial sequence analysis, a phylogenetic classification of involved organisms is also possible. By using T-RFLP, DNA is PCR amplified with one fluorescent labelled primer. Subsequently, amplicons are cut by restriction enzymes, optimally, resulting in different lengths of the fluorescent terminal restriction fragments for different organisms. The fragments can be separated by capillary electrophoresis on a genetic analyser. This method has the advantage of a quick identification of organisms as soon as restriction patterns of a clone library exist comprising main representatives of the microbial community.

16S rRNA genes vs. functional genes: A characterisation of the microbial communities based on rRNA genes as phylogenetic markers may give evidence on the role of the microbes within community but does not prove functions of microorganisms and thus their metabolic potentials. A method to determine functionality is the detection of genes encoding key enzymes of specific degradation pathways. These genes are described by the term ‚functional markers'. Concerning BTEX degradation several studies used PCR primers to detect and to quantify the presence of specific genes encoding enzymes for the aerobic metabolism such as catechol-2,3-dioxygenase (*nahH*), toluene-

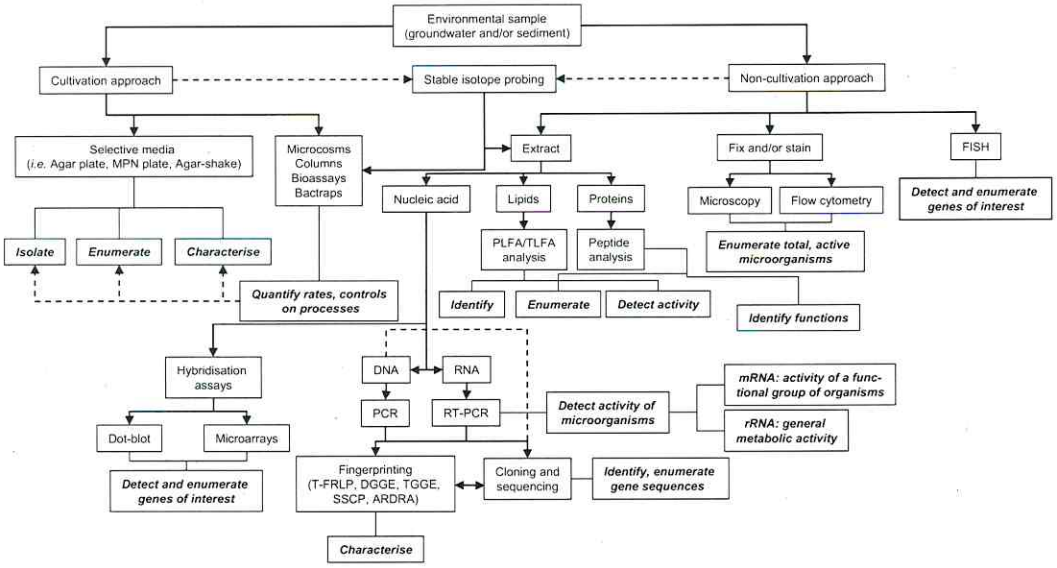


Figure 1.6: A flowchart of microbial and molecular techniques (modified according to Weiss and Cozzarelli (2008)).

dioxygenase (*todC1C2*), toluene- monooxygenase (*tomA*), and xylene-monooxygenase (*xylA*) (Hallier-Soulier *et al.* 1996; Mesarch *et al.* 2000; Baldwin *et al.* 2003). Other studies focused on field application by developing those primers as a tool to analyse the diversity and to prove the presence of those functional markers within contaminated sites (Stapleton and Sayler 1998; Ringelberg *et al.* 2001; Hendrickx *et al.* 2006). The anaerobic degradation of BTEX compounds was investigated by Winderl *et al.* (2007) by screening the intrinsic *bssA*- gene pool of three different tar oil contaminated sites with new designed primers for the detection of BssA encoding genes sequences in order to identify and compare the diversity of anaerobic BTEX-degrading organisms. Kuntze *et al.* (2008) developed primers for the detection of genes encoding 6-oxocyclohex-1-ene-1-carbonyl-CoA (6-OCH-CoA)-hydrolase, an enzyme of the lower pathway of anaerobic aromatic hydrocarbon degradation, and demonstrated on toluene and *meta*-xylene-degrading mixed cultures the potential applicability for undefined bacterial mixed communities.

Genes vs. transcripts: The pure detection of functional genes as well as 16S rRNA genes is not a definite proof whether organisms carrying those genes are active. In contrast, rRNA and mRNA molecules provide information on metabolically active members of the microbial community (Madsen 2000). Thereby, rRNA extractions reveal general metabolic activity based on the ribosome´s role in protein synthesis (Figure 1.6). Moreover, mRNA concentration is reflective of genes that are actively transcribed. Its isolation and manipulation is useful for monitoring the activity of a functional group of organisms responsible for facilitating a particular process (Fleming and Sayler 1995). Ogram *et al.* (1995) first isolated and characterised RNA from the subsurface of an aquifer contaminated with organic and inorganic chemicals. They extracted RNA and converted it to cDNA by reverse

transcriptase PCR and detected, unexpected for the sampling location, a sequence highly related to a gene encoding toluene-4-monooxygenase. mRNA analyses have also been used to examine the expression of genes involved in chlorobenzene degradation in aquifers (Alfreider *et al.* 2003). Fleming and Sayler (1995) analysed naphthalene dioxygenase mRNA in contaminated soils and showed a correlation between transcription rates and naphthalene mineralisation rates as well as soil naphthalene concentrations. Unfortunately, detection of mRNA is difficult due to its low concentrations and fast decay within less than few minutes (Siering 1998; Wilson *et al.* 1999).

Fluorescence in situ hybridisation (FISH): A method to visualise and therewith to detect and quantify active cells based on RNA content is 'fluorescence in situ hybridisation' (FISH) (see Figure 1.6). Therefore, RNA genes are used to design specific fluorescence labelled probes which hybridise with RNA of fixed bacterial cells. By choosing specific binding sites within rRNA, microorganisms of different domains, genera or groups can be visualised *in situ*. Unfortunately, this method is limited as RNA content within cells can be relatively low and correspondingly the signal strength of hybridised cells can be below detection limits. To increase the sensitivity, FISH techniques were improved by using probes either equipped with brighter fluorescence dyes (Glöckner *et al.* 1996) or multiple labelled polyribonucleotides (Pernthaler *et al.* 2002b). Another opportunity of improvement was provided by a technique based on catalysed reporter deposition (CARD) where probes equipped with the enzyme horse radish peroxidase (HRP) bind to complementary RNA-fragments within the cells. Fluorescence labelled tyramid molecules are transformed within the reaction centre of the enzyme to radicals which bind to protein structures within the cells. This results in an accumulation of fluorescence dye and an intensification of the fluorescence signal (Pernthaler *et al.* 2002a). With this method it is even possible to visualise mRNA molecules that might occur only in one copy. Within this approach a combination of probes hybridising to certain functional RNA sequences and probes hybridising to group specific 16S RNA-regions allow a linkage of functionality and phylogenetics of active cells within microbial communities. But these methods as well as all PCR based methods are limited to RNA and DNA sequences which can be identified by the designed probes and primers. Metabolic pathways where no sequences are known can not be elucidated.

Protein analysis: These restrictions do not occur by analysing proteins. Proteins as catalysers of biochemical reactions also provide a combination of phylogenetic and functional information, which make them ideal molecules for studying the structure and function of microbial cultures. In recent work, proteomic analyses, traditionally used for the study of pure cultures, were applied to detect expression profiles and provide functional insight directly from mixed microbial environmental samples. Wilmes and Bond (2004) established for the first time that a proteomics approach could be successfully applied to examine protein expression in environmental samples such as activated sludge. Since then there has been only a handful of studies describing mixed culture proteomics (termed metaproteomics) (Wilmes and Bond 2006a; Kim *et al.* 2007). These also included the examination of protein expression profiles from petroleum contaminated soil and groundwater (Benndorf *et al.* 2007).

Analyses of proteins are performed by mass spectrometry either after a separation step using 2-DE (Wilmes and Bond 2006b; Benndorf *et al.* 2007) or by shot gun approaches (Ram *et al.* 2005; Lo *et al.* 2007). These techniques provide a probe and primer independent detection, but are restricted to available amino acid sequences of databanks for identification. Furthermore, at least 10^6 cells have to be extracted to be above the detection limit for protein analysis*.

Summarising the previous paragraphs, analyses of genes (metagenomics), RNA sequences (metatranscriptomics) and proteins (metaproteomics) provide information on diversity, activity, and/or functionality of a microbial community of environmental samples. Concerning the degradation of a certain contaminant, however, they provide no definite proof which community member is responsible for this specific metabolism and what genes are expressed. One possible solution for this task is a linkage of those molecular genetic tools with stable isotope tools. Stable isotopes can be used as tracers within carbon sources exposed to the community of interest; by degrading those carbon sources and isotope incorporation into biomarker molecules (fatty acids, nucleic acids, proteins), ideally, responsible organisms and enzymes can be identified (see Figure 1.6). These techniques are summarised under the term 'stable isotope probing' (SIP) and are introduced in chapter 1.6.2.

1.6 Stabile isotope based methods for the assessment of microbial activity

Stable isotope based techniques to assess microbial activity can be divided into two main groups. The first one uses stable isotopes with their natural abundance in the environment and analyses a substrate specific fractionation of those isotopes in relation to biotic or abiotic degradation processes. Fractionation can be measured within products and residual substrates and then can be used to quantify and characterise degradation processes. The method is described by the terms 'compound-specific stable isotope analysis (CSIA)' or 'stable isotope fractionation analysis (SIFA)' and is explained in more detail within the next paragraphs.

On the other hand stable isotopes can be used as tracers. Within these methods, chemically modified substrates with an artificial amount of stable isotopes are dosed to microbial communities which are potentially able to use the substrate as carbon and/or electron source. These isotopes then might be detected in degradation products, like CO_2, which proves the microbial communities degradatively active, or within molecules of biomass (stable isotope probing SIP, see 1.6.2) which might help to find out which organisms of the community consume the substrate or to elucidate food webs. By detecting isotope labelling within degradation metabolites degradation, pathways can be elucidated. Since substrates, products, metabolites, and some biomarker molecules are detectable by gas chromatography-mass spectrometry (GC-MS) or GC-isotope ratio- mass spectrometry (GC-IRMS), stable isotope methodologies may not only provide qualitative but also quantitative information.

* Nico Jehmlich, personal communication, 12/2008

1.6.1 Compound specific isotope analysis (CSIA)

1.6.1.1 Theoretical background

Stable isotopes: Isotopes (Greek isos = "equal", topos = "site, place"; "at the same place" within the periodic table of elements) are atoms of one element with different mass numbers due to different numbers of neutrons. Neutrons are neutral nuclear particles which reduce repulsive forces between protons. They are responsible for a huge variety of stable isotopes within nature. The ratio of protons and neutrons within the nucleus of an atom (Z:N) ranges for stable isotopes from 1:1 (H to Ca) to 1:1.5 (Ca to Au). If the number of neutrons is too large one nuclear neutron (n) decays under emission of one electron (e^-) and one neutrino (υ_e) to a nuclear proton (p^+) ($n \rightarrow p^+ + e^- + \upsilon_e + 0.783\ MeV / particle$). In contrast, if the number of neutrons is smaller than the number of protons one nuclear proton can be transformed to a neutron (β^+- decay). These unstable isotopes are called radionuclides and are separately examined within the radiochemistry.

The most prominent and most simple representative of the periodic table is hydrogen with its stable isotopes hydrogen ^1H (Z = 1, N = 0) and deuterium ^2H (Z = 1, N = 1). The latter has a natural abundance of 0.0156% opposed to ^1H with 99.9844% (Table 1.3). Carbon as skeletal structure element within organic chemistry has two stable isotopes: ^{12}C and ^{13}C. The lighter one occurs with an abundance of 98.891%, the heavier one occurs with 1.11% in nature. Other structural entities of organic substances are nitrogen, oxygen, sulphur, and chloride having at least two stable isotopes (Table 1.3).

Table 1.3: Stable isotopes of selected elements, their relative natural abundances and mass differences as well as their international standards (Hoefs 1997).

Element	Relative isotope abundance	Relative mass difference	Standard
$^1H/^2H$	99.9844/0.0156	2.00	V-SMOW
$^{12}C/^{13}C$	98.891/1.11	1.08	V-PDB
$^{14}N/^{15}N$	99.64/0.36	1.07	Air
$^{16}O/^{18}O$	99.76/0.02	1.13	V-SMOW
$^{32}S/^{34}S$	94.02/4.21	1.06	V-CTD
$^{35}Cl/^{37}Cl$	75.53/24.47	1.06	24.47

V-SMOW Vienna-Standard Mean Ocean Water; V-PDB Vienna Peedee Belemnite, V-CTD Vienna-Canon Diablo Troilite

Analysis of stable isotopes: To determine the isotope ratio of a substrate in a mixtures of compounds, analytical methods are applied which combine conventional gas chromatographs (GC) or high pressure liquid chromatographs (HPLC), a chemical reaction interface, and a specialised isotope ratio mass spectrometer (IRMS) (Sessions 2006; Muccio and Jackson 2009). GC or HPLC are used to separate compounds within a mixture on a compound-specific column. The interface continuously and quantitatively converts organic matter to a common molecular form for isotopic measurement by IRMS. The element most studied is carbon. It is analysed as CO_2 derived from combustion of analytes. The measurement of carbon is most sensitive and has a precision range of 0.1-0.5‰. In contrast, hydrogen is measured as H_2 produced by pyrolysis/reduction of the compound. Its analysis has a lower

sensitivity due to the low natural abundance of deuterium which is about 50 times less than the abundance of ^{13}C (Hoefs 1997). The precision range is normally between 2 to 5‰. Due to the high mass difference of hydrogen and deuterium (see Table 1.3), however, hydrogen isotope effects are usually around two orders of magnitude higher than carbon isotope effects (Hilkert *et al.* 1999). Analysis of stable isotope ratios by mass spectrometric methods is also possible for other biological important elements, *e.g.* oxygen, nitrogen or sulphur (Muccio and Jackson 2009).

Isotope fractionation: According to classical chemistry, chemical properties of atoms or molecules only depend on the electron shell, *i.e.* the number of electrons (and protons in the nucleus, respectively), but not on the number of uncharged neutrons (Hollemann and Wieberg 1985). However, analyses by isotope specific mass spectrometry show that molecules with different isotopes of one element (isotopologues) react slightly different not only physically, but also chemically. This effect is called isotope fractionation. With respect to exchange reactions (*i.e.* reactions without net conversion) like phase transformation between water and water vapour within a closed system, it is called thermodynamic isotope fractionation. In the case of unidirectional reactions it is called kinetic isotope fractionation. Examples for latter reactions are evaporation of water and instant removal of formed water vapour within an open system, adsorption and diffusion of gases, and irreversible chemical reactions like carbonate precipitation or microbial degradation of organic material. Commonly, kinetic isotope fractionation exceeds thermodynamic isotope fractionation.

Kinetic isotope effects (KIE) (see eq. 1.1) are related to different dissociation energies and are thus associated with different reaction rates (k) of different isotopes of an element that occur within molecular bonds:

$$KIE = \frac{{}^{l}k}{{}^{h}k} \qquad \text{eq. 1.1}$$

where 'l' stands for the light and 'h' for the heavy isotope.

Bonds containing lighter isotopes of the same element (*e.g.* $^{12}C - {}^{12}C$, $^{12}C - {}^{1}H$) have a slightly higher zero-point energy (E_0) compared to bonds containing heavier isotopes (*e.g.* $^{12}C - {}^{13}C$, $^{12}C - {}^{2}H$, $^{13}C - {}^{1}H$; see Figure 1.7). E_0 is the lowest possible energy an atomic system may have and is defined as:

$$E_0 = \frac{1}{2} h \nu_0 \qquad \text{eq. 1.2}$$

with 'h' as Planck´s constant and 'ν_0' as oscillation frequency of an oscillating atomic system. The oscillation frequency is defined as:

$$\nu_0 = \frac{1}{2\pi}\sqrt{\frac{k}{\mu}} \qquad \text{eq. 1.3}$$

with 'k' as spring constant and 'μ' as reduced mass of a two atomic system defined as $\mu = m_1 \times m_2 / (m_1 + m_2)$. Thus, the higher the mass of one of the two atoms is, the smaller are the

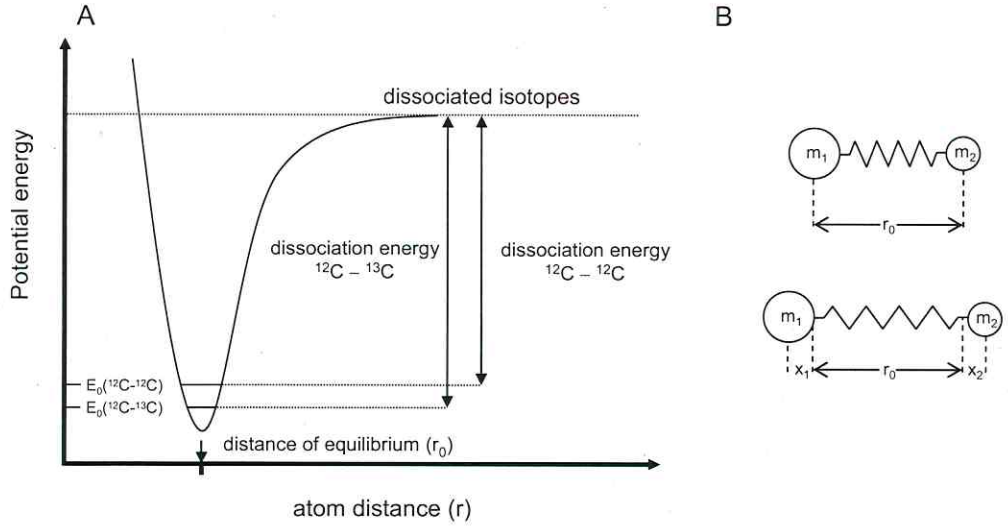


Figure 1.7: A) Morse potential of the anharmonic oscillation of a two-atom-system for the examples of $^{12}C - ^{12}C$- and $^{12}C - ^{13}C$-bonds, respectively. B) Ball model to describe oscillation of a two-atom-molecule (modified according to Steinbach (2003)).

oscillation frequency and the zero-point energy, respectively. This also influences the dissociation energy of a bond, since this is defined as the difference between zero-point energy and an energy level at which a bond breaks (see Figure 1.7).

Thus, dissociation of heavier isotopologues requires more energy than dissociation of lighter isotopologues. This implies that lighter bonds ($^{12}C - ^{12}C$, $^{12}C - ^{1}H$, $^{1}H - ^{1}H$) are preferentially cleaved within both biotic and abiotic degradation processes. This results in an enrichment of heavier isotopologues within the residual, non-degraded substrate. Elements involved in a reaction in which a specific bonds is broken will generally show *normal* isotope effects (KIE > 1, see eq. 1.1). Inverse isotope effects (KIE < 1) may be observed in cases in which bonds are strengthened or formed (Elsner *et al.* 2005).

Kinetic isotope effects can be separated into primary and secondary KIE. In most cases, pronounced kinetic isotope effects are only observable within primary effects which emerge if the heavy isotope is directly involved in the rate-limiting reaction step(s) of cleavage or formation of the chemical bond. Secondary isotope effects may also occur if a heavier neighbouring atom changes the chemical properties of the site of the reaction but does not directly participate in the reaction (Meckenstock *et al.* 2004a; Elsner *et al.* 2005). This is especially the case for deuterium due to the big mass difference compared to hydrogen (see Table 1.3). Normally, secondary effects are one or two orders of magnitude lower than primary effects for elements with lower mass differences between isotopes (Meckenstock *et al.* 2004a).

1.6.1.2 Determining degradation pathways by CSIA

Determination of compound-specific stable isotope signatures: In principle, CSIA is applicable to any isotopic element (X) in an organic compound. Using gas and liquid chromatography coupled to isotope ratio mass spectroscopy (Hayes *et al.* 1990; Sessions 2006), isotope ratios of hydrogen, carbon, nitrogen, oxygen, sulphur, and chlorine can be measured in individual organic compounds to date. Since the abundance of rare isotopes (y) and the variations in isotope ratios of those elements (R_{sample} = $^{2}H/H^{1}$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$, or $^{37}Cl/^{35}Cl$) are generally very small, isotope ratios are given in "delta notation", $\delta^{y}X$, in "per mil" relative to an internationally defined standard ($R_{standard}$, see Table 1.3).

$$\delta^{y}X_{sample}\,[‰] = \left(\frac{R_{sample} - R_{\text{standard}}}{R_{\text{standard}}}\right) \times 1000 \qquad \text{eq. 1.4}$$

The more positive the δ-value is, the stronger is the enrichment of heavier isotopes within the examined compound.

The mathematical relation between the change in concentration of the degraded compound and the change in isotope composition of the residual, non-degraded compound can be described with the Rayleigh equation (Rayleigh 1896; Mariotti *et al.* 1981) by the isotope fractionation factor α or the enrichment factor ε, respectively.

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{(\alpha-1)} \quad or \quad \frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \qquad \text{eq. 1.5}$$

R_t is the isotope signature of a determined element of a molecule of interest at time t, R_0 is the initial isotope signature, C_0 and C_t are the initial concentration of the substrate and a concentration at a given time t, respectively. Considering delta notation, these equations can be expressed as:

$$\frac{C_t}{C_0} = \left(\frac{\delta^{y}X_t + 1000}{\delta^{y}X_0 + 1000}\right)^{\frac{1}{(\alpha-1)}} \quad or \quad \frac{C_t}{C_0} = \left(\frac{\delta^{y}X_t + 1000}{\delta^{y}X_0 + 1000}\right)^{\frac{1000}{\varepsilon}} \qquad \text{eq. 1.6}$$

The kinetic isotope fractionation factor 'α' is calculated assuming that the observed isotope fractionation in the bulk organic compound is caused by intermolecular isotopic competition between molecules containing exclusively n isotopically light atoms of element X (*i.e.* the light isotopologue) versus isotopologues containing one heavy and (n – 1) light isotopes (Melander and Saunders 1980). For example, ^{13}C-fractionation during anaerobic toluene degradation is due to competition between isotopologues containing a ^{12}C atom at the methyl moiety versus isotopologues containing a ^{13}C atom at this position. The fractionation factor is a constant for a reaction at given experimental conditions and can be obtained from experiments by plotting $\ln(R_t/R_0)$ over $\ln(C_t/C_0)$ for the sampling points t (Hoefs 1997). For notation of isotope fractionation the enrichment factor 'ε' is commonly used, which

is defined as $\varepsilon = (\alpha - 1) \times 1000$ and results in values more easily to compare than α (Clark and Fritz 1997).

$$\ln\left(\frac{\delta^{y}X_0 + \Delta\delta^{y}X_t + 1000}{\delta^{y}X_0 + 1000}\right) = \frac{\varepsilon_{bulk}}{1000} \times \ln\left(\frac{C_t}{C_0}\right) \qquad \text{eq. 1.7}$$

where $\delta^{y}X_0$ is the initial isotope signature and $\Delta\delta^{y}X_0$ is its change relative to the initial value. While ε_{bulk} values are used to assess the fractionation of entire molecules, this parameter is not suited to comparing isotope effects and reaction mechanisms among different compounds. For the latter, kinetic isotope effects at the reacting bond are necessary. To convert ε_{bulk} values into apparent kinetic isotope effect, ε values are calculated as isotope enrichment factors at the reactive position of the molecule, $\varepsilon_{reactive\ position}$.

$$\ln\left(\frac{\delta^{y}X_0 + \frac{d}{e}\Delta\delta^{y}X_t + 1000}{\delta^{y}X_0 + 1000}\right) = \frac{\varepsilon_{reactive\ position}}{1000} \times \ln\left(\frac{C_t}{C_0}\right) \qquad \text{eq. 1.8}$$

The correction for 'd' isotopic atoms of the determined element X present in the molecule and 'e' the number of isotopes located at the reactive sites takes into account that (i) nonreactive atoms "dilute" the observed isotopic fractionation, whereas (ii) multiple reactive sites can increase it (Elsner *et al.* 2005). Finally, intramolecular isotopic competition can result in a depletion of molecules that contain heavy isotopes without leading to isotope fractionation. This is the case if a molecule reacts at the isotopically light reactive site instead of the one carrying the heavy isotopes. For example, in the case of ^{2}H-fractionation during anaerobic toluene degradation two hydrogen atoms of the methyl moiety compete against one deuterium atom. The concentration of isotopologues with two deuterium atoms is, according to a binomial distribution of light and heavy isotopes, too small to contribute to the measurable isotope fractionation. Correction for z competing reactive sites leads to the apparent kinetic isotope effect

$$AKIE = \left(\frac{^{l}k}{^{h}k}\right)_{apparent} = \frac{1}{1 + z \times \frac{\varepsilon_{reactive\ position}}{1000}} \qquad \text{eq. 1.9}$$

The expression of the KIE in a (bio)chemical reaction is not only influenced by inter- and intramolecular isotope competition, but also depends on kinetic limitations associated with bond changes and rate limitations of non-fractionating reaction steps preceding the bond cleavage which have been summarised under the term 'commitment to catalysis' (Northrop 1981; Elsner *et al.* 2005). Thus, the apparent kinetic isotope effect (AKIE) of a (bio)chemical reaction can be much smaller compared to the KIE of bond cleavage. The often observed variability of bulk enrichment factors (ε_{bulk}) and AKIEs for specific contaminant degradation pathways is thought to be caused by commitment to catalysis. For example, Kampara *et al.* (2008) observed variable hydrogen isotope

fractionation factors during toluene degradation by *Pseudomonas putida* mt-2 depending on the substrate bioavailability; a decrease in toluene concentration resulted in a decrease in fractionation. Also Tobler *et al.* (2008) hypothesised that the smaller isotope fractionation observed during anaerobic toluene degradation by *Geobacter metallireducens* in suspension of a solid Fe(III)-phase compared to solutions of Fe(III)-citrate is due to toluene transport limitations to the cells at surfaces of solid Fe(III)-phases. Morasch *et al.* (2001b) discovered that isotope effects can be influenced by temperature. During degradation of toluene by *P. putida* mt-2, isotope fractionation was slightly stronger in cultures incubated at 20°C compared to 37°C. Nijenhuis *et al.* (2005) reported a correlation between the increase of isotope fractionation during reductive dechlorination of PCE by *Sulfurospirillum multivorans* and the decrease of cell integrity. The isotope fractionation of PCE increased from culture experiments with whole cells to *in vitro* assays with crude extracts and even more with purified PCE reductive dehalogenase. Mancini *et al.* (2006) attributed varying hydrogen and carbon isotope fractionation during toluene degradation by *P. putida* mt-2 to concentration differences of key constituents (trace elements) of involved enzymes (methyl monooxygenase) which affected the enzyme binding. The authors explained the increase of enrichment factors at low iron concentrations with a slower enzyme-catalysed substrate conversion step (k_2) relative to the enzyme-substrate binding step (k_{-1}).

Recently, two-dimensional compound-specific isotope analysis (2D-CSIA) has been suggested as a tool for characterising rate-determining reaction steps of biodegradation pathways more precisely (Elsner *et al.* 2005; Zwank *et al.* 2005; Fischer *et al.* 2007). 2D-CSIA relates the isotope signature changes ($\Delta\delta^{v}X$) of two elements within a 2D-plot. The slope of this plot is a constant named Λ.

$$\Lambda = m_{2D-plot} \approx \frac{\varepsilon^{2}H}{\varepsilon^{13}C} \qquad \text{eq. 1.10}$$

Λ nearly conforms to the ratio of the enrichment factors for the both elements used within the 2D-approach. Commitment to catalysis is eliminated assuming that isotopes for both elements are comparably affected.

$$\Lambda_{reactivepositive} = m_{2D-plot} \approx \frac{\varepsilon_{reactive\ position}{}^{2}H}{\varepsilon_{reactive\ position}{}^{13}C} \qquad \text{eq. 1.11}$$

The ratio of $(AKIE_C - 1) / (AKIE_H - 1)$ considers intramolecular competition (Abe *et al.* 2009).

1.6.1.3 Isotope fractionation in the course of contaminant (BTEX) degradation

One of the first studies dealing with isotope fractionation of BTEX degradation was done by Stahl (1980) and focused on aerobic degradation of crude oil constituents in seawater while looking on the isotope composition of the residual, not yet degraded substrates. Although they could detect a carbon fractionation for alkanes, they were not able to show isotope fractionation of benzene. A similar result was published by Sherwood Lollar *et al.* (1999) who investigated the isotope fractionation during

anaerobic trichloroethylene and aerobic toluene degradation. Carbon isotope fractionation within latter reaction could not be detected. Isotope fractionation of BTEX compounds was first demonstrated by a field study of Kelley *et al.* (1997), who investigated CSIA as a tool to monitor bioremediation analysing groundwater of a BTEX-contaminated site within several monitoring wells. They showed a slight shift of the ^{13}C-isotope signature of toluene and benzene, but pointed out that the shift also could have been caused by different contamination sources. A direct linkage between isotope fractionation and BTEX degradation was first demonstrated with the sulphate-reducing bacterial strain TRM1 by Meckenstock *et al.* (1999a), where a pronounced enrichment of ^{13}C during toluene degradation was observed. Since then many studies have been performed on toluene degradation (Meckenstock *et al.* 1999b; Ahad *et al.* 2000; Morasch *et al.* 2001b; Morasch *et al.* 2002; Richnow *et al.* 2003b; Steinbach *et al.* 2004; Mancini *et al.* 2006). Carbon and hydrogen isotope fractionation during benzene degradation was studied by Hunkeler *et al.* (2001) for oxic conditions and by Mancini *et al.* (2003) for nitrate- and sulphate-reducing as well as methanogenic conditions leading to a wide range of values for enrichment factors for different redox conditions. So far only a couple of studies investigated isotope fractionation of ethylbenzene and the isomers of xylene, mainly focusing on carbon isotopes (Wilkes *et al.* 2000; Morasch *et al.* 2002; Richnow *et al.* 2003b; Meckenstock *et al.* 2004a; Morasch *et al.* 2004a; Steinbach *et al.* 2004). See Table 1.4 for an overview of presently determined enrichment factors for microbial degradation of BTEX compounds.

1.6.1.4 Quantification of *in situ* biodegradation by isotope fractionation

By knowing enrichment factors specific for the conditions of an aquifer of interest, it is possible to quantify the extent of *in situ* biodegradation (Meckenstock *et al.* 1999b; Meckenstock *et al.* 2004a). The percentage of biological degradation (B) is calculated by the following relation of the Rayleigh equation (Meckenstock *et al.* 2002):

$$B[\%] = 100 \times \left(1 - \frac{C_t}{C_0}\right) = \left(1 - \left(\frac{R_t}{R_0}\right)^{\frac{1000}{\varepsilon}}\right) \times 100 \qquad \text{eq. 1.12}$$

In recent years, CSIA was more and more used to characterise the *in situ* degradation potential for BTEX compounds at contaminated field sites (Mancini *et al.* 2002; Richnow *et al.* 2003b; Griebler *et al.* 2004b; Vieth *et al.* 2005). For applying CSIA in the course of biodegradation quantification, it is necessary to choose the right enrichment factor for the examined field. To gain reliable data, intensive studies on enrichment factors for certain conditions have to be performed beforehand. Previously mentioned masking effects may complicate an application. A conservative estimation of *in situ* biodegradation is still possible as long as calculation is done by using the highest enrichment factors determined for the examined conditions, to avoid an overestimation of degradation potential (Rosell *et al.* 2007; Fischer *et al.* 2008).

Table 1.4: Enrichment factors (ε) for isotope fractionation of carbon (C) and hydrogen (H) isotopes during *in vitro* degradation of BTEX compounds for distinct bacteria and redox conditions to the stand of 2008.

Compound	ε_C	ε_H	Conditions	Bacteria	References
benzene	-1.5±0.1	-13±0.7	oxic	*Acinetobacter* sp.	Hunkeler *et al.* (2001)
benzene	-3.5±0.3	-11±1.8	oxic	*Burkholderia* sp.	Hunkeler *et al.* (2001)
benzene	-2.4±0.1	-29±4	NO_3^--reducing	enrichment culture	Mancini *et al.* (2003)
benzene	-2.2±0.1	-35±6	NO_3^--reducing	enrichment culture	Mancini *et al.* (2003)
benzene	-3.6±0.3	-79±4	SO_4^{2-}-reducing	enrichment culture	Mancini *et al.* (2003)
benzene	-1.9±0.1	-60±3	methanogenic	enrichment culture	Mancini *et al.* (2003)
benzene	-2.1±0.1	-59±4	methanogenic	enrichment culture	Mancini *et al.* (2003)
benzene	-0.8±0.2	-34±8	methanogenic	enrichment culture	Mancini *et al.* (2008)
benzene	-1.1±0.1	-38±6	methanogenic	enrichment culture	Mancini *et al.* (2008)
benzene	-2.6±0.6	-47±11	NO_3^--reducing	enrichment culture	Mancini *et al.* (2008)
benzene	-2.8±0.6	-47±4	NO_3^--reducing	enrichment culture	Mancini *et al.* (2008)
benzene	-1.9±0.7	-31±7	NO_3^--reducing	enrichment culture	Mancini *et al.* (2008)
ethylbenzene	-2.2		NO_3^--reducing	strain EBN1	Meckenstock *et al.* (2004a)
ethyltoluene	-3.7		SO_4^{2-}-reducing	enrichment culture	Wilkes *et al.* (2000)
toluene	n.s.		oxic	Microcosms	Sherwood Lollar *et al.* (1999)
toluene	-3.3±0.3	-905 ±71	oxic	*Pseudomonas putida strain* mt-2	Morasch *et al.* (2002)
toluene	-1.1±0.2	-16±5	oxic	*Ralstonia pickettii* strain PKO1	Morasch *et al.* (2002)
toluene	-0.4±0.3	-28±10	oxic	*Pseudomonas putida* strain F1	Morasch *et al.* (2002)
toluene	-1.7		NO_3^--reducing	*Thauera aromatica*	Meckenstock *et al.* (1999b)
toluene	-1.8		Fe^{3+}-reducing	*Geobacter metallireducens*	Meckenstock *et al.* (1999b)
toluene	-0.8		SO_4^{2-}-reducing	enrichment culture	Ward *et al.* (Ward *et al.* 2000)
toluene	-1.5		SO_4^{2-}-reducing	column experiment	Meckenstock *et al.* (1999b)
toluene	-2.2		SO_4^{2-}-reducing	*Desulfobacterium cetonicum*	Morasch *et al.* (2001b)
toluene	-1.7		SO_4^{2-}-reducing	strain TRM1	Meckenstock *et al.* (1999b)
toluene		-728	SO_4^{2-}-reducing	strain TRM1	Morasch *et al.* (2001b)
toluene		-198	SO_4^{2-}-reducing	*Desulfobacterium cetonicum*	Morasch *et al.* (2001b)
toluene	-0.5		methanogenic	enrichment culture	Ahad *et al.* (2000)
toluene		-12	methanogenic	Consortium	Ward *et al.* (2000)
toluene		-65	methanogenic	Consortium	Ward *et al.* (2000)
toluene	-1.7±0.1	-77±4	oxic	*Pseudomonas putida* strain mt-2	Mancini *et al.* (2006)
toluene	-2.5±0.3	-159 ±11	oxic	*Pseudomonas putida* strain mt-2	Mancini *et al.* (2006)
toluene	-1.1		SO_4^{2-}-reducing	column experiment	Richnow *et al.* (2003b)
m-xylene	-1.7 ±0.1		oxic	*Pseudomonas putida* strain mt-2	Morasch *et al.* (2002)
m-xylene	-1.8±0.2		SO_4^{2-}-reducing	strain OX39	Morasch *et al.* (2004a)
p-xylene	-2.3±0.3		oxic	*Pseudomonas putida* strain mt-2	Morasch *et al.* (2002)
o-xylene	-1.5±0.1		SO_4^{2-}-reducing	strain OX39	Morasch *et al.* (2004a)
o-xylene	-1.1		SO_4^{2-}-reducing	column experiment	Richnow *et al.* (2003b)
o-xylene	-3.2		SO_4^{2-}-reducing	enrichment culture	Wilkes *et al.* (2000)

n.s. not significant, e.d. enrichment detected

1.6.2 Stabile isotope probing (SIP)

A method to link phylogenetic and functional properties of heterogeneous bacterial communities comprises tracer experiments with isotopically labelled substances (^{13}C, ^{15}N) that are used as source of carbon or nitrogen. The process of biomass labelling is called 'stable isotope probing' (SIP) and can be detected by analysis of biomarker molecules such as fatty acids, (desoxy-) ribonucleic acids, and proteins.

1.6.2.1 Nucleic acid-based stable isotope probing

Since the 1950s investigations with SIP-technologies have been performed. Meselson and Stahl (1958) first used ^{15}N-labelled substrates and were able to discover the replication cycle of DNA. Since nucleic acids mainly consist of carbon and hydrogen, it was soon discovered that a labelling of RNA and DNA could be gained also by using heavy isotopes of carbon and hydrogen (Schildkraut 1967; Radajewski *et al.* 2003).

With the beginning of the 21st century a new application field for the SIP-technologies was discovered: the identification of organisms involved in the degradation of hydrocarbons within a microbial community. Precursor of a large number of studies was an investigation of Radajewski *et al.* (2000), who identified organisms involved in the C1-metabolism. Within this study the term DNA-stable isotope probing (DNA-SIP) was first used. Radajewski *et al.* (2000) incubated microbial communities within microcosms with ^{13}C-methanol, extracted DNA and separated light from heavy DNA by equilibrium centrifugation within a CsCl-density gradient. 16S rRNA genes of the heavy fraction were amplified and identified by cloning and sequencing. Soon after, Manefield *et al.* (2002a; 2002b) published the first application of RNA-SIP and the separation by using a CsTFA-density gradient. Initially, degradation experiments were performed for C1-compounds like ^{13}C-methanol, -methane or -carbon dioxide. Meanwhile, a large number of investigations were conducted in order to identify key organisms involved in the metabolism of several aromatic and chlorinated hydrocarbons (Manefield *et al.* 2002a; Jeon *et al.* 2003; Padmanabhan *et al.* 2003; Mahmood *et al.* 2005; Yu and Chu 2005; Kasai *et al.* 2006; Kunapuli *et al.* 2007). Kasai *et al.* (2006) used RNA-based SIP to identify organisms of a petroleum contaminated aquifer able to degrade benzene with nitrate as electron acceptor. Organisms within the genus of *Azoarcus* were identified to be the key organisms and were subsequently isolated (strain DN11 and AN9) being, next to the isolated *Dechloromonas* strains (Coates *et al.* 2001b), the only organisms able to degrade benzene anaerobically in pure culture. Kunapuli *et al.* (2007) used RNA-based SIP to analyse the carbon fluxes in an enrichment culture gained from a field of a former coal gasification site which was able to degrade benzene under iron(III)-reducing conditions. They discovered two dominant organisms and postulated an unusual syntrophy, with members of *Clostridia* initially oxidising benzene and sharing gained electrons with members of *Desulfobulbaceae* which remove hydrogen formed during benzene oxidation as syntrophic partners. Sulphate-reducing mixed cultures were analysed by Oka *et al.* (2008) using DNA-

SIP and subsequent T-RFLP-analysis to identify benzene degraders. They found a dominant organism within the heavy-DNA fraction which clustered to sulphate-reducing δ-*Proteobacteria.* A field based stable isotope probing experiment was performed by Liou *et al.* (2008) identifying organisms closely related to *Pelomonas* to be involved in degradation at a coal-tar waste contaminated site.

The disadvantage of nucleic acid-based stable isotope probing is the low sensitivity of this technique. To get an effective separation of labelled and unlabelled nucleic acids a labelling of over 20% and 50% is needed for RNA and DNA, respectively (Radajewski *et al.* 2003). Due to semi-conservative replication of DNA two cell divisions are necessary to obtain one of two daughter cells with fully labelled DNA. High labelling yields mostly require unusually high substrate concentrations – possibly stimulating growth of atypical organisms within the microbial community – or long incubation times which support cross feeding effects (Gray and Head 2001). Substrate sharing may additionally lower the label incorporation if more than one organism is present that is able to degrade the substrate of interest. The same effect may occur if variable carbon sources are present. RNA faster incorporates ^{13}C-label than DNA since new synthesised molecules – independent of cell division – are fully labelled as long as the ^{13}C-labelled substrate is the only carbon source (Manefield *et al.* 2002a; Radajewski *et al.* 2003). However, a disadvantage of RNA-SIP is the lability of RNA which necessitates on the one hand a well developed extraction protocol to receive adequate amounts of RNA with a high purification degree from co-extracts and on the other hand a very careful treatment to avoid RNA losses due to RNase digestion.

1.6.2.2 Fatty acid-based stable isotope probing

A much more sensitive method to analyse ^{13}C-incorporation into biomass is stable isotope probing based on fatty acids. These biomarker molecules can be analysed by (isotope ratio-) mass spectroscopy which enables the detection of very low ratios of incorporated heavy isotopes (Mottram and Evershed 2003). A separation of heavy and light biomass is not necessary, which allows the concentration of isotopically labelled compound to be kept low – appropriate to environmental conditions – as well as incubation times (Boschker *et al.* 1998; Boschker and Middelburg 2002). Fatty acids are important cell components particularly within the cell membrane (double lipid layer). They can be separated into three main groups of membrane lipids: phospho-, glyco-, and neutral lipids. Best suited biomarker molecules among the lipids are phospholipid fatty acids (PLFA). They represent living biomass and additionally allow (to a certain extent) a phylogenetic classification of the analysed community on the basis of distinct PFLA-profiles representing specific phylogenetic organism groups. PLFA-based SIP has implicated more soil microbes in environmental processes of interest than any other form of SIP (see Table 1.5 for a survey), although the phylogenetic resolution of PLFA-SIP is by far not as high as the resolution based on nucleic acids (Manefield *et al.* 2006).

Table 1.5: Survey of chosen biomarkers of certain organisms/organism groups modified according to Zelles (1999), Green and Scow (2000), Boschker and Middelburg (2002), and Kaur *et al.* (2005). Fatty acids are named according to the ω-nomenclature A:BωC with A number of carbon atoms, B number of double bonds, C location of closest double bond starting with aliphatic chain of molecules, c and t stand for *cis* or *trans* configuration of carbon atoms that are bound in the double bond; prefixes *iso* (*i*) and *anteiso* (*a*) mark position of methyl branching.

Organism	Biomarker	References
Bacteria	*i*15:0, *a*15:0, 15:0, 16:1ω5, *i*17:0, 17:0, 18:1ω7	Tunlid and White (1992)
Gram-positives	branched PFLAs (br17:0, br18:0, *i*17:0, *i*16:0, *i*16:1, 10Me16:0, 10Me17:0) *i*15:0, *a*15:0 monounsaturated fatty acids < 20% (16:1ω9, 16:1ω7c, 16:1ω5, 18:1ω7c, 19:1)	Harwood and Russel (1984), O'leary and Wilkinson (1988)
Gram-negatives	Cyclopropan-Fettsäuren (cy17:0, cy19:0) β-hydroxy fatty acids of cell walls monounsaturated fatty acids > 20% (16:1ω9, 16:1ω7c, 16:1ω5, 18:1ω7c, 19:1)	Parker *et al.* (1982), Guckert *et al.* (1985), Wilkinson (1994), White (1988)
Sulphate reducers	*Desulfobacter* (10Me16:0, cy18:0(ω7,8) *Desulfovibrio* (*i*17:1ω7c, *i*15:1ω7c, *i*19:1ω7c) *Desulfobulbus* (17:1ω6, 15:1)	Scheuerbrandt and Bloch, Parker *et al.* (1962), Taylor and Parkes (1983), Edlung *et al.* (1985), Parkes and Calder (1985), Dowling *et al.* (1986)
Methanotrophes	Typ I (16:1ω8c, 16:1ω6c) Typ II (18:1ω8c, 18:1ω8t, 18:1ω6c)	Makula (1978), Nichols *et al.* (1987), Bowman *et al.* (1993), Sundh *et al.* (1995), Börjesson *et al.* (1998)
Actinomycetes	(10Me17:0, 10Me18:0)	
Fungi	polyunsaturated fatty acids (18:2ω6, 18:3ω6, 18:3ω3c)	Federle (1986), Vestal and White (1989), Frostegard and Bååth (1996)
Protozoa	20:2ω6, 20:3ω6, 20:4ω6	White *et al.* (1983), White (1988)
Algaes	20:5ω3, 18:3ω3	

Note that most experiments proving microbial degradation of organic pollutants by using SIP technologies were performed within batch cultures or soil, sediment and groundwater microcosms due to high prices of labelled substrates (Hanson *et al.* 1999; Pelz *et al.* 2001a; Pelz *et al.* 2001b; Mauclaire *et al.* 2003).

Recently, a concept of *in situ* microcosms supplemented with ^{13}C-tracers was developed which opened up the opportunity to use SIP technologies also in the field. Herein used microcosms contain so called Bio-Sep® beads which are composed of activated carbon. These beads are used to adsorb ^{13}C-labelled hydrocarbons. Introduced to groundwater of contaminated aquifers, the beads release the labelled substrates which then may be degraded by indigenous microorganisms. Additionally, the beads function as settlement material for microorganisms. Subsequently, growing microbes can be extracted and analysed for ^{13}C-incorporation. Extensive information on the concept of *in situ* microcosms with Bio-Sep® beads is provided by Stelzer *et al.* (2006). Using this concept, first investigations were performed at a BTEX-contaminated site in order to analyse benzene and toluene

degradation with PLFA-SIP (Geyer *et al.* 2005). Later studies reported its applicability to other substrates and other biomarker molecules (DNA) (Kästner *et al.* 2006; Stelzer 2008).

1.6.2.3 Protein-based stable isotope probing

Next to nucleic acids and fatty acids, proteins can also serve as marker molecules for SIP approaches. Although stable isotope techniques had been widely used, mainly in eukaryotic systems, for quantitative proteomics (for an overview see Ong and Mann (2005)), labelling of proteins in order to identify certain metabolisms is a very recently developed technique. A first report on protein-SIP described the expression of genes involved in the central carbon metabolisation of *Sulfolobus solfataricus* (Snijders *et al.* 2006). The authors used stable isotope-incorporation into proteins provided by a ^{15}N-containing medium to quantify the expression and thus to elucidate the carbon metabolism of the hyperthermophilic crenarchaeon. Jehmlich *et al.* (2008a; 2008b) first reported a protein-SIP-approach to identify proteins expressed during metabolisation of ^{13}C-labelled contaminants. The authors demonstrated the applicability of their method on a pure strain approach by analysing stable isotope incorporation within peptides of *Pseudomonas putida* strain ML2 after ^{13}C-benzene and ^{15}N-ammonium consumption (Jehmlich *et al.* 2008a). The subsequent study proved the method to be transferable to more complex systems by analysing an artificial mixed culture. There, they could identify the responsible species for anoxic toluene degradation after growing on gluconate and $^{13}C_7$-toluene under nitrate reducing conditions (Jehmlich *et al.* 2008b). Stable isotope incorporation into proteins was detected by matrix-assisted laser ionisation/desorption mass spectrometry (MALDI-MS) which measures the change of isotopic distribution resulting in a higher diisotope peak. According to the authors, a ^{13}C-incorporation of 1 – 2 atom% is still detectable. This is a one order of magnitude higher sensitivity compared to RNA/DNA-SIP. A limitation for protein-SIP so far is the availability of protein-sequences for protein-identification. Furthermore, the need of at least 10^6 cells per analysis might restrict applicability for aquifer organisms growing under anaerobic conditions since the cell number is usually very low. However, the protein-SIP-technology promises to be the most suitable methodology for combining a specific metabolic activity and phylogenetic information within microbial communities.

1.7 Outline of the thesis

Although several studies concerning BTEX degradation have been performed (refer to the previous sections in this chapter), there are still gaps in the knowledge about anaerobic BTEX degradation. In particular, the bacteria involved in anaerobic benzene degradation and the benzene degradation pathway have still not been elucidated. The objective of the research presented in this thesis was to gain more insight in the degradation of BTEX compounds in a highly contaminated anaerobic aquifer close to the city Zeitz. In particular, the physiology and phylogeny of the bacteria responsible for the degradation were studied.

Chapter 2 describes a new method to capture and enrich microorganisms of the aquifer able to degrade benzene under sulphate-reducing conditions. Therefore, *in situ* microcosms filled with different solids were initially exposed to the sulphidogenic zone of the aquifer and subsequently cultivated in the laboratory. These cultures were analysed regarding their physiological properties and ecological composition by comparison of microbial community patterns using the PCR based fingerprinting method SSCP and subsequent partial sequence analysis.

Chapter 3 describes the identification of organisms involved in the initial attack of benzene under sulphate-reducing conditions by means of DNA-based SIP and subsequent T-RFLP analysis.

In chapter 4, the extent of carbon and hydrogen isotope fractionation of benzene was determined for aerobic strains with known biodegradation pathways in order to distinguish between isotope patterns for mono- or dioxygenase-catalyzed reactions. Additionally, 2D-CSIA was applied to elucidate benzene biodegradation pathways of a new isolate able to use chlorate as well as oxygen as electron acceptor and for a sulphate-reducing enrichment culture enriched from the aquifer of Zeitz, respectively.

In chapter 5, carbon and hydrogen isotope fractionation of Bss-initiated degradation pathways for xylene isomers were explored in order to obtain information on reaction mechanisms and to assess the variability of isotope fractionation processes associated with Bss. Therefore, mixed cultures enriched from the aquifer of Zeitz and pure cultures were investigated which were able to degrade either *m*-xylene, *o*-xylene, or *p*-xylene under sulphate- or nitrate-reducing conditions.

Chapter 6 investigates air-sparging as a strategy to enhance natural attenuation in the source zone of the test site Zeitz. A combination of different methods (hydrogeochemical groundwater and soil air analyses; electron donor/acceptor monitoring; MPN-analysis; isotope fractionation of degradation products and residual substrate; fatty acid-based stable isotope probing) is used to evaluate the sufficiency of air-sparging and the extent of bioremediation.

Finally, the main conclusions are summarised and discussed in chapter 7.

2

Enrichment of anaerobic benzene-degrading microorganisms by *in situ* microcosms

Steffi Herrmann[1], Sabine Kleinsteuber[2], Thomas R. Neu[3], Hans Hermann Richnow[1], and Carsten Vogt[1]

[1]Department of Isotope Biogeochemistry, UFZ – Helmholtz Centre for Environmental Research, Leipzig, Germany

[2]Department of Environmental Microbiology, UFZ – Helmholtz Centre for Environmental Research, Leipzig, Germany

[3]Department of River Ecology, UFZ – Helmholtz Centre for Environmental Research, Magdeburg, Germany

Published in FEMS Microbiology Ecology, 2008, vol. 63, p. 94-106

Abstract

Microcosms filled with different solids (sand, lava, Amberlite XAD-7) were exposed for 67 days in the sulfidic part of a groundwater monitoring well downstream of the source zone of a benzene-contaminated aquifer and subsequently incubated in the laboratory. Benzene was repeatedly degraded in several microcosms accompanied by production of sulfide, leading to stable benzene-degrading enrichment cultures. In control microcosms without filling material, benzene was initially degraded, but the benzene-degrading capacity could not be sustained. The results indicate that long-term physiologically active benzene-degrading microorganisms were attached to surfaces of the solids. The biodiversity and attachment behavior of microorganisms in the *in situ* microcosms were assessed by confocal laser scanning microscopy and single-strand conformation polymorphism (SSCP) analysis, followed by sequencing of dominant SSCP bands. The microbial community was composed of several different *Bacteria*, representing members of *Clostridia*, *Bacteroidales*, all subgroups of the *Proteobacteria*, *Verrucomicrobia*, *Nitrospira*, *Chloroflexi* and *Chlorobi*. Only a few archaeal sequences could be retrieved from the communities. The majority of phylotypes were affiliated to bacterial groups with a possible functional relationship to the bacterial sulfur cycle, thus indicating that the microbial community in the investigated aquifer zone depends mainly on inorganic sulfur compounds as electron donors or acceptors, a finding that corresponds to the geochemical data.

2.1 Introduction

Petroleum hydrocarbons are frequent soil and groundwater contaminants world wide, mostly caused by leaking pipes and underground fuel tanks. Aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes (BTEX), which make up a substantial percentage of gasoline, are toxic and also mobile in the saturated and vadose zone of an aquifer, due to their relatively high water solubility and volatility, thus creating a major health risk. Among the BTEX compounds, benzene is the most mobile and most toxic (Aksoy 1985). Under aerobic conditions, all BTEX compounds are easily biodegraded by ubiquitous bacteria (van Agteren *et al.* 1998). However, due to the low water solubility and rapid microbial consumption of oxygen, contaminant plumes generally become anoxic. It is now widely accepted that all BTEX compounds can also be mineralized in the absence of oxygen under different electron acceptor conditions (Lovley 2000; Widdel and Rabus 2001). Benzene degradation was observed in laboratory enrichment cultures under methanogenic (Vogel and Grbic-Galic 1986b; Kazumi *et al.* 1997), nitrate-reducing (Nales *et al.* 1998; Burland and Edwards 1999), iron-reducing (Lovley *et al.* 1996; Kazumi *et al.* 1997; Nales *et al.* 1998; Jahn *et al.* 2005; Botton and Parsons 2006) and sulfate-reducing conditions (Edwards and Grbic-Galic 1992; Lovley *et al.* 1995; Kazumi *et al.* 1997; Nales *et al.* 1998; Phelps and Young 1999; Vogt *et al.* 2007). So far, only nitrate-reducing bacterial strains have been reported to metabolize benzene anaerobically (Kasai *et al.* 2006).

The biochemical pathway of anaerobic benzene degradation is still not fully elucidated (Caldwell and Suflita 2000; Ulrich *et al.* 2005).

Despite being known to be degradable, benzene is considered the most resistant of all BTEX compounds under anoxic conditions: the majority of laboratory and field studies failed to demonstrate anaerobic benzene degradation (Aronson and Howard 1997; Johnson *et al.* 2003).

The question arises as to why benzene degradation under anoxic conditions has been only occasionally observed. Results from microcosm studies suggest that anaerobic benzene degraders are not ubiquitous in subsurface sediments (Kazumi *et al.* 1997; Nales *et al.* 1998; Weiner and Lovley 1998a; Phelps and Young 1999). By contrast, it has been shown that the majority of bacteria could not be cultured in the laboratory (Rappe and Giovannoni 2003; Keller and Zengler 2004). The yet uncultured fraction might include anaerobic benzene-degrading microorganisms. In most cases, successful enrichments of anaerobic benzene degraders were established using anoxic sediment taken from petroleum-contaminated sites. These samples contain the major part of microorganisms living in the subsurface (Harvey *et al.* 1984; Kölbel-Boelke *et al.* 1988; Hazen *et al.* 1991; Alfreider *et al.* 1997; Lehmann *et al.* 2000; Griebler *et al.* 2002). Attached or biofilm-forming bacteria were also shown to be taxonomically diverse and physiologically more active compared with microorganisms moving in the water phase of an aquifer (Alfreider *et al.* 1997; Lehmann *et al.* 2000; Griebler *et al.* 2002).

Hence, our work aimed to test whether anaerobic benzene-degrading bacteria can be trapped from groundwater of a monitoring well in a benzene-contaminated aquifer using *in situ* microcosms and solids (sand, lava granulate) as colonization material. Additionally, the biodiversity of the attached microbial communities was assessed by single-strand conformation polymorphism (SSCP) as a cultivation-independent, 16S rRNA gene-based method. The selected test site was an anoxic benzene-contaminated aquifer with large parts of ongoing sulfate reduction. Recently, benzene degradation under anoxic conditions was qualitatively and quantitatively demonstrated at the site (Vieth *et al.* 2005; Fischer *et al.* 2007; Vogt *et al.* 2007).

2.2 Materials and Methods

2.2.1 Description of the field site

The contaminated test site is located in the area of a former coal hydrogenation and benzene production plant near Zeitz (Saxony-Anhalt, Germany). The contamination was caused by several leaks, damage and accidents during the operation of the plant between 1960 and 1990. The site has been intensively investigated in recent years with respect of monitoring of natural attenuation (NA) processes (Vieth *et al.* 2005; Fischer *et al.* 2006; Gödeke *et al.* 2006; Schirmer *et al.* 2006; Stelzer *et al.* 2006; Alfreider and Vogt 2007; Fischer *et al.* 2007; Vogt *et al.* 2007). Two aquifers are present, separated by a lignite and clay layer. Both aquifers are heterogeneous and hydrogeologically connected due to discontinuities of the lignite–clay layer. The aquifers are composed of river gravel

and sand sediments, which contain more than 95% quartz. The general groundwater flow direction is to the north. The groundwater of both the upper and the lower aquifer is heavily contaminated, with benzene concentrations of up to 13 mM in the source zone. Both aquifers are characterized by anoxic conditions. Sulfate, reaching concentrations of 10 mM, is the main electron acceptor at the site (Vieth *et al.* 2005). For the experiments described in this paper, a groundwater monitoring well in the lower aquifer (GWMS 1/99) was investigated. The well is located downstream of the source of contamination, within the sulfidic zone of the plume. Well water from 40 m depth, collected in February 2004, June 2004 and March 2005, contained 40–100 µM benzene as well as trace amounts of toluene, *m*-xylene and *p*-xylene (< 0.06 µM), 4.9 mM sulfate, 0.17 – 0.38 mM sulfide, 0.13 – 0.16 mM ammonium, and 7.3 – 7.5 mM HCO_3.

2.2.2 Preparation of *in situ* microcosms

Twenty-milliliter glass vials were completely filled with coarse sand (grain size: 2 – 3.15 mm; porosity: 0.43; 1 g sand is equal to 0.58 cm^3) or lava granules [volcanic rock from the Eifel region, Germany; under-saturated with silicon oxide (SiO_2); porosity: 0.5; 1 g lava granules is equal to 0.8 cm^3]. In addition, vials filled with sand or lava granules were spiked with 1 g Amberlite XAD-7, an adsorber resin which was successfully used for the enrichment of BTEX-degrading bacteria under different electron acceptor conditions (Morasch *et al.* 2001a; Jahn *et al.* 2005). We did not intend to use Amberlite XAD-7 as colonization material, but instead used it as a reservoir for benzene, in order to provide a stable concentration of benzene inside the microcosm during the *in situ* exposure as well as the following incubation in the laboratory. Control vials either contained only Amberlite XAD-7 or no filling material. Sand and lava granules were washed five times with distilled water to remove organic particles before use; Amberlite XAD-7 was washed as described by Morasch *et al.* (2001a). Amberlite-filled vials were prepared in five replicates, and the other approaches in four replicates (Figure 2.1). All vials were sterilized by autoclaving (20 min, 121°C), completely filled with sterile, anoxic distilled water and closed with Teflon caps and exposed in monitoring well GWMS 1/99 as follows: eight vials were placed in a round plastic jar. Three jars were used and placed one below the other in a steel cage (Figure 2.1). To facilitate the inflow of groundwater, the jars were perforated. Each jar contained at least a single replicate of each approach. Shortly before exposure in the monitoring well, all vials were opened by tongs, and the steel cage was immediately lowered by means of a steel cable to a final depth of 40 m. After an exposure for 67 days, the jars were recovered and the vials were immediately closed with Teflon-coated butyl septa (ESWE Analysentechnik, Gera, Germany). One replicate from each different approach was used for colonization and biodiversity studies; these vials were all taken from the jar which was hanging in the monitoring well right at the bottom (Figure 2.1). For these studies, the microcosms were processed immediately after recovery from the groundwater well. They were divided into three different depth-oriented zones (Figure 2.1). Material for SSCP analyses was frozen at -80°C until further processing; material for confocal laser

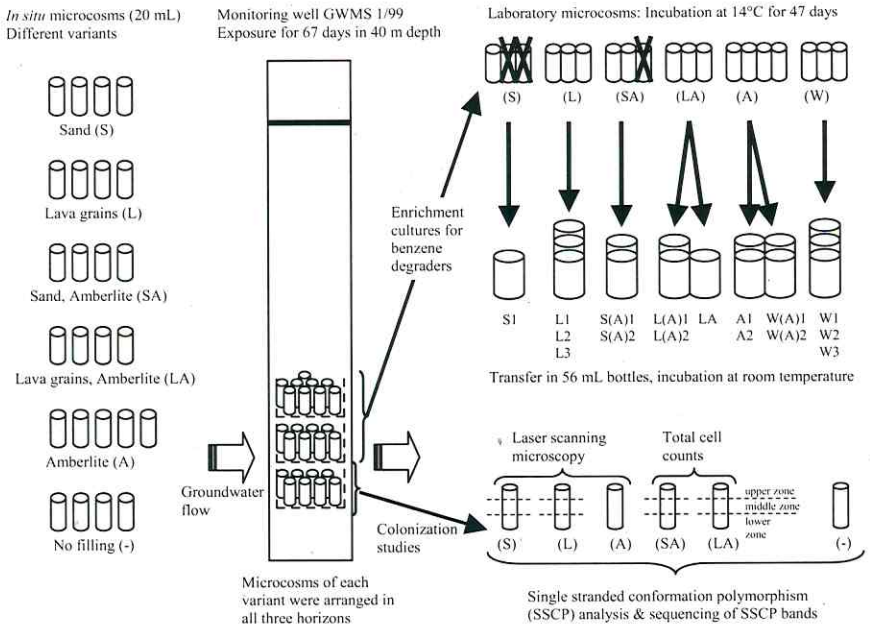


Figure 2.1: Flow chart of the *in situ* microcosm experiment described in this study.

scanning microscopy (CLSM) was anoxically transferred in anoxic mineral salt medium (Vogt *et al.* 2007) and examined under the microscope within 24 h; material for total cell count determinations was processed within 6 h after extraction.

The residual vials were incubated statically in the dark for 47 days at 14°C. Subsequently, the contents of each vial were transferred in larger bottles (56 mL; Glasgerätebau Ochs, Bovenden-Lenglern, Germany) and filled up to a final volume of *c.* 50 mL with anoxic mineral salt medium (Vogt *et al.* 2007) spiked with benzene from an anoxic stock solution (5 mM) to final concentrations of 100 – 200 µM benzene. The microcosms were sampled in regular time intervals for benzene and sulfide concentrations as previously described (Vogt *et al.* 2007) and respiked with benzene if benzene concentrations were low or not detectable. If sulfide concentrations became too high (5 – 8 mM), most of the liquid phase (over three-quarters) was carefully replaced by fresh anoxic mineral salt medium. For evaluating abiotic losses of benzene during the sampling and incubation procedure, sterile controls were prepared using microcosm set up as with each microcosm variant, but autoclaved on 3 consecutive days (121°C, 20 min) before being spiked with benzene. All steps for preparing enrichment cultures were done in an anaerobic glove box (gas atmosphere: 95% nitrogen, 5% hydrogen; Coy Laboratory Products Inc.).

2.2.3 Chemical analyses

BTEX were determined as published elsewhere (Alfreider *et al.* 2003). *m*-Xylene and *p*-xylene could not be separated by the method used and were evaluated as a summed parameter. Sulfide was

determined spectrophotometrically according to Cline (1969) with the following modifications: samples (25 – 200 µL) were dissolved in 1 mL zinc acetate dehydrate solution (20 g L^{-1}) for fixing sulfide immediately after sampling. Subsequently, 4 mL demineralized water and 400 µL N, N-dimethyl-*p*-phenylendiammoniumdichloride (DMPD) were added. After 20 min reaction time absorption values were measured. Concentrations were calculated using standards prepared from an anoxic sulfide stock solution.

2.2.4 Molecular-genetic analyses

DNA was extracted according to Maher *et al.* (2001) either directly from the sand and lava granules or from membrane filters (ISOPORE™ 0.2 µm GTTP, Millipore, Germany) after filtration of groundwater. The V4 – V5 region of the bacterial 16S rRNA gene was amplified using primers UniBac515f (5′-GTGCCAGCAGCCGCG-3′) and 5′-phosphorylated UniBac927r-Ph (5′-Ph-CCCGTCAATTYMTTTGAGTT- 3′). The V4 – V5 region of the archaeal 16S rRNA gene was amplified in a seminested PCR using primers UniArch21f (5′-TTCYGKTTGATCCYGSCRG-3′) and Uni-Arch958r (5′-YCCGGCGTTGAVTCCAATT-3′) for the first PCR and primers UniArch571f-Ph (5′-Ph-GCYTAAAGCRICCGTAGC-3′) and UniArch958r for the second PCR. PCR was performed in 50-µL reaction mixtures containing 25 µL *Taq* PCR Master Mix (Qiagen, Germany), 3 mM $MgCl_2$, 5 pmol of each primer (supplied by MWG Biotech, Germany) and 2 µL diluted DNA in a PTC-200 Thermal Cycler (MJ Research, MA). Cycle conditions for the PCR reactions were as follows: initial denaturation at 94°C for 3 min; 30 cycles at 94°C for 20 s, 53°C for 30 s and 72°C for 60 s; and a final elongation for 10 min at 72°C. After DNA purification (E.Z.N.A.® cycle pure kit, peqLab Biotechnologie GmbH, Germany) 15 µL of purified product was digested with 6 U Lambda exonuclease (New England Biolabs, Germany) within 2 h at 37°C. After addition of 1 volume 2 × SSCP sample buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol FF) the samples were denatured for 2 min at 95°C, immediately chilled on ice for 5 min and stored at -20°C until electrophoresis. SSCP electrophoresis was run on a 0.6% MDE gel (Biozym, Germany) in a TGGE Maxi chamber (Biometra, Germany) in 1 × TBE buffer (Sambrook and Russell 2001) at 400 V and 26°C for 16 – 20 h. The gel was silver stained according to Bassam *et al.* (1991) with the modification that 3% NaOH was used as developing bath.

After drying, the gel was scanned and the image was analyzed using the PHORETIX 1D software (Nonlinear Dynamics, UK). A hierarchical cluster analysis of the SSCP pattern was performed applying the Jaccard similarity index and the UPGMA algorithm for calculating a similarity dendrogram.

Bands of interest were cut out from the gel and the DNA was extracted as described previously (Frost and Guggenheim 1999). The extraction procedure was repeated, the supernatants were pooled, and DNA was precipitated with ethanol and glycogen (Sambrook and Russell 2001) and dissolved in 10 µL bidistilled water. For reamplification, 1 – 4 µL of the DNA was applied as template in a 50-µL

reaction mixture as described above, with the exception that the reverse primer UniBac927r was not phosphorylated and 2.5 µL dimethyl sulfoxide was added. Reamplification was performed with 35 cycles according to the protocol described above. After agarose gel electrophoresis and purification the PCR products were used for direct sequencing or cloning. Cloning was done using the PCR Cloning^{plus} Kit (Qiagen) according to the suppliers' protocol. Recombinant clones were picked and screened for the appropriate insert size by PCR using the vector-specific primers M13uni(-21) and M13rev(-29). DNA sequencing of cloned 16S rRNA gene amplicons was performed using the BigDye RR Terminator AmpliTaq^{TM} FS Kit version 3.1 (Applied Biosystems, Germany) and the vector-specific primers M13uni(-21) and M13rev(-29), respectively, as sequencing primers. Direct sequencing of reamplified SSCP bands was done with primers 530f (Lane, 1991) and UniBac927r. Capillary electrophoresis and data collection were carried out on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Data were analyzed via ABI PRISM® DNA sequencing analysis software, and sequences of both complementary strands were assembled by the ABI PRISM® AUTOASSEMBLER software. The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST; (Altschul *et al.* 1990)) was used to search for similar sequences in the GenBank database, and the Sequence Match tool was used to search for similar sequences compiled by the Ribosomal Database Project – II Release 9.4 (http://rdp.cme.msu.edu; (Cole *et al.* 2005)). The 16S rRNA gene sequences determined were deposited in the GenBank database (www.ncbi.nlm.nih.gov/Genbank) under accession numbers EF417493 – EF417542.

2.2.5 Confocal laser scanning microscopy (CLSM)

Native sand, lava granules and Amberlite particles were first examined without any staining in order to exclude autofluorescence. The particles were then placed in a 5-cm Petri dish and stained with a few droplets of SYTO 9 (Molecular Probes, Eugene, OR). After 5 min the Petri dish was flooded with water and immediately subjected to CLSM. The samples were examined using a true confocal scanner spectral photometer (TCS SP1) in combination with an upright microscope (Leica, Heidelberg, Germany). The instrument was controlled by the Leica confocal software, version 2.61 Build 1537174191. Biofilm samples were observed with × 20 0.4-NA and × 63 0.9-NA water-immersible lenses. Excitation was done with the 488-nm line of an argon laser. Emission signals were recorded at 480 – 490 nm (reflection of particles) and 500 – 550 nm (SYTO 9 nucleic acid stain). Images were projected using the microscope software and IMARIS version 4.06 (Bitplane, Zurich, Switzerland).

2.2.6 Determination of total cell counts

Total cell counts for bacteria attached to surfaces were determined by the method of Griebler *et al.* (2001).

2.3 Results and discussion

2.3.1 Enrichment of benzene-degrading microorganisms

The goal of our study was to establish anaerobic benzene-degrading enrichment cultures starting from the colonized sand particles and lava granules inside the *in situ* microcosms after exposure. This was done in order to test the hypothesis that anaerobic benzene-degrading microorganisms living at the Zeitz site prefer a sessile life style. We came to this hypothesis for several reasons. First, mineralization of benzene under sulfate-reducing conditions in columns percolated with groundwater from the lower aquifer of the site had been shown (Vogt *et al.* 2007). These columns were filled with coarse sand or lava granules. Furthermore, *in situ* microcosm experiments using ^{13}C-labeled activated carbon as colonization material indicated that benzene and toluene can be metabolized under *in situ* conditions in the upper aquifer (Geyer *et al.* 2005; Stelzer *et al.* 2006). By contrast, microcosms comprising only groundwater taken from several different monitoring wells of the upper and lower aquifer of the Zeitz site have not been degrading benzene for years (data not shown), indicating that the benzene degraders cannot develop under those conditions. Generally, the cultivation of benzene-degrading bacteria under sulphate-reducing conditions is difficult. Only a few anaerobic benzene-degrading enrichment cultures have been described hitherto that use sulfate as terminal electron acceptor. These were all enriched from anaerobically incubated sediment or sludge samples (Edwards and Grbic-Galic 1992; Lovley *et al.* 1995; Phelps *et al.* 1996; Kazumi *et al.* 1997; Nales *et al.* 1998; Phelps and Young 1999; Vogt *et al.* 2007). This also indicates that the initial enrichment of anaerobic benzene degraders under sulfate-reducing conditions might be facilitated by the presence of surfaces, thus allowing the attachment of bacteria.

In the sand-filled microcosms, approximately the upper half of the sand particles became black-colored, indicating iron sulfide coatings and, hence, sulfidic conditions during exposure. Sulfide could either originate from the surrounding sulfidic groundwater or be produced *in situ* by sulfate-reducing bacteria inside the microcosms. The same blackening effect has been observed in benzene-degrading, sulfate-reducing reactors filled with sand (Vogt *et al.* 2007). After retrieval from the well, benzene concentrations inside the vials varied between 10 and 100 µM (data not shown). One to four replicates of each variant were incubated at 14°C in the laboratory for another 47 days (Figure 2.1). Three sand-filled microcosms (one with additional Amberlite XAD-7) broke immediately after retrieval, and thus only a single sand-filled vial and two vials filled with sand and Amberlite were incubated further. Benzene concentrations in all of the vials except the two vials without filling material decreased between 50% and 100% in the next 47 days (data not shown). In the next step, the content of each vial was transferred to 56-mL serum bottles, which were topped up with benzene-spiked anoxic mineral salt medium. To trace benzene degradation kinetics precisely, the adsorber resin Amberlite XAD-7 was not transferred except from two sand- and lava-free, but Amberlite-filled vials and from one of the lava granulate/Amberlite-filled vials (Figure 2.1).

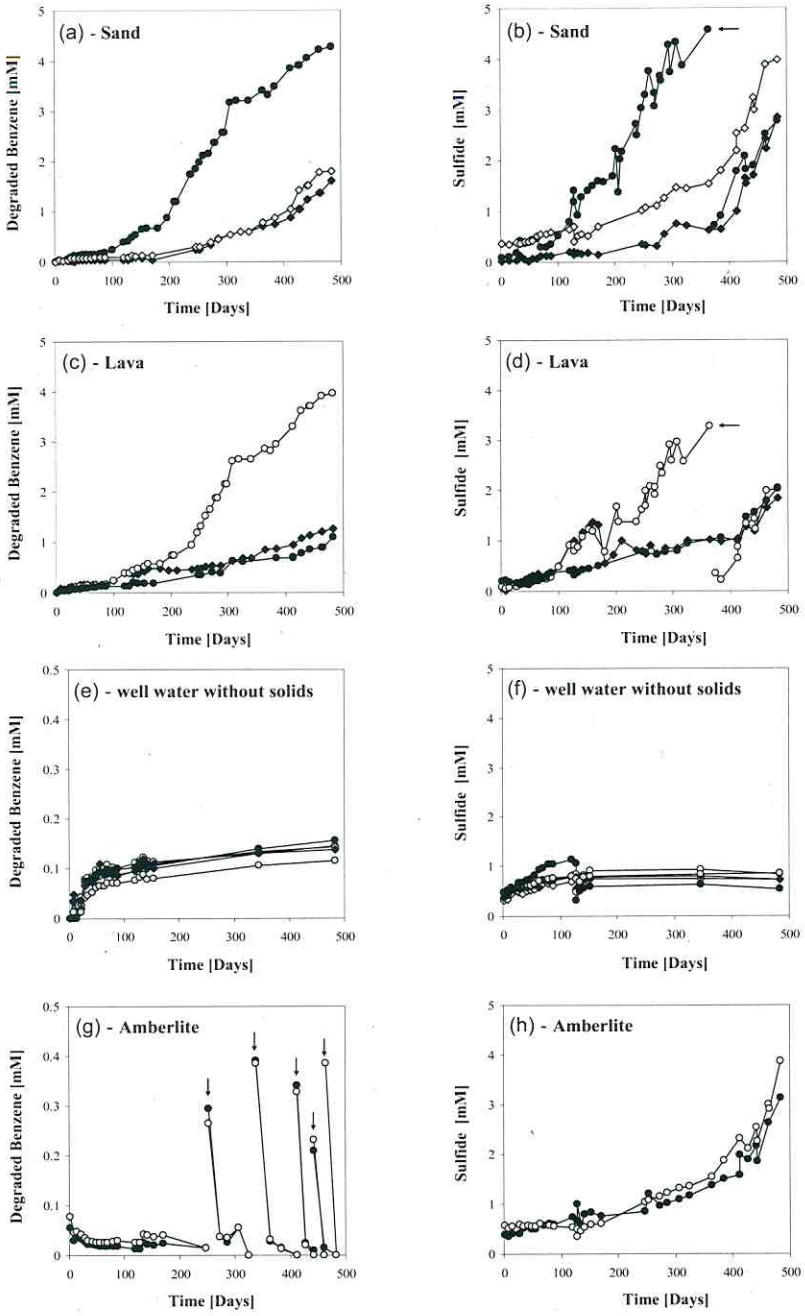


Figure 2.2: Time courses for benzene degradation (diagrams on the left side: a, c, e, g) and sulfide production (diagrams on the right side: b, d, f, h) in several individual laboratory microcosms within the first 483 days of incubation. (a, b) Microcosms filled with coarse sand: S1, black circles; S(A)1, black diamonds; S(A)2, white diamonds; (c, d) microcosms filled with lava granules: L(A)1, black circles; L3, white circles; L1, black diamonds; (e, f) microcosms without filling material (groundwater mixed with mineral salt medium): W1 – W3, circles in black, gray or white; W(A)1, white diamonds; W(A)2, black diamonds; (g, h) microcosms filled with Amberlite XAD-7: A1, black circles; A2, white circles. Exchange of anoxic mineral salt medium (for lowering the sulfide concentrations) is indicated by arrows. Benzene data are cumulative except for diagram (g).

Thereafter, all microcosms were incubated from this time at room temperature in the dark. Stable benzene-degrading, sulfide-producing enrichment cultures were established from all *in situ* microcosms filled with sand and from three microcosms filled with lava granules within a year (Figure 2.2a-d). Lag-phases and benzene degradation rates differed considerably between the microcosms during this initial stage of enrichment. One sand-filled microcosm (S1) and one lava-filled microcosm (L3) started earlier and degraded benzene faster than their respective counterparts; in these two microcosms degradation rates increased up to more than 20 μM day^{-1} in the course of the enrichment (Figure 2.2a and c). Attempts to establish a planktonic benzene-degrading culture using cells previously removed from the surfaces of the solids failed (data not shown). Precise time courses for benzene degradation were determined for all stable benzene-degrading enrichments 780 days after their set up in the laboratory (Figure 2.3). After these 780 days, the degradation rates leveled off between 2 and 8 μM benzene day^{-1}. Abiotic losses of benzene during sampling and incubation were negligible, as confirmed by sterile controls. Before beginning the experiment, the lava granules of one single microcosm were divided into three, thus leading to three new lava granule-filled microcosms, each containing only one-third of the original colonization material (Figure 2.4a: L1, L1a and L1b). These microcosms degraded benzene with similar rates. Hence, it is possible to set up new enrichments by simply splitting the solid material. The rates for benzene degradation are comparable with those already described for benzene-degrading microcosms under sulfate-reducing conditions, established from filling material of benzene-degrading columns running at the site (Vogt *et al.* 2007), and also comparable with those reported by others for benzene-degrading microcosms under sulfate-reducing conditions (Edwards and Grbic-Galic 1992; Nales *et al.* 1998). Benzene degradation in the sand- and lava-filled microcosms was always accompanied by sulfide production.

During the first 483 days of incubation, the sulfide to benzene ratio was only 36.5 – 65.2% of that expected for the mineralization of benzene with sulfate as electron acceptor (Table 2.1), a reaction described by the following theoretical equation:

$$C_6H_6 + 3H_2O + 3.75SO_4^{2-} \rightarrow 6HCO_3^- + 1.88HS^- + 1.87H_2S + 0.37H^+ \qquad \text{eq. 2.1}$$

Some of the sulfide produced might have been precipitated as FeS or Fe_2S coatings on sand particles or lava granules, as previously observed (Vogt *et al.* 2007), which would at least partially explain the unbalanced stoichiometry between benzene and sulfide in this early stage of enrichment. In contrast, more sulfide than theoretically expected was released in six out of eight sand- and lava-filled microcosms after 780 days of incubation (Table 2.2). This is probably caused by hydrogen, which entered the bottles during the preparation in the glove box, assuming that no sulfide precipitated in this later phase of enrichment. The benzene-degrading bacterial community tolerated sulfide in concentrations of up to 7.6 mM (Table 2.2), a value which has sometimes been observed as being toxic for BTEX-degrading, freshwater sulfate-reducing species (Beller and Reinhard 1995; Morasch *et al.* 2004b).

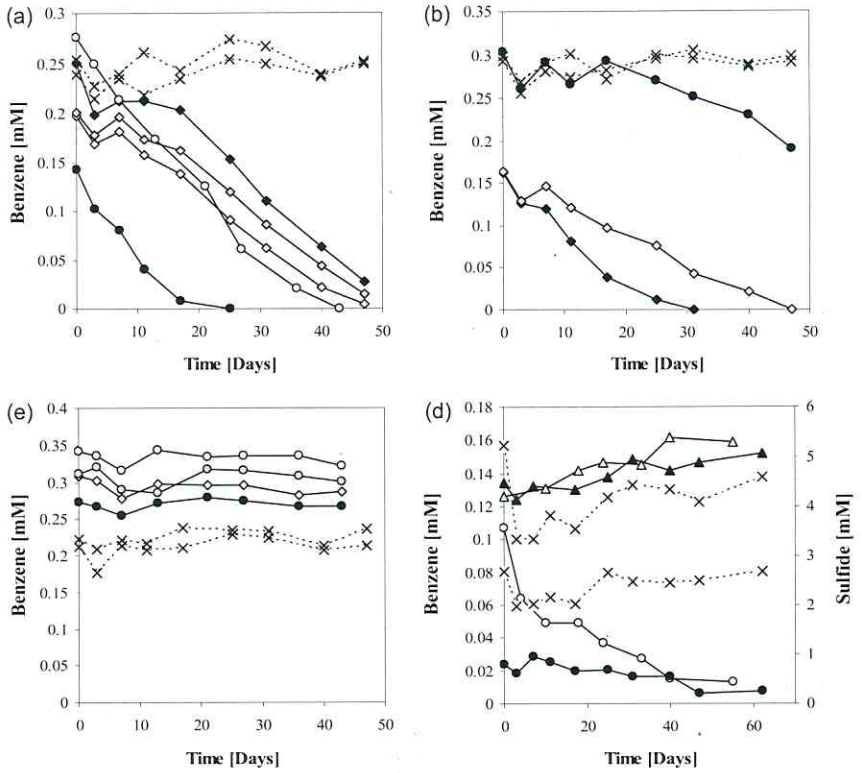


Figure 2.3: Time courses for benzene concentrations in individual laboratory microcosms established from different *in situ* microcosms. The experiment was carried out 780 days after set up of the microcosms. (a) Microcosms filled with lava granules: L(A)1, black circles; L3, white circles; L1, black diamonds; L1a, white diamonds; L1b, gray diamonds; (b) microcosms filled with coarse sand: S1, black circles; S(A)1, black diamonds; S(A)2, white diamonds; (c) microcosms without filling material (groundwater mixed with mineral salt medium): W1 – W3, circles in black, gray or white; W(A)1, white diamonds; (d) microcosms filled with Amberlite XAD-7: A1, benzene = black circles, sulphide = black triangles; A2, benzene = white circles, sulphide = white triangles. Data for sterile controls for each variant (in duplicate; only for benzene concentrations) are indicated by dashed lines and crosses.

The enrichments made of only well water mixed with mineral salt medium – three of them prepared from *in situ* microcosms without filling material (W1 – W3) and two of them prepared from *in situ* microcosms originally equipped with Amberlite XAD-7 [W(A)1 and W(A)2] – initially produced sulfide and degraded up to 100 µM benzene. After *c.* 50 days, benzene degradation slowed and benzene and sulfide concentrations remained almost stable from that time on (Figure 2.2e and f). Benzene concentrations remained constant in the well water enrichments during the degradation experiment set up after 780 days of incubation (Figure 2.3c), indicating that it was not possible to enrich benzene-degrading bacteria under sulfate-reducing conditions in 'empty' bottles within more than 2 years.

In the two bottles equipped only with Amberlite XAD-7, respiked benzene repeatedly disappeared in both enrichments after an incubation for more than 200 days and was correlated with increasing sulfide concentrations, indicating the degradation of benzene under sulfate-reducing conditions

(Figure 2.2g and h, and 2.3d). Here, calculating degradation rates was not possible due to sorption of benzene to the resin. Thus, besides sand and lava granules, also Amberlite XAD-7 beads facilitated the enrichment of benzene degraders under sulfate-reducing conditions. It is not clear how the Amberlite beads interact with the anaerobic benzene degraders. The beads could be colonized by microorganisms including benzene degraders, as observed for the sand and lava granules, supporting the hypothesis that the benzene-degrading bacteria are generally favored by the attachment to surfaces. However, the colonization of Amberlite beads could not be confirmed by CLSM. Amberlite causes low and stable benzene concentrations ((Morasch *et al.* 2001a; Jahn *et al.* 2005); Figure 2.2h and 2.3), which might also be beneficial for the development of anaerobic benzene degraders.

2.3.2 Microbial colonization of sand and lava granules during *in situ* exposure

For colonization examination, the particles were taken from three different depth-oriented zones in a single microcosm variant (Figure 2.1). Sand and lava granules taken from the upper microcosm zone were most densely colonized by microorganisms, compared with particles from the middle and lower microcosm zones. This was confirmed by CLSM (Figure 2.4) and total cell count determinations. We did not examine the same microcosm variant by CLSM and total cell count determinations (see Figure 2.1); therefore, the results for the two methods are not directly comparable, although they corresponded quite well. Total cell counts ranged from 3.46×10^5 cells g^{-1} (upper zone, lava granules) to 8.4×10^4 cells g^{-1} (lower zone, sand particles). Lava granules were slightly more colonized than sand particles (Figure 2.4); this might be due to the high surface area of the lava granules, which support the attachment of bacteria. Attached bacteria differed in shape and size and were distributed mainly as single cells. Using CLSM, no microorganisms were observed on Amberlite XAD-7 spheres (data not shown). Either Amberlite XAD-7 was not colonized *in situ* or the attached microorganisms dropped completely off from the Amberlite spheres during preparation for the CLSM. The unequal distribution of cells in the lava- and sand-filled microcosms is reasonable due to the general set-up of the experiment: the vials were open only at one side, so that microorganisms had to cross a distance of *c.* 4 cm to reach the middle zone and up to 7 cm to reach the lower zone. The vial set-up was chosen in order to exclude any oxygen contamination after retrieval from well exposure, as traces of oxygen could poison or even kill obligate anaerobic bacteria, including anaerobic benzene degraders. The cell counts reported here are lower than those observed by Griebler *et al.* (2002) for the *in situ* colonization of sterile sediment samples in three different types of oxic groundwater, ranging from ~10^7 to ~4×10^8 cells cm^{-3} after 6 weeks of exposure. An important finding of Griebler *et al.* (2002) is that in all groundwater variants the sediments were maximally colonized after 6 – 10 weeks of exposure; therefore, the exposure time used in our study (67 days) might be long enough to trap a major part of the surrounding bacterial community.

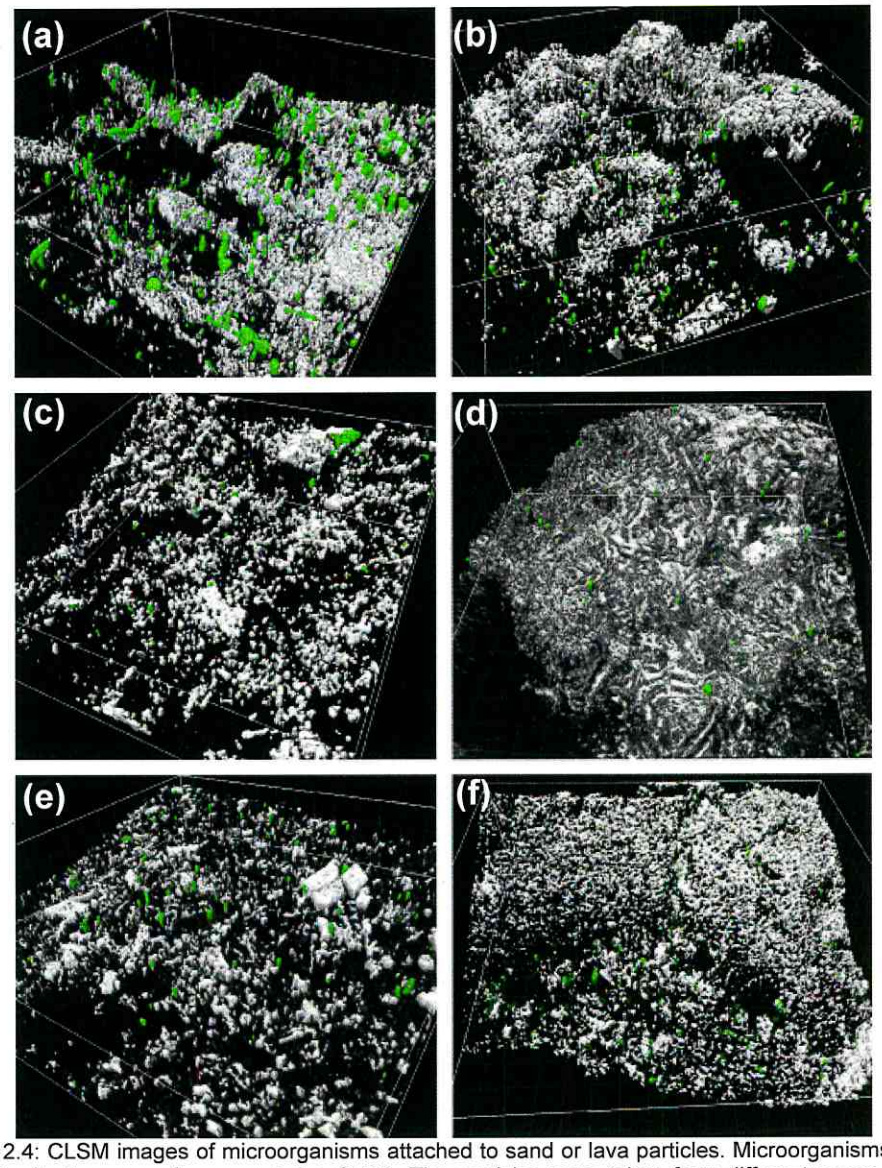


Figure 2.4: CLSM images of microorganisms attached to sand or lava particles. Microorganisms were stained with the green fluorescent dye Syto9. The particles were taken from different zones of the respective *in situ* microcosm (see Figure 2.1). (a) Lava, upper horizon; (b) sand, upper horizon; (c) lava, middle horizon; (d) sand, middle horizon; (e) lava, lower horizon; (f) sand, lower horizon (Grid size = 10 µm).

Table 2.1: Stoichiometries of degraded benzene and produced sulfide in three lava granule-containing [L1, L3, L(A)1], three coarse sand-containing [S1, S(A)1, S(A)2] and five solid-free microcosms (W) during the first 483 days of incubation (see also Figure 2.2). For the five solid-free microcosms, the mean average and standard deviation are given.

Microcosm	L1	L3	L(A)1	S1	S(A)1	S(A)2	W*
Benzene oxidized (mM)	1.08	3.56	1.1	4.13	1.24	1.6	0.14±0.02
Sulfide produced (mM)	2.63	4.87	2.02	7.84	2.64	3.66	0.7±0.11
Sulfide produced of theoretical amount (%)†	65.2	36.5	49	50.7	56.8	61.1	134.2±16

*Mean average and SD of the microcosms W1 – W3, W(A)1 and W(A)2.

†According to the following equation: $C_6H_6 + 3\ H_2O + 3.75\ SO_4^{2-} \rightarrow 6\ HCO_3^- + 1.87\ HS^- + 1.88\ H_2S + 0.37\ H^+$

Table 2.2: Benzene degradation rates and stoichiometries of oxidized benzene and produced sulfide in five lava granule-containing [L1, L3, L(A)1, L1a, L1b] and three sand-containing [S1, S(A)1, S(A)2] microcosms. The experiment was carried out 780 days after set-up of the microcosms (see also Figure 2.3).

	L1	L3	L(A)1	L1a*	L1b*	S1	S(A)1	S(A)2
Benzene oxidized (mM)	0.224	0.25	0.136	0.163	0.186	0.114	0.163	0.164
Benzene degradation rate (µM day^{-1})	4.8	7.7	7.9	3.5	4.0	2.4	6.1	3.5
Sulfide (mM) before adding benzene	4.91	4.91	4.53	3.12	3.82	4.76	5.71	6.03
Sulfide (mM) after consumption (> 90%) of benzene	1.47	0.84	5.64	3.95	4.56	5.37†	7.36	7.61
Sulfide produced (mM)	1.47	0.84	1.11	0.83	0.74	0.61	1.65	1.58
Sulfide produced of theoretical amount (%)‡	175	90	218	136	106	144	285	258

*Subcultures of microcosm L1 (see text).
†Only 37% benzene oxidized (see Figure 2.3b).
‡According to the following equation: $C_6H_6 + 3\ H_2O + 3.75\ SO_4^{2-} \rightarrow 6\ HCO_3^- + 1.87\ HS^- + 1.88\ H_2S + 0.37\ H^+$

2.3.3 Phylogenetic diversity of colonizing microbial communities

Bacterial 16S rRNA gene amplicons were retrieved from all but the middle zone of the sand-filled microcosm. Most SSCP bands appeared from well water and lava granule samples (Figure 2.5), indicating that lava granules were colonized by a higher number of phylotypes than sand particles. Cluster analysis of SSCP patterns confirmed that the community in well water was at most 40% similar to the communities found in all samples of colonization material (data not shown); hence, the lava and sand particles were apparently colonized not by all, but by specific bacteria of the surrounding groundwater. The lava variants showed the highest similarity within the different depth zones in the same microcosms, indicating that most phylotypes attached to lava granules could indeed reach deeper zones of the microcosms within the 67 days of exposure. In the sand variants, the SSCP patterns of the different zones were less similar. However, the upper horizons of both sand- and lava-filled microcosms showed nearly identical SSCP patterns, indicating that (1) both sand and lava granules could in principle be colonized by the same phylotypes, and (2) the differences in SSCP patterns of sand and lava microcosms could be due to the limited transport of bacteria in deeper zones of the sand microcosms. In SSCP patterns of the Amberlite-spiked microcosms, a specific band was observed in five out of six samples (Figure 2.5, lanes 7 – 12), which was found to represent a phylotype affiliated to the sulfate reducer genus *Desulfomicrobium* (Table 2.3).

From the SSCP gel, 22 different bands (indicated by numbers) were reamplified and sequenced (Figure 2.5). The results are shown in Table 3. Fifteen bands could be assigned to a single phylotype. Seven bands could be sequenced only after cloning; here, the sequencing results revealed the presence of more than one phylotype (2 – 4 sequences). Five cut bands could not be reamplified, and two of the cloned sequences (BAC8-3 and BAC10-3) were not evaluated because of low sequencing quality. In most cases, bands at identical positions from different microcosm variants represented nearly identical phylotypes; thus, phylogenetic information could be, at least to some extent, transferred to unidentified bands in identical positions. Bacterial sequences were related to several phylogenetic groups, including members of the *Clostridia*, *Bacteroidales*, all subgroups of the *Proteobacteria*, as well as *Verrucomicrobia*, *Nitrospira*, *Chloroflexi* and *Chlorobi*. Similarities to known phylotypes ranged from 84% to 100% BLAST identity (Table 2.3).

Table 2.3: Sequencing results of SSCP bands from bacterial 16S rRNA amplicons.

SSCP clone (bp) and origin of the DNA	Accession no.	Highest BLAST hit (accession no.)/Identity	Taxonomic affiliation according to RDP (S_{AB})
BAC1 (411), LA-u	EF417502	*Desulfomicrobium* sp. STP16 (AJ006610)/99%	*Desulfomicrobium* sp. (0.967)
BAC2 (411), LA-m	EF417503	*Desulfomicrobium* sp. STP16 (AJ006610)/99%	*Desulfomicrobium* sp. (0.967)
BAC3 (399), LA-l	EF417504	*Desulfomicrobium* sp. STP16 (AJ006610)/100%	*Desulfomicrobium* sp. (0.984)
BAC4 (378), L-l	EF417505	*Acetobacterium wieringae* (X96955)/100%	*Acetobacterium* sp. (1.000)
BAC5 (407), S-l	EF417506	*Acetobacterium wieringae* (X96955)/99%	*Acetobacterium* sp. (0.982)
BAC6 (392), L-m	EF417507	Uncultured *Cytophagales* bact. VIBAC-1 (AJ240007)/100%	Phylum *Bacteroidetes* (0.900)
BAC7 (336), LA-u	EF417508	Uncultured *Cytophagales* bact. VIBAC-1 (AJ240007)/99%	Phylum *Bacteroidetes* (0.877)
BAC8-1 (414), L-m	EF417509	Candidate division OD1 clone 02D2Z76 (DQ330672)/84%	Unclassified *Bacteria* (0.491)
BAC8-2 (410), L-m	EF417510	*Desulfovibrio* sp. Mlhm (AF193026)/99%	*Desulfovibrio* sp. (0.965)
BAC8-4 (410), L-m	EF417511	Uncultured bacterium HDBW-WB42 (AB237705)/95%	Phylum *Bacteroidetes* (0.792)
BAC8-5 (410), L-m	EF417512	Uncultured *Cytophagales* bact. VIBAC-1 (AJ240007)/98%	Phylum *Bacteroidetes* (0.834)
BAC9-1 (396), L-l	EF417513	Uncultured bacterium U35-5 (DQ137939)/93%	*Magnetobacterium* sp. (0.693)
BAC9-2 (417), L-l	EF417514	Uncultured δ-proteobacterium MSB-5D12 (DQ811831)/88%	*Bacteria* (0.515)
BAC9-3 (386), L-l	EF417515	*Desulfocapsa* sp. Cad626 (AJ511275)/98%	*Desulfocapsa* sp. (0.934)
BAC10-1 (410), L-m	EF417516	Uncultured *Sphingobacterium* (AY510256)/94%	Unclassified *Bacteroidales* (0.734)
BAC10-2 (401), L-m	EF417517	Uncultured α-proteobacterium (AJ583167)/99%	Unclassified *Alphaproteobacteria* (0.962)
BAC10-4 (410), L-m	EF417518	*Spirochaeta zuelzerae* (M88725)/96%	*Spirochaetaceae* (0.769)
BAC10-5 (414), L-m	EF417519	Uncultured *Chloroflexi* bacterium (DQ463707)/87%	Unclassified *Bacteria* (0.506)
BAC11 (406), W	EF417520	Uncultured bacterium SO3 (AF507684)/92%	Unclassified *Bacteria* (0.612)
BAC12 (390), WA	EF417521	Uncultured ε-proteobacterium FC1_c118 (DQ295648)/99%	*Arcobacter* sp. (0.984)
BAC13 (406), WA	EF417522	*Acetobacterium wieringae* (X96955)/99%	*Acetobacterium* sp. (0.980)
BAC14-1 (415), L-m	EF417523	Uncultured bacterium AD_C08 (AY644475)/96%	Unclassified *Clostridiales* (0.476)
BAC14-2 (421), L-m	EF417524	Uncultured bacterium KNA6-NB29 (AB179680)/91%	Unclassified *Bacteria* (0.489)
BAC14-3 (410), L-m	EF417525	*Spirochaeta zuelzerae* (M88725)/97%	*Spirochaetaceae* (0.812)
BAC14-4 (412), L-m	EF417526	Uncultured bacterium SR_FBR_L83 (AY340841)/96%	Unclassified *Bacteria* (0.760)
BAC15 (414), W	EF417527	Uncultured δ-proteobacterium (AY921969)/99%	*Geobacter* (0.970)
BAC16 (409), W	EF417528	Uncultured ε-proteobacterium FC1_c118 (DQ295648)/99%	*Arcobacter* sp. (0.973)
BAC17-1 (414), W	EF417529	*Idiomarina ramblicola* (AY526862)/97%	*Idiomarina* sp. (0.889)
BAC17-2 (415), W	EF417530	Uncultured bacterium SO-07 (AB181500)/97%	Unclassified γ-*proteobacteria* (0.843)
BAC18 (381), W	EF417531	Uncultured bacterium SLB220 (DQ787680)/99%	*Sulfuricurvum* sp. (0.984)
BAC19-1 (413), LA-u	EF417532	Uncultured bacterium FW48 (AF524014)/98%	*Anaerolinea* sp. (0.910)
BAC19-2 (413), LA-u	EF417533	Uncultured soil bacterium PBS-III-26 (AJ390455)/99%	Unclassified *Bacteria* (0.650)
BAC19-3 (410), LA-u	EF417534	*Desulfocapsa* sp. Cad626 (AJ511275)/98%	*Desulfocapsa* sp. (0.919)
BAC19-4 (414), LA-u	EF417535	*Clostridium pascui* DSM 10365 (X96736)/96%	*Clostridium* (0.484)
BAC19-5 (410), LA-u	EF417536	Uncultured bacterium 1013-1-CG48 (AY532556)/98%	*Anaerophaga sp. (0.915)*
BAC20-1 (414), S-u	EF417537	*Desulfocapsa* sp. Cad626 (AJ511275)/99%	*Desulfocapsa* sp. (0.975)
BAC20-2 (414), S-u	EF417538	Uncultured bacterium PBS-III-26 (AJ390455)/99%	Unclassified *Bacteria* (0.638)
BAC20-3 (413), S-u	EF417539	*Acinetobacter lwoffii*^{T} DSM2403 (X81665)/100%	*Acinetobacter* sp. (1.000)
BAC20-4 (410), S-u	EF417540	Uncultured bacterium; 1013-1-CG48 (AY532556)/99%	*Anaerophaga* sp. (0.957)
BAC21 (335), LA-l	EF417541	Uncultured bacterium lka12a (EF467585)/97%	*Desulfocapsa* sp. (0.889)
BAC22 (416), S-l	EF417542	Uncultured bacterium lka12a (EF467585)/99%	*Desulfocapsa* sp. (0.950)

In the case that more than one database entry displayed the highest BLAST score or S_{AB} value, only one representative is given. The similarity score S_{AB} represents the number of (unique) oligomers shared between the query sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences. The sequenced bands are indicated by numbers in Figure 2.5; each number corresponds to the numbers of each SSCP clone given in the table. Some bands were composed of two or more different sequences; these sequences are indicated by subnumbers beginning with 1 (*e.g.* BAC8-1, BAC8-2). For clarity, in the first row of the table the source of the sequence is also indicated. L, lava; S, sand; A, Amberlite; W, well water; u, upper horizon; m, middle horizon; l, lower horizon.

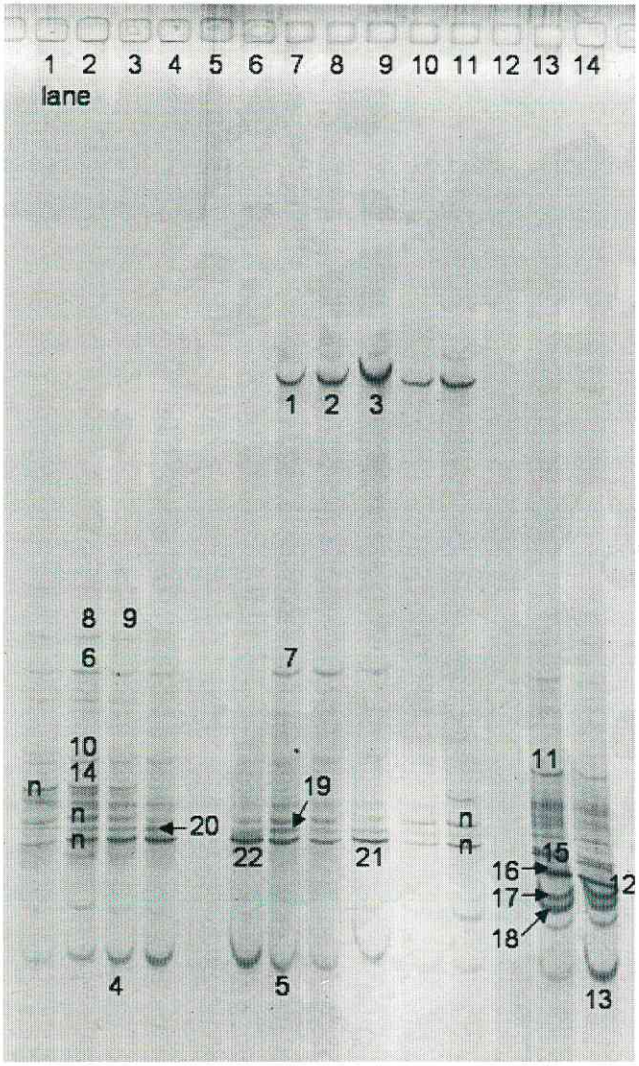


Figure 2.5: SSCP patterns of V4 – V5 regions of the bacterial 16S rRNA genes. Lanes 1 – 3: lava; upper, middle and lower microcosm zone; 4 – 6: sand; upper, middle and lower zone; 7 – 9: lava+Amberlite XAD-7; upper, middle and lower zone; 10 – 12: sand+Amberlite XAD-7; upper, middle and lower zone; 13: well water; 14: well water+Amberlite XAD-7. Numbers 1 – 22: cut, reamplified and sequenced bands (see Table 2.3); n: unreamplifiable bands.

Several groups of *Deltapoteobacteria*, at least three of them highly similar to known sulfate-reducing bacteria, were identified: *Desulfomicrobium*, *Desulfovibrio*, *Desulfocapsa, Geobacter* and *Syntrophobacteraceae*. The presence of various phylotypes of sulfate reducer underlines the importance of sulfate as terminal electron acceptor in the investigated aquifer zone. Four sequences are related to the freshwater species *Desulfocapsa thiozymogenes* (Table 2.3: BAC9-3, BAC19-3, BAC21 and BAC22). *Desulfocapsa thiozymogenes* is known for its ability to disproportionate inorganic sulfur compounds to sulfide and sulfate (Janssen et al., 1996); hence, besides sulfate reduction, the disproportionation of inorganic sulfur compounds might play a distinct role as a

bacterial metabolic process *in situ*. We also found two different phylotypes representing *Epsilonproteobacteria*, a bacterial group which comprises a large number of uncultured representatives, but which has recently been recognized as ubiquitous, especially in sulfidic habitats (Campbell *et al.* 2006). As phylotypes related to sulfur-driven processes were frequent, we conclude that the bacterial communities of the microcosms and surrounding groundwater are highly adapted to the use of inorganic sulfur compounds as electron acceptors or donors. Besides sulfate reducers, other obligate anaerobic bacteria were detected: members of the genera *Anaerolinea*, *Anaerophaga* and *Acetobacterium*. The genus *Acetobacterium* indicates that acetogenesis might also occur in the investigated aquifer zone. However, considering the physiological heterogeneity of most bacterial groups, we cannot definitely derive their possible function or impact in the system based exclusively on analysis of their 16S rRNA gene sequences.

Archaeal 16S rRNA genes could be amplified from all zones of the lava microcosms, from the lava plus Amberlite approach (upper and middle zone), from sand (only upper zone), from sand plus Amberlite (only lower zone), from well water and from well water plus Amberlite. SSCP patterns of archaeal 16S rRNA gene amplicons showed only a few, nonspecifically distributed bands (data not shown), indicating that archaea did not play a major role in the microbial communities of the microcosms. Nine different bands were reamplified and sequenced; the phylotypes were related to uncultured archaea, and some of them showed only low similarity to any known sequence (data not shown).

A striking feature of the identified sequences is that the majority represent phylotypes that have not yet been cultivated. Additionally, several phylotypes showed only low similarity to known bacterial species. At first glance, this is not surprising, as the majority of bacteria in environmental samples cannot yet be cultivated, but detected by molecular–biological methods only; furthermore, the entire bacterial diversity is currently not understood (Pace 1997; Hugenholtz *et al.* 1998). The microcosm method used in this study might enable the detection of groundwater bacteria that are adapted to a sessile lifestyle and therefore are not detectable by conventional groundwater sampling.

In summary, the results suggest that it could be favorable to use solids for the *in situ* enrichment of anaerobic benzene-degrading bacteria, a strategy that might be generally useful for the cultivation of bacteria today considered to be hardly or 'not cultivable'.

Acknowledgements

This work forms part of the internal research and development program of the UFZ as well as the SAFIRA project. We thank Jörg Ahlheim, Ralf Trabitzsch and Werner Kletzander of the Department of Groundwater Remediation for help with the field experiment, and Kathleen M. Schleinitz for proofreading of the manuscript. Special thanks are addressed to Prof. Michael Schlömann (Technical University of Freiberg, Germany) for giving S.H. the opportunity to carry out her Diploma Thesis at

the Helmholtz Centre for Environmental Research. Part of the work of S.H. was funded by the BMBF project BEOQUE, grant number 02 WN 0651.

Note for reviewers

S. Herrmann performed all experimental work for this manuscript under supervision of C. Vogt. S. Kleinsteuber supervised molecular biological analysis. T. Neu contributed with CLSM imaging of microbial communities. S. Herrmann performed data analysis and contributed to interpretation and manuscript preparation.

3

Identification of anaerobic benzene-assimilating organisms by DNA stable isotope probing

Steffi Herrmann[1], Sabine Kleinsteuber[2], Antonis Chatzinotas[2], Steffen Kuppardt[3], Tillmann Lueders[4], Hans-Hermann Richnow[1], and Carsten Vogt[1]

[1]Department of Isotope Biogeochemistry, UFZ - Helmholtz Centre for Environmental Research, Leipzig, Germany

[2]Department of Environmental Microbiology, UFZ - Helmholtz Centre for Environmental Research, Leipzig, Germany

[3]Department of Bioremediation, UFZ - Helmholtz Centre for Environmental Research, Leipzig, Germany

[4]Institute of Groundwater Ecology, Helmholtz Centre Munich, Neuherberg, Germany

Subitted to Environmental Microbiology

Abstract

The carbon flow under strictly anoxic conditions within a benzene-mineralising enrichment culture was analysed using fully labelled $^{13}C_6$-benzene. More than 90% of added ^{13}C-benzene was released as ^{13}C-carbon dioxide. Sulphate was the main electron acceptor. DNA extracted from cultures which had degraded different amounts of natural benzene or ^{13}C-labelled benzene was centrifuged in CsCl density gradients to identify ^{13}C-benzene-assimilating organisms. Two phylotypes showed significantly increased relative abundances of their terminal restriction fragments in 'heavy' fractions of ^{13}C-benzene-incubated microcosms compared to a ^{12}C-benzene-incubated control: a member of the *Cryptanaerobacter/Pelotomaculum* group within the *Peptococcaceae*, and a phylotype belonging to the *Epsilonproteobacteria*. The *Cryptanaerobacter/Pelotomaculum* phylotype was the most abundant organism and has possibly a fermenting lifestyle, attacking benzene initially. The epsilonproteobacterium may assimilate fermentation products of benzene. A small amount of ^{13}C-methane was aceticlastically produced, as concluded from the linear relationship between methane production and benzene degradation and the detection of *Methanosaetaceae* as only methanogens. Other phylotypes detected but not ^{13}C-labelled belong to several genera of sulphate-reducing bacteria, which may act as hydrogen scavengers for benzene fermentation. Our results strongly support the hypothesis that benzene is mineralised by a consortium consisting of fermenters, sulphate reducers and to a minor extent of aceticlastic methanogens.

3.1 Introduction

Petroleum hydrocarbons are frequent soil and groundwater contaminants world-wide. Benzene is the most mobile and most toxic aromatic hydrocarbon, creating a major health risk (Aksoy 1985). Under aerobic conditions, benzene can be biodegraded by ubiquitous bacteria which use mono- or dioxygenases for the initial attack of the aromatic ring (for an overview see van Agteren *et al.* (1998)). Since contaminant plumes generally become anoxic due to the rapid microbial consumption of oxygen, the more important natural attenuation process might be, however, benzene degradation under anoxic conditions. This process has been only occasionally observed in several laboratory and field studies (Johnson *et al.* 2003). The enrichment of benzene-degrading anaerobic microorganisms is still a challenge and not much is known on the physiology and composition of anaerobic benzene-degrading microbial communities. Nevertheless, anaerobic benzene degradation was observed in laboratory enrichment cultures under different electron acceptor conditions: methanogenic (Vogel and Grbic-Galic 1986b; Kazumi *et al.* 1997), nitrate-reducing (Nales *et al.* 1998; Burland and Edwards 1999), iron-reducing (Lovley *et al.* 1996; Kazumi *et al.* 1997; Nales *et al.* 1998; Jahn *et al.* 2005; Botton and Parsons 2006; Kunapuli *et al.* 2007), and sulphate-reducing conditions (Edwards and Grbic-Galic 1992; Lovley *et al.* 1995; Kazumi *et al.* 1997; Phelps and Young 1999; Vogt *et al.* 2007; Herrmann *et al.* 2008; Kleinsteuber *et al.* 2008; Musat and Widdel 2008).

So far, only facultative anaerobic bacterial pure cultures have been reported to metabolise benzene anaerobically, using nitrate or chlorate as electron acceptor. Coates *et al.* (2001b) described two *Dechloromonas* strains able to mineralise benzene and other aromatic hydrocarbons, *e.g.* toluene and xylenes, with nitrate as electron acceptor. Two benzene-degrading strains, belonging to the genus *Azoarcus*, have been isolated by Kasai *et al.* (2006) under nitrate-reducing conditions; the authors used RNA-stable isotope probing (RNA-SIP) to substantiate the relevance of the respective strains directly in field samples. Weelink *et al.* (2007; 2008) isolated the bacterium *Alicycliphilus denitrificans* strain BC, which can grow on benzene with chlorate as electron acceptor. However, since oxygen is produced during chlorate reduction, the authors postulate an aerobic benzene degradation pathway for this strain.

Attempts to isolate anaerobic benzene degraders from strictly anaerobic enrichment cultures failed as yet, but some of those cultures were phylogenetically described. Phelps *et al.* (1998) and Musat and Widdel (2008) investigated cultures enriched from marine sediments under sulphate-reducing conditions. The consortium of Musat and Widdel (2008) consisted only of *Deltaproteobacteria*, and the dominant phylotype was a *Desulfobacterium* species. A closely similar phylotype, named SB-21, had been described before by Phelps *et al.* (1998) and was recently identified by DNA-SIP as the critical member of a benzene-degrading sulphate-reducing consortium (Oka *et al.* 2008). Ulrich and Edwards (2003) identified a *Desulfobacterium* species and a member of the *Peptococcaceae* as the dominant phylotypes within a methanogenic consortium enriched from the sediment of a contaminated aquifer. Selective enrichments of organisms belonging to the family *Geobacteraceae* were observed in iron-reducing benzene-degrading enrichment cultures or sediment samples by Rooney-Varga *et al.* (1999) and Botton *et al.* (2007) using 16S rRNA gene based methods. Kunapuli *et al.* (2007) demonstrated by DNA-SIP that members of the *Peptococcaceae* degraded and assimilated carbon from ^{13}C-benzene in an iron-reducing enrichment culture, and that this process involves electron sharing by an apparent syntrophic interaction with members of the *Desulfobulbaceae*. Phylotypes related to the genera *Pelomonas* and *Pseudomonas* were identified by DNA-SIP as benzene degraders in sediment microcosms incubated under anoxic conditions (Liou *et al.* 2008). Summarized, the results of these studies suggest that members of taxonomically and/or ecophysiologically different taxa may play major roles in anaerobic benzene degradation under strictly anoxic conditions.

For this study we investigated benzene degradation under sulphate-reducing conditions at a benzene-contaminated field site in Zeitz, Germany. Benzene-mineralising organisms were enriched under sulphate-reducing conditions (i) in columns percolated with benzene-containing anoxic groundwater, filled with either sand or pumice (Vogt *et al.* 2007), and (ii) by using *in situ* microcosms placed for 9 weeks within a groundwater monitoring well located downstream of the source zone of the plume (Herrmann *et al.* 2008). Recently, we analysed the microbial community composition of these benzene-degrading sulphate-reducing enrichment cultures by establishing clone libraries and using terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of PCR-amplified 16S

rRNA genes. We observed that the abundance of a *Cryptanaerobacter/Pelotomaculum* phylotype and a phylotype belonging to the *Epsilonproteobacteria* significantly increased in laboratory enrichment cultures after repeated spiking with benzene (Kleinsteuber *et al.* 2008), and proposed that both phylotypes predominantly mineralise benzene in a syntrophic relationship.

In order to prove this hypothesis, we carried out a DNA-SIP experiment adding ^{13}C-labelled benzene as substrate for carbon and energy. The rational of DNA-SIP is that 'heavy' DNA synthesised upon assimilation of a ^{13}C-labelled benzene can be separated from 'light' DNA by density gradient ultracentrifugation, thus distinct organisms assimilating a ^{13}C-labelled substrate can be identified (for an overview of DNA-SIP techniques see Friedrich *et al.* (2006) and Neufeld *et al.* (2007a; 2007b). In addition, experiments with ^{13}C-labelled compounds may allow a sensitive proof of slow degradation or mineralisation processes by tracking ^{13}C-labelled intermediates, biomolecules or mineralisation products (Richnow *et al.* 1999; Zengler *et al.* 1999; Stelzer *et al.* 2006; Morasch *et al.* 2007; Nijenhuis *et al.* 2007).

3.2 Experimental Procedures

Chemicals not indicated were either purchased from Sigma (Munich, Germany) or Merck (Darmstadt, Germany) and were of analytical grade quality. $^{13}C_6$-benzene (> 99 atom%) was obtained from Campro Scientific (The Netherlands). $^{13}C_6$-glucose (> 99 atom%) was obtained from Chemotrade (Leipzig, Germany).

3.2.1 Cultivation procedures and set up of the stable isotope probing experiment

Benzene-degrading laboratory microcosms were set up using coarse sand, colonised by anaerobic benzene degraders, taken from a benzene-degrading, sulphate-reducing column system described elsewhere (Vogt *et al.* 2007). Two 1-litre glass bottles were filled with column sand, subsequently filled with anoxic mineral salt medium (Vogt *et al.* 2007) containing 20 mM sulphate as electron acceptor and finally overlaid with 15 mL 2,2,4,6,8,8-heptamethylnonane (HMN) spiked with 150 µL benzene (HMN; Fluka, Seelze, Germany), serving as an inert, hydrophobic carrier phase (Rabus *et al.* 1993) for benzene to avoid toxic benzene concentrations in the aqueous phase. The bottles were gas-tight closed by Teflon-sealed screw-caps and regularly analysed for sulphide concentrations. If sulphide concentrations reached levels higher than 4 mM, the mineral salt medium and benzene-HMN phase were exchanged by fresh solutions, to avoid toxic effects of sulphide on the anaerobic benzene-degrading consortium. After one year incubation, the sand was distributed in 80 g portions to 240 mL serum bottles (Glasgerätebau Ochs GmbH, Bovenden, Germany) each filled with 90 mL anoxic mineral salt medium containing 15 mM sulphate, leading to a headspace of around 120 mL. Those bottles were spiked with 140 µM benzene, closed gas-tight with crimped Teflon-coated butyl rubber stoppers (ESWE Analysentechnik, Gera, Germany), and regularly sampled for benzene and sulphide concentrations. After benzene had been completely degraded in all microcosms, the SIP experiment

was started. Three microcosms were spiked with 6.5 µL $^{13}C_6$-benzene or non-labelled benzene, respectively, and regularly sampled for concentrations of sulphide and benzene as well as ^{13}C isotope signatures of carbon dioxide and methane. The sand material of individual microcosms was frozen at -20°C after degradation of a certain amount (24%, 72% and 98% ≙ 0.22 mM, 0.46 mM and 0.72 mM) of $^{13}C_6$-benzene or non-labelled (^{12}C) benzene, respectively. Control microcosms received no benzene (2 bottles) or were sterilised by autoclaving (121°C, 20 min) three times at three consecutive days before the medium was spiked with $^{13}C_6$-benzene (3 bottles). The microcosms were always incubated statically at 22°C in the dark. Medium exchange and sand transfer were carried out in an anaerobic glove box (Coy Laboratory Products Inc., USA). Samples for chemical analyses were taken by means of sterile, nitrogen flushed syringes.

3.2.2 Density gradient centrifugation of DNA

DNA was extracted as described by Maher and colleagues directly from the sand (Maher *et al.* 2001). Agarose gel electrophoresis and spectrophotometry were used for quality check. DNA was quantified using PICO-Green-DNA-quantification kit (Invitrogen, Germany) according to guidelines of manufacturers. After quantification, the extracted DNA was separated by equilibrium density centrifugation and gradient fractionation using a modified protocol of Lueders *et al.* (2004). Two micrograms of DNA were loaded onto a CsCl gradient and centrifuged in an ultracentrifuge (OPTIMA™L-90K, Beckman Coulter) equipped with a Near-Vertical Rotor (NVT 65.2, Beckman Coulter) for 46 h at 45000 rpm at 20 °C. Of each gradient, twelve fractions were harvested from the bottom of the tube by displacement with water from the top using a syringe pump with a flow rate of 1 mL min^{-1}. Subsequently, the temperature-corrected refractive index (nd-TC) of each fraction was measured with a refractometer (AR200, Reichert) and converted to buoyant density (Neufeld *et al.* 2007b). Nucleic acids were precipitated with 2 volumes of PEG solution (30% wt/vol polyethylene glycol 6000 dissolved in 1.6 M NaCl) followed by centrifugation at 14000 rpm for 30 min. After washing with 80% ethanol, the pellet was dried and resuspended in 30 µL nuclease free water. ^{13}C-labelled as well as unlabelled ^{12}C-DNA from *Haloarcula marismortui* DSM 3752 was used as a standard for the density gradient centrifugation. The strain was ordered from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The organism was grown aerobically at 30°C for 7 days in DSMZ medium 97 free of casamino acids and Na_3-citrate using 1 g L^{-1} fully ^{13}C-labelled or unlabelled glucose as carbon source. The DNA was extracted and processed as described above. It should be noted that the density shift for *H. marismortui* (0.2-0.3 g mL^{-1}) was smaller than expected for fully ^{13}C-labelling of DNA (~0.4 g mL^{-1}), for unknown reasons. The feeding of *H. marismortui* on fully labelled ^{13}C-glucose as sole source of carbon and energy should have led theoretically to almost complete labelling of DNA.

3.2.3 T-RFLP analyses

Bacterial and archaeal 16S rRNA gene fragments were PCR-amplified as described elsewhere (Kleinsteuber *et al.* 2008) using the *Bacteria*-specific primers 27F and 1492R (Lane 1991) and the *Archaea*-specific primers 21F and 958R (Delong 1992), respectively. Forward primers were labelled at the 5' end with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM). Oligonucleotides were purchased from biomers.net (Ulm, Germany). T-RFLP analysis of PCR products generated from different fractions of each CsCl density gradient (see above) was done using the restriction endonucleases *Bst*UI and *Rsa*I, respectively, as recently described (Kleinsteuber *et al.* 2008). Restriction endonucleases were purchased from New England Biolabs (Schwalbach, Germany). Terminal restriction fragments (T-RF) of bacterial 16S rRNA genes were phylogenetically assigned by comparison with cloned bacterial 16S rRNA genes (Kleinsteuber *et al.* 2008). To check the phylogenetic assignment, bacterial 16S rRNA genes from selected fractions were cloned and sequenced as described below.

3.2.4 Cloning and sequencing of 16S rRNA genes

DNA from the "light" fraction of the gradient gained from microcosms sampled after 24% and 98% of $^{13}C_6$-benzene degradation, respectively, was used to create 16S rRNA gene clone reference libraries for the phylogenetic assignment of T-RF. Bacterial and archaeal 16S rRNA gene fragments were PCR-amplified and cloned as described elsewhere (Kleinsteuber *et al.* 2006) using the bacterial primers 27F and 1492R (Lane 1991) and the archaeal primers 21F and 958R (Delong 1992), respectively. Clones were screened by amplified ribosomal DNA restriction analysis (ARDRA) using the restriction endonuclease *Rsa*I. Partial sequencing of representative clones was performed using the BigDye RR Terminator AmpliTaq FS Kit 1.1 (Applied Biosystems, Weiterstadt, Germany) and the sequencing primers 21F or 27F, respectively, and 519R (Lane 1991). Capillary electrophoresis and data collection were carried out on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Data were analysed using ABI PRISM DNA SEQUENCING ANALYSIS software, and 16S rRNA gene sequences were assembled by ABI PRISM AUTOASSEMBLER software. The BLASTN tool (www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.* 1990) was used to search for similar sequences in the GenBank database, and the Seqmatch tool was used to search for similar sequences compiled by the Ribosomal Database Project Release 10 (http://rdp.cme.msu.edu) (Cole *et al.* 2007). The sequence data have been submitted to the GenBank database under accession numbers FJ156086-FJ156091 and FJ459796-FJ459805.

3.2.5 Chemical analyses

Sulphide was determined spectrophotometrically by the method of Cline (1969) using slight modifications described elsewhere (Herrmann *et al.* 2008). Benzene was analysed by headspace gas chromatography according to Kleinsteuber *et al.* (2008). Total benzene concentrations were calculated using a Henry coefficient of 0.2 for 22°C as reported by the US-EPA under

http://www.epa.gov/athens/learn2model/part-two/onsite/esthenry.htm. The carbon isotope compositions of carbon dioxide and methane were determined using a gas chromatography-combustion-isotope-ratio-monitoring-mass-spectrometer (GC-C-IRMS) consisting of a gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA) coupled via Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Germany). Carbon dioxide and methane were separated by a Poraplot Q column (25 × 0.32 mm ID, 1 µm film; Chrompack, Middelburg, The Netherlands) isothermally at 40°C using helium as carrier gas at a flow rate of 2.0 mL min^{-1}. CO_2 and methane eluted 530 s and 460 s after injection, respectively. Samples were injected at 1:5 to 1:20 split ratios. The carbon isotope signatures are given in δ notation (per mil) relative to the Vienna Pee Dee Belemnite standard (V-PDB). For analysis, 2 mL headspace of each microcosm was taken out by sterile syringes and injected into 10 mL glass vials which had been previously evacuated and gas-tight closed with butyl rubber stoppers. Samples of 100-200 µL from those vials were injected into the GC-C-IRMS. Methane concentrations were calculated by an external calibration curve prepared with samples of known methane content. The carbon isotope signatures of CO_2 and CH_4 were converted to molar concentrations as described by Kunapuli *et al.* (2007).

3.3 Results

3.3.1 Benzene degradation pattern in microcosms spiked with $^{13}C_6$-benzene or natural benzene

Eleven individual microcosms were set up, each filled with an equal amount of coarse sand particles and mineral salt medium. The sand particles were colonised by anaerobic benzene-degrading communities as previously shown (Vogt *et al.* 2007; Herrmann *et al.* 2008; Kleinsteuber *et al.* 2008). Before adding $^{13}C_6$-benzene, the degradation rates for natural benzene in those microcosms were determined between 1.3 µM d^{-1} to 2.8 µM d^{-1}, proving that all microcosms were active. For the SIP experiment, three microcosms were spiked with either $^{13}C_6$-benzene or natural benzene in final concentrations between 588 and 744 µM; five other microcosms served as abiotic or live substrate-free controls. In the following, labelled and non-labelled benzene was degraded with rates between 3.6 µM and 5.7 µM d^{-1}, whereas benzene concentrations remained nearly constant in autoclaved control microcosms (Figure 3.1 A; data for live microcosms spiked with natural benzene are not shown). Individual live microcosms were sacrificed after 24%, 72% and 98% of the added benzene had been degraded (indicated in Figure 3.1 by the terminated line), and processed for DNA-SIP (see below).

In live microcosms, sulphide was always formed in the course of benzene degradation, whereas benzene-free control microcosms did not produce sulphide (Figure 3.1 A; data for live microcosms spiked with natural benzene are not shown). The molar ratio of formed sulphide and degraded benzene was 1.96 ± 0.33 (95% confidence interval), calculated for all live microcosm data (n = 30). The $^{13}C_6$-benzene-amended microcosms produced ^{13}C-labelled carbon dioxide in isotope signatures up to δ = + 12175‰, corresponding to 4.1 mM produced carbon dioxide; ^{13}C-enriched carbon dioxide was

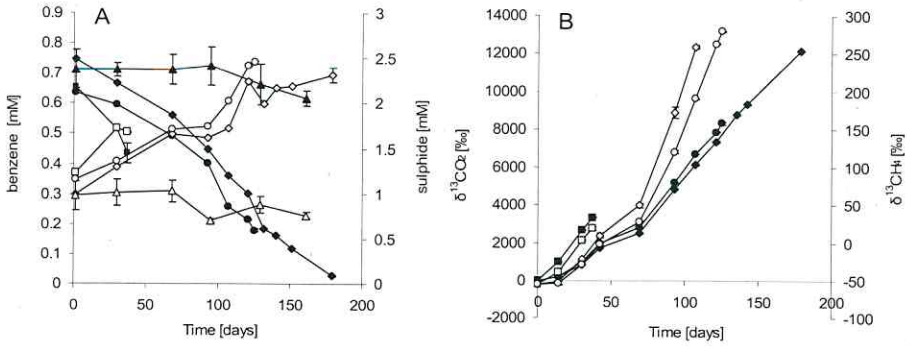


Figure 3.1: (A) Progression of benzene (filled symbols) and sulphide (open symbols) concentrations in individual microcosms amended with $^{13}C_6$-benzene. (B) Production of ^{13}C-enriched carbon dioxide (filled symbols) and ^{13}C-enriched methane (open symbols) in individual microcosms amended with ^{13}C-benzene. Diamonds: active microcosm, 0.72 mM benzene degraded; circles: active microcosm, 0.46 mM benzene degraded; squares: active microcosm, 0.22 mM benzene degraded; triangles: control experiments (filled triangles: abiotic controls, n = 3; open triangles: control without substrate, n = 2). Errors bars indicate the standard deviation of individual analyses (for carbon isotope signatures, benzene and sulphide concentrations of benzene-degrading microcosms) or the standard deviation of average data gained from different parallel microcosms (for benzene and sulphide data from autoclaved or benzene-free microcosms).

already detected after 14 days incubation (Figure 3.1 B), indicating that benzene degradation started immediately after benzene addition without lag-phase. The amount of degraded benzene highly correlated with the amount of produced carbon dioxide (Figure 3.2), demonstrating that benzene was continuously mineralised in all ^{13}C-benzene-labelled microcosms. In microcosms spiked with natural benzene, the carbon isotope signatures of carbon dioxide slightly decreased from $\delta = -18‰$ to maximal -21‰ during benzene degradation.

Besides ^{13}C-enriched carbon dioxide, also ^{13}C-enriched methane was generated in all $^{13}C_6$-benzene-amended microcosms: the carbon isotope signatures increased from $\delta = -55‰$ to values up to $\delta = +280‰$ indicating methanogenesis (Figure 3.1 B). The absolute methane concentrations were between 5 µM and 26 µM in those microcosms during the whole incubation time and slightly decreased during the experiment (data not shown), which might be caused by methane loss of the microcosms headspaces due to repeated sampling and reflecting low methane production rates. On the basis of the measured isotope signatures and methane concentrations in the individual microcosms, maximal 5 µM methane was released linked to the degradation of 100 µM benzene. As observed for carbon dioxide, benzene degradation and methane production were highly correlated (Figure 3.2).

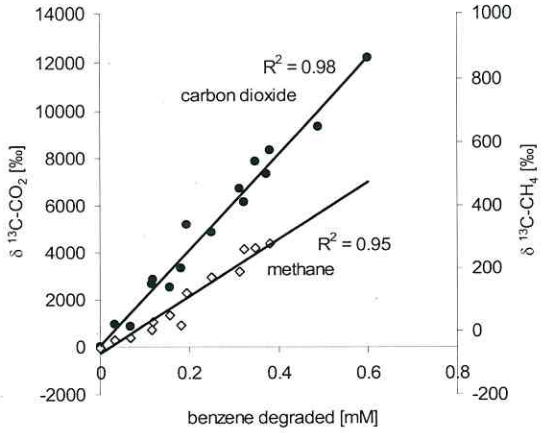


Figure 3.2: Concentrations of benzene and carbon isotope signatures of carbon dioxide and methane during the degradation experiment.

3.3.2 Identification of benzene-assimilating organisms by stable isotope probing of DNA (DNA-SIP)

DNA was extracted from each of the live benzene-amended microcosms, and equal amounts were separately ultracentrifuged in a CsCl density gradient. The DNA of the ^{13}C-benzene-incubated microcosms slightly shifted to higher buoyant densities compared to the DNA extracted from ^{12}C-benzene-incubated microcosms (Figure 3.3). However, a small peak in the 'heavy' region of the gradient, indicated by the ^{13}C-labelled control DNA of *Haloarcula marismortui* (Figure 3.3), was detected only for the DNA extracted from the microcosms sampled after degradation of 72% (0.42 mM) of the $^{13}C_6$-benzene.

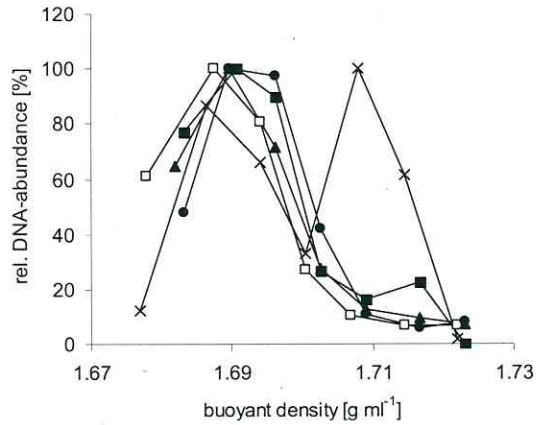


Figure 3.3: Relative abundances of DNA within buoyant density gradients of different microcosms measured fluorometrically. Figure symbols: white squares: ^{12}C-benzene (0.58 mM degraded); black circles: ^{13}C-benzene (0.72 mM degraded); black squares: ^{13}C-benzene (0.46 mM degraded); black triangles: ^{13}C-benzene (0.22 mM degraded); crosses: a 1:1 mixture of ^{13}C- and ^{12}C-DNA of *Haloarcula marismortui* forming a "light" and a "heavy" peak after density gradient centrifugation.

Within each fraction of the different gradients, the predominant bacterial and archaeal phylotypes were identified by the relative abundances of the terminal restriction fragments (T-RF) of their 16S rRNA gene amplicons produced after digestion with *Bst*UI or *Rsa*I, respectively (Table 3.1 and 3.2). Generally, the T-RFLP patterns produced by both restriction endonucleases were highly reproducible and comparable (data not shown). Bacterial T-RF were assigned to specific phylotypes on the basis of previously reported 16S rRNA gene sequences of benzene-degrading enrichment cultures (Kleinsteuber *et al.* 2008) as well as 16S rRNA gene sequences retrieved from the DNA samples used in this study. The results for the relative abundances of the T-RF in microcosms sampled after almost complete benzene degradation are summarised in Table 3.1. The most abundant bacterial T-RF was a 235 bp *Bst*UI fragment and a 458 bp *Rsa*I fragment, respectively, representing a phylotype belonging to the *Cryptanaerobacter/Pelotomaculum* group of the *Desulfotomaculum* cluster I within the family *Peptococcaceae*.

Table 3.1: Bacterial diversity within the microcosms after complete benzene degradation: Lengths of TRF obtained after *Bst*UI or *Rsa*I digestion (bp), range of relative abundances within all analysed ^{12}C-benzene or ^{13}C-benzene density gradient fractions, and phylogenetic assignment of the T-RF to cloned 16S rRNA genes reported by Kleinsteuber *et al.* (2008). The accession numbers of the according 16S rRNA gene sequences are given in parentheses.

***BstUI* T-RF (bp)**	**Relative abundance (%) ^{12}C-benzene[a]**	**Relative abundance (%) ^{13}C-benzene[b]**	**Phylogenetic affiliation (acc. no.)**
58	2 - 10	5 - 11	
60	2 - 3	-	
102	4 - 7	2 - 7	
105	5 - 6	3 - 7	*Desulfovibrio* sp. (EF613397, EF613409, EF613417, EF613418, EF613426, EF613437, EF613445, EF613458, EF613475, EF613477)
106	3 - 9	1 - 2	*Desulfuromonales* (FJ459801), *Desulfobacca* sp. (EF613479)
109	3 - 6	1 - 4	*Desulfobacteraceae* (EF613407, EF613408, EF613431, EF613448, EF613466, FJ459796)
116	3 - 7	2 - 6	*Desulfobacterium* sp. (EF613427, EF613452, EF613467)
171	2 - 4	-	
235	**18-26**	**9 - 45**	***Cryptanaerobacter/Pelotomaculum* sp. (EF613398, EF613433, EF613456, EF613470, FJ459802-459805)**
240	3 - 4	3 - 7	*Deltaproteobacteria* (FJ459798)
390	**3 - 7**	**8 - 39**	***Sulfurovum* sp. (EF613424, EF613447, EF613451, EF613455, Ef613457, EF613465, EF613474, EF613484, FJ459797, FJ459800),** unclassified bacterium (FJ459799)
392	6 - 7	3 - 7	
407	1 - 3	-	
485	1 - 2	2 - 4	
492	2 - 3	2 - 4	
***Rsa*I T-RF (bp)**	**Relative abundance (%) ^{12}C-benzene[a]**	**Relative abundance (%) ^{13}C-benzene[b]**	**Phylogenetic affilitation (acc. no.)**
55	4 - 7	3 - 5	*Desulfobacteraceae* (EF613407, EF6134408, EF613431, EF613448, EF613466)
240	2 - 3	2 - 9	*Desulfuromonales* (FJ459801)
244	3 - 4	1 - 7	*Desulfobacteraceae* (FJ459796), *Deltaproteobacteria* (FJ459798)
246	1 - 4	-	
250	1 - 4	1 - 5	
444	1 - 4	-	Unclassified bacterium (FJ459799)
449	**4 - 10**	**9 - 45**	***Sulfurovum* sp. (EF613424, EF613447, EF613451, EF613455, Ef613457, EF613465, EF613474, EF613484, FJ459797, FJ459800)**
458	**27 - 36**	**10 - 52**	***Cryptanaerobacter/Pelotomaculum* sp. (EF613398, EF613433, EF613456, EF613470, FJ459802-459805)**
460	3 - 13	2 - 11	
468	-	2 - 14	
477	2 - 17	-	
482	-	2 - 3	*Desulfovibrio* sp. (EF613397, EF613409, EF613417, EF613418, EF613426, EF613437, EF613445, EF613458, EF613475, EF613477), *Desulfobacca* sp. (EF613479)
486	2 - 4	1 - 2	*Desulfuromonas* sp. (EF613415, EF613423, EF613468)

[a] T-RF of all gradient fractions from the microcosm which had converted 0.58 mM natural benzene

[b] T-RF of all gradient fractions from the microcosm which had converted 0.72 mM 13C-benzene

This T-RF made up between 18 and 26% (*Bst*UI) and between 27 and 36% (*Rsa*I) of the total T-RF peak area within the density gradient fractions of the ^{12}C-benzene microcosms (Table 3.1 and Figure 3.4). At least five phylotypes related to different lineages of sulphate-reducing bacteria were detected, each of them with T-RF abundances of less than 10% of the total T-RF peak area. Some T-RF were present either in ^{12}C-benzene-amended or ^{13}C-benzene-amended microcosms, and some T-RF were not identified (Table 3.1).

DNA from the ^{12}C-benzene control microcosms was amplified from every gradient fraction including those representing high buoyant densities. The individual T-RF from ^{12}C-benzene microcosms always occurred in similar relative abundances in each fraction of the gradient, as shown for the *Cryptanaerobacter/Pelotomaculum*-related phylotype and the epsilonproteobacterial phylotype in Figure 3.4. This pattern was different for the three ^{13}C-benzene-amended microcosms. Compared to the gradient of the ^{12}C-benzene control microcosms, the *Cryptanaerobacter/Pelotomaculum*-related phylotype was less abundant in the lowest and highest fractions of the density gradient, but strongly enriched in the gradient fractions showing a buoyant density of around 1.7 g mL^{-1} (Figure 3.4 A and C). This effect was most pronounced for the DNA extracted from the microcosms which had consumed 0.46 and 0.72 mM of ^{13}C-benzene, respectively.

Table 3.2: Sequencing results of representative 16S rRNA gene clones and according terminal restriction fragments (T-RF).

Clone	Acc. No.	Highest BLAST hit (Acc. no.) / Sequence identity	Taxonomic affiliation according to RDP (S_{AB})	*BstUI* T-RF (bp)	*RsaI* T-RF (bp)
ZZ-bac-11-8_6 (544 bp)	FJ459796	Uncultured bacterium clone P4-35 from uranium mine sediment (AF523335) / 98%	*Desulfobacteraceae* (0.845)	109	244
ZZ-bac-11-8_14 (499 bp)	FJ459797	Uncultured bacterium clone B23 from wastewater treatment plant effluent (EU234206) / 99%	*Epsilonproteobacteria* (0.967)	389	450
ZZ-bac-11-8_15 (543 bp)	FJ459798	Bacterium clone 2BP-6 from sulfidogenic dehalogenating consortium (AF121886) / 89%	*Deltaproteobacteria* (0.648)	240	244
ZZ-bac-11-8_18 (499 bp)	FJ459799	Uncultured bacterium clone lka62 from sulfidic cave stream biofilm (EF467561) / 98%	Unclassified *Bacteria* (0.944)	390	444
ZZ-bac-11-8_24 (499 bp)	FJ459800	Uncultured bacterium clone B23 from wastewater treatment plant effluent (EU234206) / 99%	*Epsilonproteobacteria* (0.984)	389	449
ZZ-bac-11-8_31 (540 bp)	FJ459801	Uncultured bacterium clone ZZ12C11 from benzene-contaminated groundwater, Zeitz (AY214185) / 98%	*Desulfuromonales* (0.868)	106	242
ZZ-bac-11-9_41 (497 bp)	FJ459802	Uncultured *Cryptanaerobacter* sp. clone ZZ-S9D4 from benzene-mineralizing consortium (EF613456) / 100%	*Peptococcaceae* (0.961)	235	458
ZZ-bac-11-9_55 (488 bp)	FJ459803	Uncultured *Cryptanaerobacter* sp. clone ZZ-S9D4 from benzene-mineralizing consortium (EF613456) / 100%	*Peptococcaceae* (0.960)	235	458
ZZ-bac-11-9_68 (494 bp)	FJ459804	Uncultured *Cryptanaerobacter* sp. clone ZZ-S9D4 from benzene-mineralizing consortium (EF613456) / 100%	*Peptococcaceae* (1.000)	235	458
ZZ-bac-11-9_80 (514 bp)	FJ459805	Uncultured *Cryptanaerobacter* sp. clone ZZ-S9D4 from benzene-mineralizing consortium (EF613456) / 100%	*Peptococcaceae* (0.962)	235	458
ZZ-arc-9-11-2 (574 bp)	FJ156086	Uncultured archaeon clone ASN24 from petroleum-contaminated soil (AB161351) / 96%	*Thermoplasmatales* (0.840)	ND	ND
ZZ-arc-9-11-6 (470 bp)	FJ156087	Uncultured archaeon clone ASC10 from petroleum-contaminated soil (AB161325) / 99%	*Methanothrix* sp. (0.980)	336	80
ZZ-arc-11-11-1 (469 bp)	FJ156088	Uncultured archaeon clone ASC21 from petroleum-contaminated soil (AB161326) / 97%	*Thermoplasmatales* (0.834)	96	-
ZZ-arc-11-11-3 (451 bp)	FJ156089	Uncultured archaeon clone ASC36 from petroleum-contaminated soil (AB161336) / 98%	*Euryarchaeota* (0.934)	96	-
ZZ-arc-11-11-10 (469 bp)	FJ156090	Archaeon clone SJC-125a from a trichlorobenzene-transforming consortium (AJ009509) / 99%	*Methanothrix* sp. (0.989)	335	79
ZZ-arc-11-11-28 (453 bp)	FJ156091	Uncultured archaeon clone ASC36 from petroleum-contaminated soil (AB161336) / 97%	*Euryarchaeota* (0.889)	97	-

ND: not determined

The phylotype belonging to the *Epsilonproteobacteria*, which made up less than 10% of the relative T-RF areas in all fractions of the ^{12}C-benzene control microcosm, was highly abundant in the 'heaviest' fractions of the gradient, but also in the 'light' fractions after degradation of ^{13}C-benzene (Figure 3.4 B and D). Similarly to the *Cryptanaerobacter/Pelotomaculum*-related phylotype, those changing abundances were observed in all ^{13}C-benzene-amended microcosms, and the most pronounced effect was seen for the DNA extracted from the microcosm which had degraded the highest amount of ^{13}C-benzene (Figure 3.4 B and D). Other phylotypes showed no clear abundance shifts in T-RF pattern caused by ^{13}C-benzene.

Due to the observation that a small amount of ^{13}C-labelled carbon was also converted to methane (Figure 3.1 and 3-2), archaeal 16S rRNA genes were cloned and sequenced to identify the responsible methanogens. Based on ARDRA patterns of the archaeal 16S rRNA gene clones, more than 65% of the clones (40 out of 62) were assigned to two phylotypes affiliated to the *Methanosaetaceae*. The remaining 22 clones were assigned to phylotypes related to uncultured members of the *Thermoplasmatales* (Table 3.2).

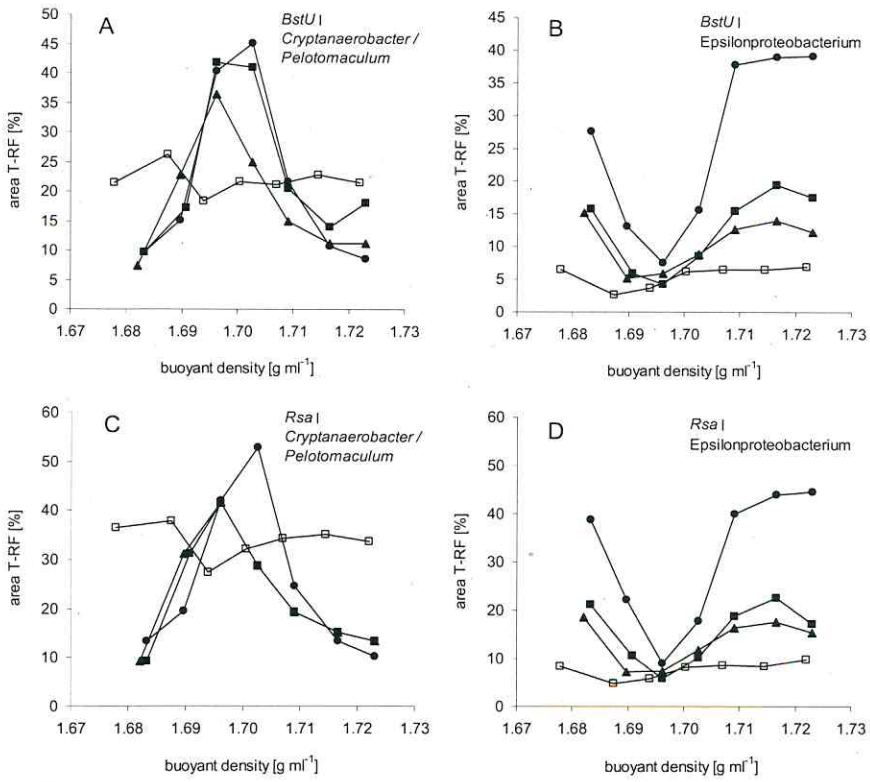


Figure 3.4: Relative abundances of T-RF, generated by either *Bst*UI (A, B) or *Rsa*I (C, D), assigned to *Cryptanaerobacter*/*Pelotomaculum* (A, C) and *Epsilonproteobacteria* (B, D) within buoyant density gradients of DNA samples extracted form different microcosms. Figure symbols: white squares: ^{12}Cbenzene (0.58 mM degraded); black circles: ^{13}C-benzene (0.72 mM degraded); black squares: ^{13}Cbenzene (0.46 mM degraded); black triangles: ^{13}C-benzene (0.22 mM degraded).

The T-RF of the *Methanosaetaceae* phylotypes were found in almost all fractions of density gradients obtained from the ^{12}C-benzene and ^{13}C-benzene degradation experiments, respectively. The relative abundances were higher than 50% (data not shown). No significant increase of the relative abundances of the archaeal phylotypes in the heavier fractions of the ^{13}C-benzene gradient was observed.

3.4 Discussion

Sulphate was the main electron acceptor used by the enrichment culture, since more than 50% of the electrons released by benzene mineralisation were channelled into sulphide assuming sulphate reduction according to the following equation:

$$C_6H_6 + 3\,H_2O + 3.75\,SO_4^{2-} \rightarrow 6\,HCO_3^- + 1.874\,HS^- + 1.875\,H_2S + 0.375\,H^+ \qquad \text{eq. 3.1}$$

$$\Delta G^{0'} = -185 \text{ KJ mol}^{-1}$$

Considering that more than 90% of benzene was oxidised to carbon dioxide, as shown by the carbon isotope signatures of produced CO_2, the sulphide values suggest that the electrons stemming from benzene oxidation were not solely used for sulphate reduction. We assume that a part of the formed sulphide precipitated as iron sulphide coatings on the sand particles within the microcosms as previously reported (Vogt *et al.* 2007; Herrmann *et al.* 2008; Kleinsteuber *et al.* 2008), hence leading to an underestimation of the extent of sulphate reduction occurring within the enrichment culture during benzene mineralization.

The microbial diversity within the enrichment culture, reflected by the high number of different T-RF observed (Table 3.1), could be merely explained by the way of cultivation, since the benzene-degrading microbial community is located on the surfaces of sand particles, forming a biofilm (Vogt *et al.* 2007; Herrmann *et al.* 2008; Kleinsteuber *et al.* 2008). All attempts to grow the culture in liquid medium with benzene as sole source of carbon and energy failed until today. Thus, we can not separate the benzene degraders from microorganisms not directly involved in anaerobic benzene degradation as it would be probably possible by stepwise transferring liquid aliquots in planktonic enrichment cultures. On the other hand, we recently observed changes in the biofilm community pattern after repeated spiking of the culture with different substrates including benzene (Kleinsteuber *et al.* 2008), hence the method of cultivation likely allows at least an enrichment of organisms primarily involved in substrate assimilation. Since the consortium was incubated for several years with benzene as main source of carbon and energy before the SIP experiment was started, the relatively high microbial diversity within the consortium might be explained by the assumption that several different organisms profit from the process of anaerobic benzene degradation, resulting in a network of carbon and energy fluxes within the community.

The consumption of ^{13}C-labelled benzene led only to a very small shift of the DNA density gradient compared to the ^{12}C-benzene control microcosm, indicating a low DNA labelling close to the detection limit of the method; at least 20 to 30 atom% of labelling of DNA from species assimilating a ^{13}C-

labelled substrate is needed for density gradient separation. For several reasons, one can expect that only species assimilating benzene to a major extent would become detectable. As shown by equation 3.1, the Gibb's free energy of benzene degradation under sulphate-reducing conditions is low and allows theoretically the synthesis of only 3 ATP per molecule benzene as compiled by Schink (1997). The growth yield of our enrichment culture was low, since more than 90% of benzene was mineralised. Assuming benzene is assimilated rather by a syntrophic consortium than by a single organism, the energy gain per involved organism likely becomes additionally lower. Due to the uncertainties about the amount of ^{13}C-benzene which had to be assimilated by the enrichment culture leading to a detectable DNA labelling, we performed DNA density gradients of enrichment cultures which had consumed different amounts of ^{13}C-benzene (0.22 mM, 0.46 mM and 0.72 mM) or ^{12}C-benzene (0.15 mM, 0.49 mM and 0.58 mM), respectively. Since RNA-SIP is more sensitive than DNA-SIP (Neufeld *et al.* 2007b), one might argue that RNA-SIP is more suitable to label anaerobic benzene degraders. In fact, we tried to extract RNA from the benzene-degrading biofilm within the microcosms, which was not possible in sufficient amounts (data not shown), probably due to the interfering matrix of the sand particles covered by iron-sulphide coatings.

Two phylotypes were significantly enriched in heavy fractions of the ^{13}C-density gradient, indicating a ^{13}C-labelling of their DNA and thus an assimilation of benzene: the *Cryptanaerobacter/Pelotomaculum*-related phylotype and an epsilonproteobacterium. The *Cryptanaerobacter/Pelotomaculum* phylotype dominated in all benzene-incubated microcosms, based on the relative abundance of its T-RF. The amount of ^{13}C-DNA-labelling for the *Cryptanaerobacter/Pelotomaculum*-related phylotype was very low, indicated by the small shift within the density gradient, which might be due to the low growth yield of this organism with benzene, as discussed above. Since this phylotype dominated the enrichment culture already at the beginning of the SIP experiment, only a limited number of cells within this population might divide in the course of the experiment, leading to a low level of ^{13}C-labelling. If DNA replication goes along with cell division, a single division of every cell within a growing population using a fully ^{13}C-labelled substrate theoretically leads to a genomic DNA labelling of only 50%. Thus, at least two rounds of cell division are necessary to obtain 100% labelled genome copies within a growing population.

The DNA of the *Epsilonproteobacteria*-phylotype peaked in both the lightest and the heaviest fractions of the ^{13}C-benzene gradients. Other phylotypes having the same relative T-RF abundances as the epsilonproteobacterium in the ^{12}C-benzene control gradient did not show pronounced changes of their T-RF abundances in any of the ^{13}C-benzene gradient fractions. Hence, we interpret the strong increase of the epsilonproteobacterial T-RF in the heavy gradient fractions as true ^{13}C-incorporation into DNA, indicating that a certain number of cells assimilate carbon derived from ^{13}C-benzene. Supporting this assumption, the effect was most pronounced in the gradient of the microcosm which had consumed the highest amount of ^{13}C benzene.

Less than 1% of labelled carbon of benzene ended up in methane, showing that besides sulphate reduction also methanogenesis occurred. Since ^{13}C-labelled methane was produced in a constant rate (Figure 3.2), we assume that methane developed aceticlastically and not by reduction of carbon dioxide; in the latter case, huge amounts of unlabelled methane are expected to be produced by preferential reduction of unlabelled carbon dioxide, which was not observed. Correspondingly, the only methanogens detected within the enrichment culture were members of the *Methanosaetaceae*; all known members of this family can not grow with hydrogen and carbon dioxide, but use acetate as the sole energy source (Kendall and Boone 2006), even in the presence of sulphate (Struchtemeyer *et al.* 2005). Hence, aceticlastic methanogens may have used small amounts of acetate formed upon ^{13}C-benzene oxidation, or may have used carbon derived from dying labelled biomass which was continuously recycled. Other archaeal phylotypes found in minor proportions were related to the *Thermoplasmatales* and therefore are not assumed to be involved in methanogenesis. Similar phylotypes have been previously found in anaerobic microcosms enriched from petroleum-contaminated soil samples (Kasai *et al.* 2005). As these phylotypes are only distantly related to cultured members of the *Thermoplasmatales*, one can only speculate about their physiological properties. Cultured representatives of the *Thermoplasmatales* are known for sulphur respiration and were typically found *e.g.* on self-heating coal refuse piles or solfatara fields (Madigan *et al.* 1997).

Our model for the syntrophic degradation of benzene within our consortium is summarised in Figure 3.5. We assume that the *Cryptanaerobacter/Pelotomaculum* phylotype may ferment benzene in the first step of the degradation pathway to hydrogen and acetate or other low-weight fermentation products usable for *Methanosaetaceae*-like archaea. The cultivated species of the genera *Pelotomaculum* and *Cryptanaerobacter* are syntrophic, heterotrophic bacteria, which live closely associated with hydrogen-consuming organisms (interspecies hydrogen transfer).

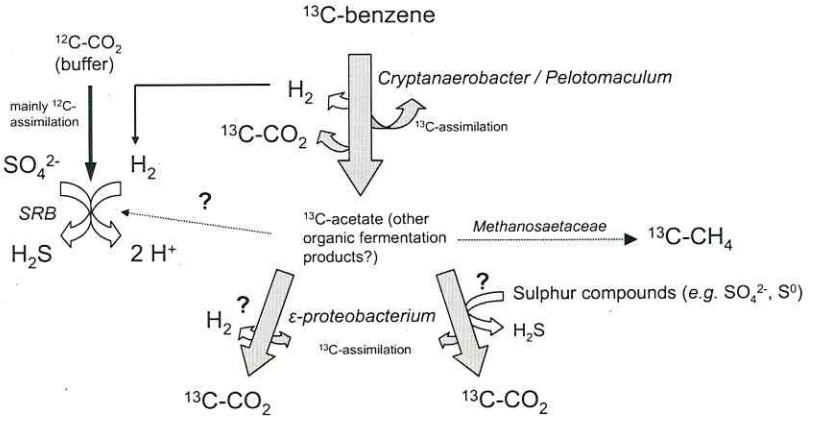


Figure 3.5: Proposed flow of carbon and redox equivalents during mineralization of benzene, based on DNA-SIP data.

Some of them were shown to degrade aromatic compounds like benzoate, phenol or phthalate (Qiu *et al.* 2003; Juteau *et al.* 2005; Qiu *et al.* 2006). Using DNA-SIP, Kunapuli *et al.* (2007) recently found that members of the *Peptococcaceae* were clearly ^{13}C-labelled and therefore responsible for benzene oxidation in an enrichment culture under iron-reducing conditions; this process involved an as-yet unexplained mode of syntrophic electron sharing with *Desulfobulbaceae*.

The function of the *Epsilonproteobacteria*-phylotype within the carbon flow network in our consortium is hard to predict based on the current knowledge about this phylogenetic lineage. Generally, the *Epsilonproteobacteria* are the most poorly characterised class within the *Proteobacteria*, most lineages are without cultured representatives (Campbell *et al.* 2006). The closest cultivated relative to our labelled phylotype is *Sulfurovum lithotrophicum* (see figure supporting information), a species isolated from a hydrothermal vent (Inagaki *et al.* 2004). More closely related environmental sequences were detected in terrestrial sulphidic habitats (Porter and Engel 2008) and a methanogenic phenol-degrading bioreactor (Zhang *et al.* 2005). The isolated species belonging to this clade of the *Epsilonproteobacteria* are usually chemolithoautotrophic, using hydrogen and/or reduced sulphur compounds as electron donors and oxygen or nitrate as electron acceptors (Campbell *et al.* 2006); the ability to use sulphate as electron acceptor has not been described yet. Notably, Koizumi *et al.* (2005) detected several sulphate reducers and *Epsilonproteobacteria* as bacterial key players in anoxic saline sediment incubations after addition of different organic and inorganic electron donors, indicating that *Epsilonproteobacteria* can use a broader range of electron donors and acceptors than currently known cultivated isolates. A common feature of sulphidophilic *Epsilonproteobacteria* is carbon fixation by the reductive tricarboxylic acid cycle (rTCA) (Campbell *et al.* 2006; Nakagawa *et al.* 2007; Nakagawa and Takai 2008), an ancient and oxygen-sensitive pathway. The rTCA can principally operate also in the oxidising direction, if low molecular weight organic molecules are present. Thus, a possible lifestyle of the epsilonproteobacterium within the benzene-degrading consortium would be to oxidise acetate by the TCA cycle while using a sulphur compound as electron acceptor, or evolving hydrogen which is then consumed by several sulphate reducers. The syntrophic oxidation of acetate is energetically unfavourable, but has been described for sulphate-reducing or methanogenic conditions (Nusslein *et al.* 2001; Zhilina *et al.* 2005; Dolfing *et al.* 2008; Hattori 2008). In such a scenario, the preferential role of sulphate reducers would be hydrogen scavenging for which they are known to be very efficient in syntrophic relationships (Cordruwisch *et al.* 1988; Elshahed *et al.* 2001a; Jackson and McInerney 2002). Nevertheless, on the basis of our data, we can not rule out the possibility the epsilonproteobacterium may also use benzene directly as carbon and energy source. It should be noted that autotrophic growth based on hydrogen oxidation and sulphate reduction likely can not result in a significant DNA labelling due to the presence of the 30 mM unlabelled-carbonate buffer used in the experiment: complete mineralisation of ^{13}C-benzene leads to a maximal ^{13}C-carbon dioxide enrichment of only 12 atom percent. Hence, organisms like hydrogenotrophic sulphate reducers using carbon dioxide as major carbon source could probably not be separated by density

gradient centrifugation due to preferential fixation of ^{12}C-carbon dioxide, explaining why we found always non-labelled T-RF of several different phylotypes of sulphate reducers within the density gradient fractions.

The current knowledge about structure and function of anoxic benzene degradation suggest that benzene can be mineralised either by a single organism, or by a consortium of organisms. A benzene-degrading consortium consisting of benzene-attacking fermenting organisms, oxidisers of low molecular weight organic fermentation products and hydrogen scavengers may always develop on the constraints of the biodiversity and environmental conditions of a habitat. As a result, a broad diversity within anaerobic benzene-degrading consortia enriched from different field sites may develop, since the ecological niche of hydrogen consumers and fermenters of low molecular weight organic compounds can be conquered by several phylogenetic and physiological different microorganisms.

3.5 Supporting information

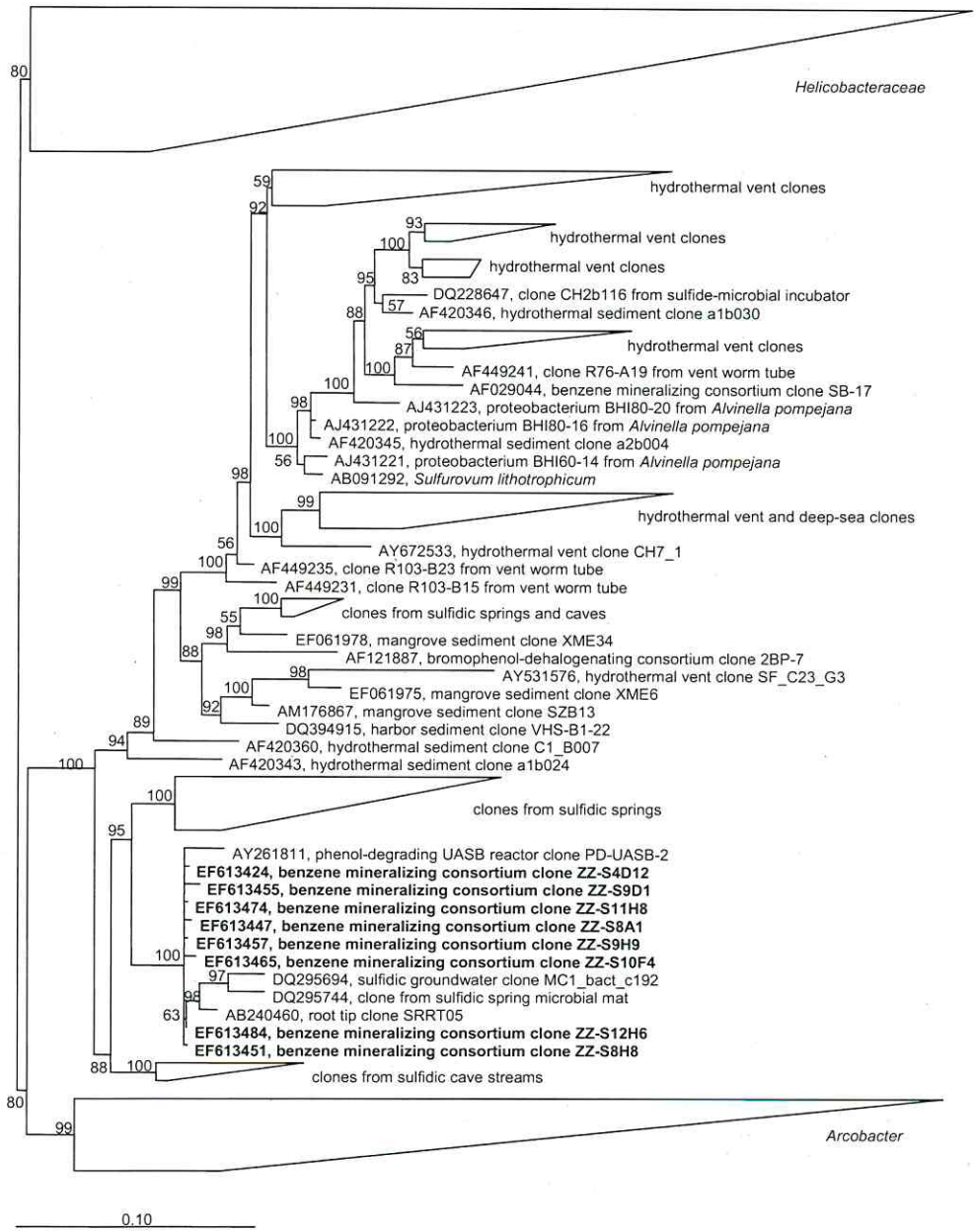


Figure 4.S1: Phylogenetic relationship of the *Sulfurovum*-related clones (bold-faced) retrieved from the benzene-degrading consortium to representatives of the *Epsilonproteobacteria* (from Kleinsteuber *et al.* (2008), supporting information).

Acknowledgements

This work is integrated in the internal research and development program of the UFZ as well as the SAFIRA I project. The authors thank Stefan Feisthauer for providing calibration data of methane, and Ute Lohse for excellent technical assistance. Steffi Herrmann thanks the Federal Ministry for Education and Research (BMBF) for funding within the project BEOQUE, grant number 02 WN 0651. We thank Matthias Gehre and Ursula Günther for assistance in our isotope laboratory.

Notes for reviewers

S. Herrmann performed all experimental work for this manuscript supervised by C. Vogt. S. Kuppardt partially established the SIP technology, which was his contribution. S. Herrmann performed data analysis supervised by C. Vogt. S. Herrmann, S. Kleinsteuber, A. Chatzinotas, T. Lüders, H.H Richnow, and C. Vogt contributed to interpretation and manuscript preparation.

4

Combined carbon and hydrogen isotope fractionation analysis for elucidating benzene biodegradation pathways

Anko Fischer[1,2], Ilka Herklotz[1], Steffi Herrmann[1], Martin Thullner[3], Sander A. B. Weelink[4], Alfons J. M. Stams[4], Michael Schlömann[5], Hans-Hermann Richnow[1], and Carsten Vogt[1]

[1]UFZ - Helmholtz Centre for Environmental Research, Department of Isotope Biogeochemistry, Permoserstraße 15, 04318 Leipzig, Germany

[2]Isodetect - Company for Isotope Monitoring, Ingolstädter Landstr. 1, 85764 Neuherberg/Oberschleißheim, Germany

[3]UFZ - Helmholtz Centre for Environmental Research, Department of Environmental Microbiology, Permoserstraße 15, 04318 Leipzig, Germany

[4]Laboratory of Microbiology, Wageningen University, de Dreijen 10, 6703 HB, Wageningen, The Netherlands.

[5]Interdisciplinary Ecology Centre, TU Bergakademie Freiberg, Freiberg, Germany

Published in Environmental Science and Technology, 2008, vol. 42, p. 4356-4363

Abstract

Recently, combined carbon and hydrogen isotope fractionation investigations have emerged as a powerful tool for the characterization of reaction mechanisms relevant for the removal of organic pollutants. Here, we applied this approach in order to differentiate benzene biodegradation pathways under oxic and anoxic conditions in laboratory experiments. Carbon and hydrogen isotope fractionation of benzene was studied with four different aerobic strains using a monooxygenase or a dioxygenase for the initial benzene attack, a facultative anaerobic chlorate-reducing strain as well as a sulfate-reducing mixed culture. Carbon and hydrogen enrichment factors (ε_C, ε_H) varied for the specific pathways and degradation conditions, respectively, so that from the individual enrichment factors only limited information could be obtained for the identification of benzene biodegradation pathways. However, using the slope derived from hydrogen vs. carbon isotope discriminations or the ratio of hydrogen to carbon enrichment factors ($\Lambda = \Delta_H/\Delta_C \approx \varepsilon_H/\varepsilon_C$), benzene degradation mechanisms could be distinguished. Although experimentally determined Λ values partially overlapped, ranges could be determined for different benzene biodegradation pathways. Specific Λ values were < 2 for dihydroxylation, between 7 and 9 for monohydroxylation, and > 17 for anaerobic degradation. Moreover, variations in Λ values suggest that more than one reaction mechanism exists for monohydroxylation as well as for anaerobic benzene degradation under nitrate-reducing, sulfate-reducing, or methanogenic conditions. Our results show that the combined carbon and hydrogen isotope fractionation approach has potential to elucidate biodegradation pathways of pollutants in field and laboratory microcosm studies.

4.1 Introduction

Benzene is one of the most abundant organic groundwater pollutants (Plumb 1987; Arneth *et al.* 1989). It is toxic to humans and can cause various forms of cancer (Smith 1999; Korte *et al.* 2000). Since benzene represents a significant health risk due to its high abundance and toxicity, it is a pollutant of particular concern. Therefore, it is important to investigate its fate in the environment including the assessment of natural attenuation processes. It has been shown that benzene can be biodegraded under oxic and anoxic conditions and that biodegradation is the most significant process of benzene removal in groundwater and soil (see Gibson and Subramanian (1984) and Lovely (2000) for an overview).

Aerobic bacterial benzene degradation can be initiated by dihydroxylation or monohydroxylation. The first step of dihydroxylation is an addition of both atoms of dioxygen to the aromatic nucleus to form *cis*-benzene dihydrodiol (Gibson *et al.* 1968). The oxygen-dependent monohydroxylation of benzene is catalyzed by monooxygenases with rather broad substrate specificities: toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1, the toluene 3-monooxygenase (T3MO) of

Ralstonia pickettii PKO1, and the toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4, all converting benzene to phenol (Tao *et al.* 2004).

Anaerobic benzene degradation has been shown to take place under nitrate-reducing, sulfate-reducing, iron(III)- reducing, and methanogenic conditions (see Lovely (2000) for an overview). However, the initial step of the degradation pathway under anoxic conditions is still not elucidated. It has been proposed that benzene could be methylated to form toluene, carboxylated to form benzoate, or hydroxylated to form phenol (Chakraborty and Coates 2005; Ulrich *et al.* 2005).

For the characterization and assessment of biodegradation of groundwater contaminants, stable isotope fractionation investigations are a valuable tool (see Meckenstock *et al.* (2004a) for an overview). The method relies on the fractionation of stable isotopes during enzymatically catalyzed reactions, leading to an enrichment of heavier isotopes in the residual fraction of the reacting compound. Generally, the extent of isotope fractionation depends on the type of transformation reaction. Thus, isotope fractionation investigations might be used as an indicator for distinct biodegradation pathways.

Compound-specific stable isotope analysis (CSIA) does not distinguish between different isotopomers; therefore, measured data only provide bulk isotope fractionation factors and no information on specific kinetic isotope effects (KIE) at different reactive position(s). For calculating apparent kinetic isotope effects (AKIE), bulk isotope fractionation data have to be corrected regarding nonreactive molecule positions as well as intramolecular isotopic competition (Elsner *et al.* 2005). By the use of AKIE, mechanistic information about the initial step of biodegradation pathways can be revealed (Elsner *et al.* 2005).

However, significant isotope effects can only be observed when the first irreversible step of a reaction is isotope-sensitive and represents the rate-determining step in the overall biotransformation process. If the bond conversion is preceded by non or slightly isotope fractionating processes (*e.g.*, uptake and transport of a substrate to the reactive site of enzyme, binding of substrate to form enzyme–substrate complexes), isotope fractionation will be lowered and may become even insignificant. Therefore, AKIE calculated from the observable isotopic enrichment of a particular element during degradation not only reflects the chemical mechanism according to the transition state theory but is also modulated by rate limitation of preceding reaction steps of the overall biological transformation. This may complicate the interpretation of isotope effects in biological systems (Northrop 1981). The tendency of the biologically catalyzed reaction to continue forward and become irreversible has been described as "forward commitment to catalysis" (Northrop 1981). Thus, the observable isotope fractionation of an element can significantly vary either because of a different chemical mechanism associated with the actual bond cleavage or because of nonisotope-sensitive steps of a particular biodegradation pathway, as in the "forward commitment to catalysis". Hence, the characterization of the degradation pathway as well as the assessment of biodegradation using stable isotope fractionation investigations of only one element can lead to bias in the interpretation of

experimental data. However, since "commitment to catalysis" affects the isotope composition of different elements involved in an enzymatic reaction similarly, the correlation of isotope fractionation of these elements may be used to characterize the reaction mechanism more precisely than by isotope fractionation investigations of only one element (Elsner *et al.* 2005). Thus, the two-dimensional isotope fractionation approach (TDIFA) is a valuable tool for determining biodegradation pathways or conditions (Elsner *et al.* 2005). Recently, predominant redox conditions under which methyl *tert*-butyl ether (MTBE) and benzene were biodegraded in contaminated aquifers could be identified by measuring carbon and hydrogen isotope signatures (Kuder *et al.* 2005; Zwank *et al.* 2005; Fischer *et al.* 2007). Furthermore, in laboratory experiments pathways for benzene, toluene and MTBE biodegradation have been evaluated using two-dimensional isotope fractionation investigations (Hunkeler *et al.* 2001; Mancini *et al.* 2003; Mancini *et al.* 2006; Elsner *et al.* 2007; Rosell *et al.* 2007).

In order to characterize *in situ* biodegradation by TDIFA, the extent of isotope fractionation for appropriate elements has to be known for the expected reaction mechanisms of biotransformation. In recent laboratory experiments, carbon and hydrogen isotope fractionation have been determined for aerobic and anaerobic benzene biodegradation under nitrate-reducing, sulfate-reducing, and methanogenic conditions (Hunkeler *et al.* 2001; Mancini *et al.* 2003). However, it was not known whether the tested aerobic strains used a mono- or dioxygenase for the initial step of benzene transformation. Moreover, the proposed initial steps for anaerobic benzene biodegradation – methylation, carboxylation, or hydroxylation (Chakraborty and Coates 2005; Ulrich *et al.* 2005) – had not yet been linked to carbon and hydrogen isotope fractionation investigations.

In this study, the extent of carbon and hydrogen isotope fractionation was determined for aerobic strains with known biodegradation pathways in order to distinguish between isotope patterns for mono- or dioxygenase-catalyzed reactions. Additionally, we applied TDIFA for elucidating benzene biodegradation pathways of a new isolate able to use chlorate as well as oxygen as electron acceptor and for a sulfate-reducing enrichment culture, respectively. The results were interpreted with respect to the variability of isotope fractionation for investigating benzene biodegradation pathways under *in situ* redox conditions and for enrichment or pure cultures.

4.2 Materials and Methods

4.2.1 Microorganisms and cultivation conditions

The aerobic pure cultures used in this study were kindly provided by the following people or institutions: *Pseudomonas putida* strain ML2 (Tan and Mason 1990) by Jeremy Masons (King's College, London, England), *Ralstonia pickettii* strain PKO1 (Olsen *et al.* 1994) by Christian Griebler (Helmholtz Zentrum München - German Research Center for Environmental Health, Munich, Germany), *Cupriavidus necator* ATCC 17697 (Vandamme and Coenye 2004) by the strain collection of the Interdisciplinary Ecology Centre (TU Bergakademie Freiberg, Germany), and *Rhodococcus opacus* strain B-4 (Na *et al.* 2005) by Junichi Kato (Hiroshima University, Hiroshima, Japan). For the

isotope fractionation experiments, the strains were cultivated in modified Brunner mineral salt medium (Vogt *et al.* 2002) spiked with benzene as the sole source of carbon and energy. *Alicycliphilus denitrificans* strain BC was isolated from an enrichment culture that degrades benzene with chlorate (Weelink *et al.* 2007). The bacterium grows on benzene with oxygen and chlorate. Although the bacterium is able to grow with acetate and nitrate, it is not able to grow with benzene and nitrate (Weelink et al., unpublished). *A. denitrificans* was cultivated anoxically in a chlorate-containing mineral salts medium described elsewhere (Weelink *et al.* 2007) using benzene as the sole source of carbon and energy. For aerobic cultivation of *A. denitrificans*, the same medium – but without chlorate – was used. For purity control and maintenance, strains were plated on peptone agar medium (German collection of microorganisms and cell cultures medium number 83) and R2A agar medium (Reasoner and Geldreich 1985) every two months. The sulfate-reducing and benzene-degrading mixed culture was enriched as described elsewhere (Vogt *et al.* 2007). The different media used contained all 7.5 μM iron.

The degradation experiments and analytical methods are described in detail in the Supporting information.

4.2.2 Quantification of isotope fractionation

Carbon and hydrogen enrichment factors (ε_C, ε_H) were determined using the logarithmic form of the Rayleigh equation,

$$\ln\left(\frac{(\delta_t + 1000)}{(\delta_0 + 1000)}\right) = \frac{\varepsilon}{1000}\ln\left(\frac{C_t}{C_0}\right) \qquad \text{eq. 4.1}$$

plotting changes in concentrations $\ln(C_t/C_0)$ versus changes in isotope ratios $\ln[(\delta_t + 1000)/(\delta_0 + 1000)]$ and obtaining ε from the slope of the linear regression. δ_t, C_t and δ_0, C_0 are the stable isotope ratios in delta notation (see Supporting information, eq. 4.S4) and concentrations of a compound at a given point in time and at the beginning of a transformation reaction, respectively.

For a general mechanistic interpretation of the isotope discrimination, enrichment factors have to be converted to AKIE. According to Elsner *et al.* (2005), AKIE for benzene should be calculated according to the following equation:

$$AKIE = \frac{1}{1 + z \cdot \varepsilon_{reactive\ position}/1000} \qquad \text{eq. 4.2}$$

where z is the number of atoms of an element in identical reactive positions . If only a single carbon or hydrogen atom of the benzene ring takes part in the overall reaction, as suggested for benzene degradation under anoxic conditions (Chakraborty and Coates 2005; Ulrich *et al.* 2005), z is 6 for both elements. In the case of dioxygenase-catalyzed reactions, two adjacent carbon and hydrogen atoms of the aromatic ring take part in the reaction (Gibson *et al.* 1968); hence, for dioxygenases, z is 3. The reaction mechanism for monooxygenases is less well understood. It has been reported that the first

step might be initiated by (1) loss of a hydride, a hydrogen atom, or a proton to form a readily oxidized carbonium ion, a radical, or a carbanion (abstraction mechanism); (2) transfer of the equivalent of a singlet oxygen atom (insertion mechanism); (3) transfer of a singlet oxygen atom to a formal aromatic double bond (direct addition mechanism); or (4) addition and rearrangement of a triplet oxygen or related species without obligatory formation of an epoxide (addition – rearrangement mechanism) (Tomaszewski *et al.* 1975; Mitchell *et al.* 2003). Therefore, it is expected that one or two carbon and hydrogen atoms are involved in benzene monohydroxylation and that carbon as well as hydrogen AKIE should be calculated using z of 3 and 6, respectively.

The derivation of enrichment factors and AKIE is given in detail in the Supporting information.

4.3 Results and Discussion

4.3.1 Carbon isotope fractionation

Enrichment factors for carbon isotope fractionation were calculated using the Rayleigh equation (Figure 4.1 A,C,E,G; summarized in Table 4.1). The relatively high correlation between concentration and isotope composition with correlation factors (R^2) between 0.86 and 0.99 suggests that carbon isotope fractionation during aerobic and anaerobic benzene degradation can be modeled by the Rayleigh equation. In sterile controls, benzene concentrations as well as benzene carbon isotope signatures were stable (data not shown).

The degradation experiments with the strains *P. putida* ML2 and *Rh. opacus* B-4, both using a dioxygenase for the initial attack of the benzene ring, resulted in low carbon enrichment factors (ε_C) of -0.7 ± 0.1‰ and -1.3 ± 0.2‰, respectively. The corresponding apparent kinetic carbon isotopic effects ($AKIE_C$) were 1.002 ± 0.0006 and 1.004 ± 0.0006. The initial step of the dioxygenase-catalyzed reaction is an interaction of the reactive oxygen species, bound to Fe(III) in the reaction center of the enzyme, with the π-electron system of the benzene ring, leading finally to the formation of *cis*-benzene dihydrodiol (Bagneris *et al.* 2005). Reactions involving interactions with π-electron systems cause only minor carbon isotope effects as has been shown for the dioxygenase catalyzed ring activation of toluene and chlorobenzenes (Morasch *et al.* 2002; Griebler *et al.* 2004a; Kaschl *et al.* 2005).

The strains *R. pickettii* and *C. necator*, both using a monooxygenase for the initial benzene ring attack, revealed higher ε_C values (-1.7 ± 0.2‰ and -4.3 ± 0.4‰, respectively) and $AKIE_C$ (1.005 ± 0.0006 to 1.026 ± 0.0025, respectively) compared to the strains using a dioxygenase. The carbon enrichment factor for *C. necator* was more than twice as high as that for *R. pickettii*, indicating that the initial reaction mechanisms of the monooxygenases may not be identical. The ε_C values of *R. pickettii* and *C. necator* are similar to values obtained with *Burkholderia* sp. and *Acinetobacter* sp. for the aerobic biodegradation of benzene, ranging between -1.5 ± 0.1‰ and -3.5 ± 0.3‰, reported by Hunkeler *et al.* (2001). It is not known which pathways these strains use for aerobic benzene degradation.

Compared to the carbon isotope fractionation during dihydroxylation, biodegradation of benzene by *A. denitrificans* under both oxic and chlorate-reducing conditions was accompanied by a higher carbon isotope fractionation, with ε_C of -2.6 ± 0.8‰ ($AKIE_C$ = 1.008 ± 0.0024 to 1.016 ± 0.005) and -1.5 ± 0.5‰ (AKIEc = 1.005 ± 0.0015 to 1.009 ± 0.0031), respectively. Similar to *Burkholderia* sp. and *Acinetobacter* sp., the pathways of benzene degradation for *A. denitrificans* under both oxic and chlorate-reducing conditions are yet not known. However, there are strong indications that benzene is also degraded by *A. denitrificans* under chlorate-reducing conditions by means of an aerobic degradation pathway, since oxygen is produced during the reduction of chlorate (Rikken *et al.* 1996). Primer sets designed to analyze distinct evolutionary branches in the catabolic families predicted to be involved in benzene biodegradation indicated that monooxygenases, but not dioxygenases, are present in *A. denitrificans* (Weelink et al., unpublished).

For the sulfate-reducing mixed culture, ε_C of -1.9 ± 0.3‰ was determined for benzene biodegradation, leading to an $AKIE_C$ of 1.011 ± 0.0018. These data were in the range of ε_C (-1.9 ± 0.1‰ to -3.6 ± 0.3‰) and $AKIE_C$ (1.010 ± 0.0012 to 1.022 ± 0.0019) previously derived from anaerobic benzene degradation experiments under nitrate-reducing, sulfate-reducing, and methanogenic conditions (Mancini *et al.* 2003).

Our results demonstrate that $AKIE_C$ values for dihydroxylation of benzene are smaller compared to those for monohydroxylation and anaerobic degradation. Thus, a differentiation of benzene dihydroxylation from other reaction mechanisms seems to be possible by means of $AKIE_C$. Elsner *et al.* (2005) re-evaluated ε_{bulk} from literature data for the estimation of reaction-specific AKIE. For C = C bond epoxidation, $AKIE_C$ values were calculated from 1.00 to 1.01, which are similar to our results for benzene dihydroxylation. Hence, the results strongly indicate that the aromatic nucleus has been epoxidated by the dioxygenases investigated in this study. A C = C bond epoxidation-like mechanism (direct addition mechanism) can also be expected for benzene monohydroxylation (Tomaszewski *et al.* 1975; Mitchell *et al.* 2003). The observed $AKIE_C$ for monohydroxylation by *R. pickettii* was in the theoretical range expected for C = C bond epoxidation, indicating that the monooxygenase of *R. pickettii* may add oxygen directly to the benzene ring. In contrast, benzene monohydroxylation by *C. necator* gave an $AKIE_C$ significantly higher than assumed for C = C bond epoxidation. $AKIE_C$ of *C. necator* is in the range expected for C – H bond cleavage, which is between 1.01 and 1.03 (Elsner *et al.* 2005). This indicates that a cleavage of C – H bond occurs in the rate-limiting step of benzene monohydroxylation by *C. necator* which has been supposed for ring monooxygenase reactions (Tomaszewski *et al.* 1975; Mitchell *et al.* 2003). $AKIE_C$ values for C – H bond cleavage from literature (Elsner *et al.* 2005) are in a good agreement with the values for anaerobic benzene degradation derived from our experiments and from a previous study where C – H bond cleavage was considered as initial step (Mancini *et al.* 2003). However, $AKIE_C$ values for monohydroxylation and anaerobic benzene degradation are similar. Therefore, a distinction between these two degradation pathways by the determination of only $AKIE_C$ is not possible.

4.3.2 Hydrogen isotope fractionation

With exception of strains using a dioxygenase for benzene biodegradation (*Rh. opacus* and *P. putida*), hydrogen enrichment factors (ε_H) were derived from a ln–ln plot of the Rayleigh equation (Figure 4.1 B,D,F,H; summarized in Table 4.1). In sterile controls, hydrogen isotope signatures for benzene remained constant over time, concomitant with benzene concentrations (data not shown). Benzene dihydroxylation did not lead to significant hydrogen isotope fractionation for *Rh. opacus* and *P. putida*. Since the correlation of concentrations and isotope shifts was poor (R^2 between 0 and 0.29), the Rayleigh equation could not be applied to determine hydrogen enrichment factors for *Rh. opacus* and *P. putida*. It can be expected that ε_H values for these strains are within the error range for hydrogen isotope measurements (±5‰, see Supporting information). The insignificant hydrogen isotope fractionation can be explained by the mechanism of the benzene dihydroxylation reaction, where dioxygen is added to the aromatic ring in the irreversible reaction step without a hydrogen bond cleavage (Gibson *et al.* 1968; Kaschl *et al.* 2005). Thus, only a secondary hydrogen isotope effect can be considered for benzene dihydroxylation (Morasch *et al.* 2002).

For benzene degradation under sulfate-reducing conditions, the determined hydrogen enrichment factor was significantly higher than that observed for aerobic benzene degradation (ε_H = -59 ± 10‰; $AKIE_H$ = 1.548 ± 0.144). The ε_H and $AKIE_H$ values obtained in our study are similar to results from benzene biodegradation under methanogenic and sulfate-reducing conditions (ε_H = -59‰ and = -79‰, respectively) reported by Mancini *et al.* (2003). The anaerobic benzene transformation involves a primary hydrogen isotope effect implying that a C – H bond is broken in the rate-limiting step of the reaction (Hunkeler *et al.* 2001; Mancini *et al.* 2003). Carboxylation, methylation, or hydroxylation of benzene are likely to cause the cleavage of a C – H bond under anoxic conditions (Chakraborty and Coates 2005; Ulrich *et al.* 2005). Typical $AKIE_H$ values for C – H bond cleavage are in the range of 2 to 23 (Elsner *et al.* 2005). These values are higher than observed for anaerobic benzene degradation in our study. The variability of $AKIE_H$ values might result from the influence of commitment to catalysis leading to a lowering of AKIE.

ε_H values for benzene monohydroxylation were -11 ± 4‰ ($AKIE_H$ = 1.036 ± 0.012 to 1.071 ± 0.0028) for *R. pickettii* and -17 ± 11‰ ($AKIE_H$ = 1.057 ± 0.037 to 1.114 ± 0.082) for *C. necator*. Changes in concentrations ($\ln(C_t/C_0)$) and isotope compositions ($\ln[(\delta_t + 1000)/(\delta_0 + 1000)]$) showed a relatively good correlation with correlation factors (R^2) of 0.86 and 0.89, respectively. Compared to dihydroxylation and anaerobic degradation, benzene monohydroxylation resulted in moderate $AKIE_H$ values. Small or even negligible hydrogen isotope effects were determined for aromatic monohydroxylation by mammalian, methane, toluene-3, and toluene-4 monooxygenases. This was explained by the direct addition mechanism as the initial step, for which no hydrogen bond cleavage occurs (Tomaszewski *et al.* 1975; Hanzlik *et al.* 1984; Hinson *et al.* 1985; Wilkins *et al.* 1994; Morasch *et al.* 2002; Mitchell *et al.* 2003).

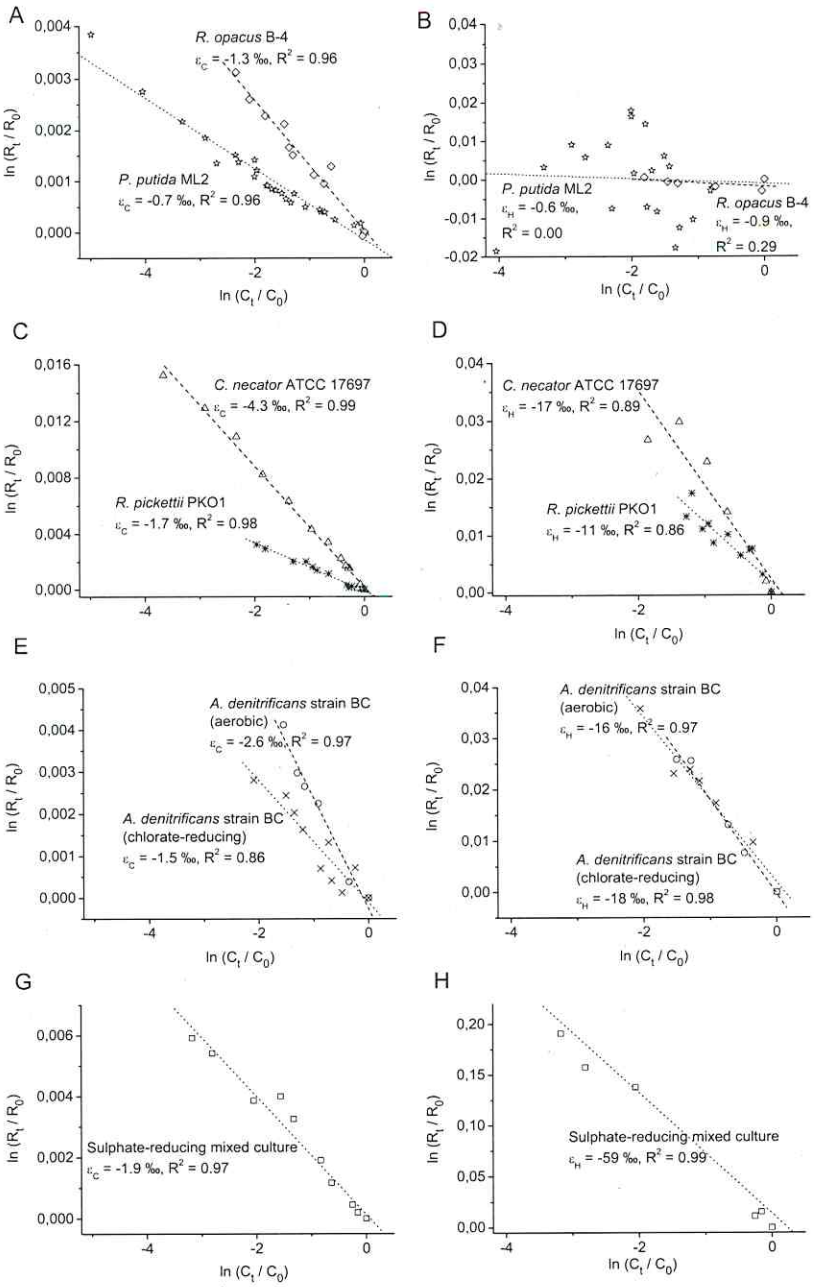


Figure 4.1: Double logarithmic plot according to the Rayleigh equation (eq. 4.1) of changes in isotope ratios and changes in concentrations from benzene biodegradation experiments using aerobic strains and a sulfate-reducing mixed culture. The reproducibility of carbon and hydrogen isotope measurements was always better than 0.5‰ and 5‰, respectively. The uncertainty of benzene concentration analyses was less than 5%. The lines correspond to a linear regression model: (A) carbon and (B) hydrogen isotope fractionation of *Rh. opacus* B-4 (◇) and *P. putida* ML2 (☆); (C) carbon and (D) hydrogen isotope fractionation of *C. necator* ATCC 17697 (Δ) and *R. pickettii* PKO1 (∗); (E) carbon and (F) hydrogen isotope fractionation of *A. denitrificans* strain BC under aerobic (O) and chlorate-reducing (×) conditions; (G) carbon and (H) hydrogen isotope fractionation of sulfate-reducing mixed culture (□).

Table 4.1: Comparison of carbon and hydrogen enrichment factors (ε_C, ε_H) and derived apparent kinetic isotope effects ($AKIE_C$, $AKIE_H$) as well as Λ values for benzene biodegradation.

culture	enzymatic pathway	ε_C [‰]	$AKIE_C$	ε_H [‰]	$AKIE_H$	Λ	reference
Rh. opacus B-4	dihydroxylation	-1.3 ± 0.2	1.004±0.0006[a]	no enrichment (±5‰)[c]	0.985 to 1.015[a,c]	±5[d]	this work
P. putida ML2	dihydroxylation	-0.7 ± 0.1	1.002±0.0003[a]	no enrichment (±5‰)[c]	0.985 to 1.015[a,c]	±7[d]	this work
R. pickettii PKO1	monohydroxylation	-1.7 ± 0.2	1.005±0.0006[a]	-11 ± 4	1.036±0.012[a]	3 ± 1	this work
			1.010±0.0012[b]		1.071±0.028[b]		
C. necator ATCC 17697	monohydroxylation	-4.3 ± 0.4	1.013±0.0012[a]	-17 ± 11	1.057±0.037[a]	11 ± 6	this work
			1.026±0.0025[b]		1.114±0.083[b]		
Burkholderia sp.	unknown	-3.5± 0.3	1.011±0.0009[a]	-11 ± 2	1.036±0.006[a]	3 ± 1	Hunkeler *et al.* (2001)
			1.021±0.0019[b]		1.071±0.014[b]		
Acinetobacter sp.	unknown	-1.5 ± 0.1	1.005±0.0003[a]	-13 ± 1	1.043±0.003[a]	9 ± 1	Hunkeler *et al.* (2001)
			1.009±0.0006[b]		1.085±0.007[b]		
A. denitrificans strain BC (aerobic)	unknown	-2.6 ± 0.8	1.008±0.0024[a]	-16 ± 4	1.053±0.013[a]	5 ± 2	this work
			1.016±0.0050[b]		1.106±0.029[b]		
A. denitrificans strain BC (chlorate-reducing)	unknown	-1.5 ± 0.5	1.005±0.0015[a]	-18 ± 6	1.061±0.020[a]	10 ± 4	this work
			1.009±0.0031[b]		1.121±0.045[b]		
nitrate-reducing mixed culture	unknown	-2.2 ± 0.4	1.013±0.0025[b]	-29 ± 4	1.211±0.035[b]	12 ± 3	Mancini *et al.* (2003)
		-2.4 ± 0.1	1.014±0.0006[b]	-35 ± 6	1.266±0.058[b]	16 ± 3	
sulfate-reducing mixed culture	unknown	-3.6 ± 0.3	1.022±0.0019[b]	-79 ± 4	1.901±0.087[b]	22± 3	Mancini *et al.* (2003)
sulfate-reducing mixed culture	unknown	-1.9 ± 0.3	1.011±0.0018[b]	-59 ± 10	1.548±0.144[b]	28 ± 3	this work
methanogenic mixed culture	unknown	-1.9 ± 0.1	1.011±0.0006[b]	-60 ± 3	1.563±0.044[b]	28 ± 3	Mancini *et al.* (2003)
		-2.1 ± 0.1	1.013±0.0006[b]	-59 ± 4	1.548±0.058[b]	28 ± 3	
		-2.0 ± 0.1	1.012±0.0006[b]	-59 ± 3	1.548±0.043[b]	32 ± 3	

[a] $z = 3$ for eq. 4.2. [b] $z = 6$ for eq. 4.2. [c] Expected range for ε_H given by the uncertainty of hydrogen isotope analysis. Λ values from our study, except for dihydroxylation, were derived from the slope of the plot of carbon vs. hydrogen isotope discriminations (Δ_H/Δ_C). Λ values from the literature and from the dihydroxylation experiments were calculated by $\varepsilon_H/\varepsilon_C$. [d] Expected Λ range derived from the ε_H range given by the uncertainty of hydrogen isotope analysis. Uncertainties of enrichment factors and Λ values from our study, in the form of a 95% confidence interval, were determined by linear regression analysis as described by Elsner *et al.* (2007). Uncertainties of AKIE factors and Λ values from the literature were estimated by error propagation: error of AKIE = $|\partial AKIE/\partial\varepsilon|$ × error of ε and error of $\Lambda = |\partial\Lambda/\partial\varepsilon_H|$ × error of $\varepsilon_H + |\partial\Lambda/\partial\varepsilon_C|$ × error of ε_C, respectively.

Higher hydrogen isotope effects were considered for aromatic monohydroxylation during the abstraction, insertion, and addition-rearrangement mechanism, respectively (Tomaszewski *et al.* 1975; Korzekwa *et al.* 1989; Mitchell *et al.* 2003). However, the reaction mechanism of the benzene monooxygenases of *R. pickettii* and *C. necator* can definitely not be elucidated by determining and comparing $AKIE_H$ values; here, further biochemical studies are needed. Compared to *R. pickettii* and *C. necator*, similar ε_H values were reported for the aerobic benzene degradation by *Burkholderia* sp. ($\varepsilon_H = -11 \pm 2$‰) and *Acinetobacter* sp. ($\varepsilon_H = -13 \pm 1$‰) (Hunkeler *et al.* 2001). For benzene degradation by *A. denitrificans*, nearly the same enrichment factors were determined under aerobic

and chlorate-reducing conditions (ε_H = -16 ± 4‰ and -18 ± 6‰, respectively). The experiments revealed relatively good correlations between concentrations ($\ln(C_t/C_0)$) and isotope compositions, with correlation factors (R^2) in the range of 0.97 and 0.98. The enrichment factors observed for *Burkholderia* sp., *Acinetobacter* sp., and *A. denitrificans* were in the same range as that observed for the monooxygenase-catalyzed reactions of *R. pickettii* and *C. necator*. Therefore, it might be possible that all these strains use monooxygenases for the initial hydroxylation step, since dioxygenase-catalyzed benzene ring attack leads to insignificant hydrogen isotope effects as shown in this and in a previous study (Morasch *et al.* 2002).

4.3.3 Two-dimensional isotope fractionation investigations

Isotope fractionation investigations of a single element are not always reliable for identifying biodegradation pathways, since enrichment factors for a specific transformation reaction can significantly differ due to the influence of non or slightly fractionating rate-limiting steps preceding the bond cleavage (commitment to catalysis) (Nijenhuis *et al.* 2005; Mancini *et al.* 2006; Elsner *et al.* 2007). TDIFA can improve the interpretation of isotope effects which are considerably masked by commitment to catalysis (Elsner *et al.* 2005). Here, Λ is given by the slope of the plot of carbon vs. hydrogen isotope discrimination ($\Delta_C = \delta_t{}^{13}C - \delta_0{}^{13}C$, $\Delta_H = \delta_t{}^{2}H - \delta_0{}^{2}H$) (see Supporting information). Alternatively, the ratio between hydrogen and carbon enrichment factors ($\Lambda \approx \varepsilon_H/\varepsilon_C$) is used for elucidating reaction mechanisms (Elsner *et al.* 2007; Rosell *et al.* 2007). Λ values derived from enrichment factors and isotope discrimination, respectively, are approximately equal. It has to be taken into account that for $\varepsilon_H >> -100‰$, the relationship between Δ_C and Δ_H exhibits a slightly nonlinear behavior (see Supporting information). However, in this case, the slope of carbon vs. hydrogen isotope discrimination derived from lower ranges of biodegradation is approximately equal to Λ values calculated from $\varepsilon_H/\varepsilon_C$.

In our study, Λ values show large variations for different initial benzene-attacking mechanisms to a large extent (Table 4.1). Although Λ values overlapped due to the uncertainty of calculations, significant ranges could be derived for distinct benzene biodegradation pathways. Specific Λ values were < 2 for dihydroxylation, between 7 and 9 for monohydroxylation, and > 17 for anaerobic degradation. With these Λ ranges, it can be reasoned that *Acinetobacter* sp. uses a monooxygenase for the initial attack on the benzene ring. Although *Burkholderia* sp. and *A. denitrificans* under oxic conditions exhibited Λ values similar to those determined for monohydroxylation by *R. pickettii*, because of overlapping Λ values it cannot be excluded that these strains may use a dioxygenase for the initial benzene attack. However, molecular biology studies indicated that monooxygenases but not dioxygenases are present in *A. denitrificans*. Thus, benzene monohydroxylation can be expected for this strain under oxic conditions.

The variability in Λ values for benzene monohydroxylation could be caused by different initial monooxygenase reactions. Mechanisms leading to a primary hydrogen isotope effect should yield

higher Λ values compared to mechanisms involving a secondary hydrogen isotope effect. Hence, benzene monohydroxylation showing high Λ values may be initiated by mechanisms which can be expected to have a primary hydrogen isotope effect such as abstraction, addition – rearrangement, or insertion mechanisms (Tomaszewski *et al.* 1975; Korzekwa *et al.* 1989; Mitchell *et al.* 2003). Benzene monohydroxylation exhibiting low Λ values might be due to reactions for which a secondary hydrogen isotope effect is expected, such as a direct addition mechanism (Tomaszewski *et al.* 1975; Korzekwa *et al.* 1989; Mitchell *et al.* 2003).

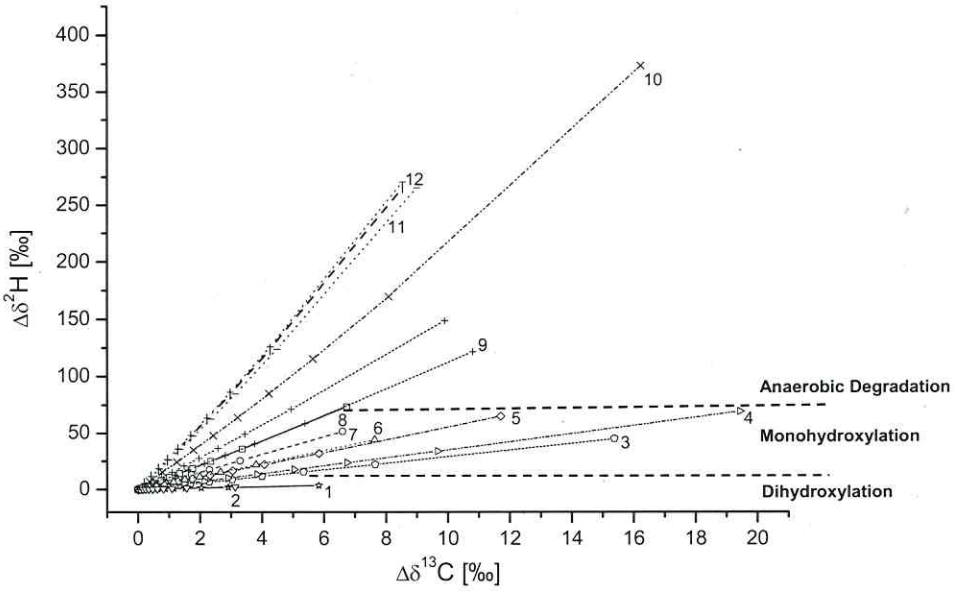
Λ values for anaerobic benzene degradation ranged between 10 and 32. The lowest values were determined for degradation under nitrate-reducing ($\Lambda = 12 \pm 3$ to $\Lambda = 16 \pm 3$) and chlorate-reducing ($\Lambda = 10 \pm 4$) conditions, respectively. For *A. denitrificans*, it is assumed that benzene degradation under chlorate-reducing conditions is initiated by an oxygen-dependent enzymatic reaction, since oxygen is produced during the reduction of chlorate (Rikken *et al.* 1996). In this case, the observed Λ value strongly indicates that benzene is attacked by a monooxygenase under chlorate-reducing conditions and not by a dioxygenase. This was also confirmed by molecular biology investigations.

For benzene degradation by methanogenic and sulphate-reducing mixed cultures, the Λ values are significantly higher compared to nitrate- and chlorate-reducing cultures, indicating that the initial step of benzene degradation under strictly anoxic conditions involves a C – H cleavage (Mancini *et al.* 2003). Additionally, the different Λ values observed under anoxic conditions (Table 4.1) may indicate that more than one reaction mechanism exists for the first enzymatic step of anaerobic benzene degradation. This assumption is supported by the identification of different metabolites (toluene, benzoate, phenol) during anaerobic benzene degradation, indicating that methylation, carboxylation, or hydroxylation are possible benzene-activating reactions under anoxic conditions (Chakraborty and Coates 2005; Ulrich *et al.* 2005).

4.3.4 Environmental implications

We propose that TDIFA can be used for elucidating benzene degradation pathways, which opens opportunities for characterizing the fate of benzene in contaminated environments. Furthermore, this approach maybe used to indicate whether a not characterized benzene-degrading enrichment or pure culture uses a mono- or dioxygenase reaction to initialize the aerobic benzene biodegradation pathway. For a preliminary survey of aerobic benzene degradation mechanisms it may be reasonable to perform two-dimensional isotope fractionation investigations before starting time-consuming biochemical and complex molecular biology test series.

For the quantification of *in situ* pollutant biodegradation via the Rayleigh equation approach, it is important to select an appropriate enrichment factor (see Meckenstock *et al.* (2004a) for a review). TDIFA may be a reliable tool for determining predominant degradation pathways in the environment, helping in the selection of an appropriate enrichment factor. Recently, TDIFA was used to analyze anaerobic benzene degradation under *in situ* conditions (Fischer *et al.* 2007).



1) —⋆— *R. opacus* B-4, 2) -- ▽-- *P. putida* ML2, 3) ---○--- *Burkholderia* sp. (*32*),
4) ---▷--- *C. necator* ATCC 17697, 5)---◇--- *A. denitrificans* strain BC (aerobic),
6) ---△--- *R. pickettii* PKO1, 7) -- ○-- *Acinetobacter* sp. (*32*),
8) —□— *A. denitrificans* strain BC (chlorate-reducing), 9) ---+--- Nitrate-reducing mixed culture (*31*),
10) ---×--- Sulphate-reducing mixed culture (*31*), 11) ---- Methanogenic mixed culture (*31*),
12) - - | - - Sulphate-reducing mixed culture

Figure 4.2: Plot of hydrogen versus carbon isotope discrimination for aerobic and anaerobic benzene biodegradation derived from the Rayleigh equation (eq. 4.3). The symbols of each curve give benzene biodegradation for 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 99%. Since no significant hydrogen isotope fractionation was observed for benzene dihydroxylation by *Rh. opacus* B-4 and *P. putida* ML2, the mean value of the expected enrichment factor range (ε_H of 0‰) was chosen for the calculation of hydrogen isotope discrimination by these two strains.

However, even if the pathway is identified by TDIFA, enrichment factors for a specific pathway can vary, leading to an uncertainty in the quantification of *in situ* biodegradation. This issue is illustrated in Figure 4.2 where Δ_C and Δ_H were calculated for each culture based on measured enrichment factors and hypothetical concentration changes using a simplified Rayleigh equation (Mariotti *et al.* 1981):

$$\Delta \approx \delta_t - \delta_0 \approx \varepsilon \cdot \ln(C_t / C_0) \qquad \text{eq. 4.3}$$

The length of the different curves representative for a microbial culture with known enrichment factors represents the carbon and hydrogen isotope discrimination (Δ_C, Δ_H) for 99% biodegradation. The symbols of the curves show the benzene biodegradation between 0 and 99% in steps of 10%. The data indicate that, for similar Λ values, a given carbon or hydrogen isotope discrimination corresponds to varying amounts of biodegradation. Hence, the amount of *in situ* benzene biodegradation calculated from stable isotope analysis can vary significantly for a specific pathway due to different enrichment

factors. To achieve a conservative assessment of biodegradation, the enrichment factor representing highest isotope fractionation for the predominant pathway should be chosen for the Rayleigh equation approach (Elsner *et al.* 2007). Generally, a selection of an appropriate enrichment factor by TDIFA should only be carried out if field data are consistent with a specific Λ range for a distinct pathway. If the field data are equal to overlapping Λ ranges, the highest known enrichment factor of all degradation pathways should be chosen for the assessment of biodegradation, otherwise there is a risk of overestimating the *in situ* biodegradation.

Generally, two-dimensional isotope fractionation investigations can also be applied for contaminants other than benzene. For example, analysis of carbon and hydrogen isotope fractionation has been performed in order to characterize pathways for fuel oxygenates (Kuder *et al.* 2005; Zwank *et al.* 2005; Elsner *et al.* 2007; Rosell *et al.* 2007). In the future, it is envisioned that compound-specific isotope analysis will be routinely available for measuring oxygen, chlorine, sulfur, or nitrogen isotope signatures of pollutants. Hence, it should be possible to extend the TDIFA to multidimensional isotope fractionation investigations for elucidating unknown degradation pathways. This may open further perspectives for detailed analysis of the fate of organic pollutants in field studies.

4.4 Supporting information

This material is available free of charge via the Internet at http://pubs.acs.org.

4.4.1 Degradation experiments

For the aerobic degradation experiments, each strain was first pre-grown in 118 mL serum bottles (Glasgerätebau Ochs, Germany) filled with 50 mL modified Brunner medium (or mineral salt medium for *A. denitrificans* - see publication). For inoculation, a single colony of bacteria previously grown on peptone agar or R2A agar, respectively, was taken. After adding benzene (3 µL), the bottles were closed with butyl rubber stoppers, crimped, and incubated on a rotary shaker (115 rotations per minute) at room temperature. After benzene degradation and growth had occurred, a second pre-culture was incubated under the same conditions as described above, using an inoculum of 5 mL for 45 mL medium, until the benzene was consumed and cells were grown again. The whole contents of two of these cultures (100 mL) served as inoculum for each separate isotope fractionation experiment. For the degradation experiment with *A. denitrificans* under chlorate-reducing conditions, precultures were prepared similarly, but using anoxic chlorate-containing mineral salt medium spiked with benzene.

The isotope fractionation experiments were performed in 2 L bottles filled with 900 mL Brunner medium (or mineral salt medium for *A. denitrificans*) and sealed using butyl rubber stoppers and aluminium crimps. The medium was spiked with benzene, sealed, and shaken overnight for equilibration. Afterwards, a single bottle was inoculated with 100 mL pre-culture (see above) and incubated at 25°C on a rotary shaker (115 rotations per minute). Initial benzene concentrations were 67 mg L^{-1} for the degradation experiment with *Rh. opacus* B-4, 56 mg L^{-1} with *P. putida* ML2, 92 mg

L^{-1} with *C. necator* ATCC 17697, 69 mg L^{-1} with *R. pickettii* PKO1, 47 mg L^{-1} with *A. denitrificans* strain (oxic conditions) and 36 mg L^{-1} with *A. denitrificans* strain (anoxic conditions), respectively. Bottles not inoculated with bacteria served as sterile controls. In these controls, changes in concentrations and isotope ratios were in the range of the analytical uncertainty of the method used (see "Analytical methods" section). For sampling, aliquots of the culture medium were regularly taken by means of syringes for GC analysis of benzene (1 mL), measurement of the optical density at 600 nm for following growth (1 mL), and stable isotope analysis (14 mL). The removed volume was always balanced by sterile air (or nitrogen for *A. denitrificans* incubated anoxically) in order to avoid negative pressure inside the bottles. Samples for isotope analysis were conserved with 0.5 mL concentrated hydrochloric acid and overlaid with 1 mL *n*-pentane for extraction of benzene, using 16 mL vials closed with Teflon-coated screw-caps (Supelco). GC samples were conserved using sulfuric acid (see analytical methods). Fixed samples were stored at 4°C until analysis.

Degradation experiments with a sulfate-reducing mixed culture were performed at 20°C. The origin of the culture and the general culture conditions are described in Vogt *et al.* (2007): the benzene degrading microorganisms are attached to coarse sand particles immersed in anoxic mineral salt medium. The experiment was carried out in a 2 L bottle filled with a mixture of coarse sand and anoxic mineral salt medium in a ratio of approximately 1:3. Initial benzene concentrations of 15 mg L^{-1} were achieved by adding pure benzene. Control bottles were triply autoclaved (121°C, 20 minutes, once on each of three consecutive days) after benzene was added. All culture bottles were incubated statically in the dark. Sampling was performed as described for the aerobic strains, but the syringes were flushed with nitrogen before use and the volume was balanced with nitrogen in order to exclude the intrusion of oxygen. For the sterile controls, changes in concentrations and isotope ratios were in the range of the analytical uncertainty (see "Analytical methods" section).

4.4.2 Analytical methods

Benzene was analyzed by means of automated headspace gas chromatography with a Varian 3800 gas chromatograph (Varian, USA) equipped with a CP SIL 5 CB capillary column (25 m × 0.12 mm ID × 0.12 µm FD; Varian, Germany) and a flame ionization detector. The chromatographic conditions were as follows: injector temperature 250°C (split 1:50); detector temperature 260°C; and an oven temperature program consisting of 70°C for 2 min, followed by an increase at a rate of 10°C min^{-1} up to 90°C and then at a rate of 60°C min^{-1} until 220°C. Helium (1 mL min^{-1}) was used as carrier gas. Liquid test samples (diluted 1:10 or 1:20 in 1.6 mM H_2SO_4 to prevent bacterial activity; final volume, 10 mL) were prepared in 20 mL glass vials. The samples were incubated for 30 min at 70°C in an agitator (rotation regime: 250 rpm for 5 s and no rotation for 2 s) prior to analysis, and 1 mL of each sample's headspace was injected. For calibration, diluted standards of benzene prepared from stock solutions were treated in the same way as the samples. The stock solutions were prepared in pure

methanol. The relative error of the benzene concentration analyses was less than 5% of the determined concentration.

The carbon and hydrogen isotope ratios of benzene were analyzed by means of gas chromatograph-isotope ratio mass spectroscopy (GC-IRMS) described elsewhere (Fischer *et al.* 2007). For the analyses, 14 mL of the medium from degradation experiment was extracted with 1 mL *n*-pentane. With this extraction method, it was possible to measure reliable carbon isotope ratios for samples with concentrations > 0.25 mg L^{-1} and reliable hydrogen isotope ratios for samples with concentrations > 2 mg L^{-1}. The isotope ratios were expressed in the delta notation ($\delta^{13}C$ and $\delta^{2}H$) in per mil (‰) units according to eq. 4.S1.

$$\delta^{13}C_{sample} \quad or \quad \delta^{2}H_{sample}\,[‰] = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \cdot 1000 \qquad \text{eq. 4.S1}$$

In eq. 4.S1, R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$- or $^{2}H/^{1}H$-ratios of the sample and an international standard, respectively. Vienna Pee Dee Belemnite (VPDB) was used as the standard for the analysis of carbon isotope signature and Vienna Standard Mean Ocean Water (VSMOW) was used as the standard for the detection of hydrogen isotope ratios (Gonfiantini *et al.* 1995; Coplen *et al.* 2006).
All samples were measured in at least 3 replicates. The reproducibility of $\delta^{13}C$ values and $\delta^{2}H$ values was always better than 0.5‰ and 5‰, respectively.

4.4.3 Quantification of isotope fractionation

For the description of isotope fractionation of biochemical reactions the Rayleigh equation can be expressed in its most general form (Mariotti *et al.* 1981):

$$\frac{R_t}{R_0} = \left(\frac{\frac{C_t}{C_0}}{\frac{R_t + 1}{R_0 + 1}}\right)^{\frac{\varepsilon}{1000}} \qquad \text{eq. 4.S2}$$

In most natural systems the assumption $R+1 \approx 1$ is valid which leads to a simplified version of the Rayleigh equation most commonly used for the assessment of biodegradation processes:

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \qquad \text{eq. 4.S3}$$

where R_t, C_t and R_0, C_0 are the stable isotope ratios and concentrations of a compound at a given point in time and at the beginning of a transformation reaction, respectively. The enrichment factor ε [‰] correlates the changes in stable isotope ratios (R_t/R_0) with the changes in the concentrations (C_t/C_0). Using the delta notation, the Rayleigh equation can be expressed as:

$$\frac{(\delta_t + 1000)}{(\delta_0 + 1000)} = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \qquad \text{eq. 4.S4}$$

The enrichment factor (ε) can be determined from the logarithmic form of the Rayleigh equation,

$$\ln\left(\frac{(\delta_t+1000)}{(\delta_0+1000)}\right)=\frac{\varepsilon}{1000}\ln\left(\frac{C_t}{C_0}\right) \qquad \text{eq. 4.S5}$$

by plotting $\ln(C_t/C_0)$ versus $\ln[(\delta_t+1000)/(\delta_0+1000)]$ and obtaining ε from the slope of the linear regression (m):

$$m=\frac{\varepsilon}{1000}. \qquad \text{eq. 4.S6}$$

Direct comparison of enrichment factors is only meaningful, if the same specific compound is considered. For a general mechanistic interpretation of the isotope discrimination per reacting position, enrichment factors have to be converted to AKIE of the bond cleavage. Therefore, the enrichment factors calculated from isotopic changes of the chemical (ε_{bulk}) are used to calculate the enrichment factors specific for the reactive position ($\varepsilon_{reactive\ position}$) in order to determine AKIEs (Elsner *et al.* 2005). According to Elsner *et al.* (2005) equations 4.S7 and 4.S8 should be applied to calculate the AKIE for benzene:

$$\varepsilon_{bulk} = \varepsilon_{reactive\ position} \qquad \text{eq. 4.S7}$$

since benzene is a symmetric molecule (Elsner *et al.* 2005). The potentially reactive six carbon and six hydrogen atoms are in equivalent positions requiring corrections for intramolecular isotope competition (Elsner *et al.* 2005). Equation 4.S8 is used to calculate the AKIE considering the intramolecular competition of carbon and hydrogen atoms,

$$AKIE=\frac{1}{1+z\cdot\varepsilon_{reactive\ position}/1000} \qquad \text{eq. 4.S8}$$

where z is the number of atoms of an element in identical reactive positions.

4.4.4 Two-dimensional isotope fractionation approach

Combining the Rayleigh equation for C and H with the definition of Λ leads to the expression:

$$\frac{\Lambda\cdot\varepsilon_C}{1000}\cdot\left(\ln\left(\frac{R_{C,t}+1}{R_{C,0}+1}\right)-\ln\left(\frac{R_{H,t}+1}{R_{H,0}+1}\right)\right)+\Lambda\cdot\ln\left(\frac{R_{C,t}}{R_{C,0}}\right)=\ln\left(\frac{R_{H,t}}{R_{H,0}}\right) \qquad \text{eq. 4.S9}$$

where $R_{C,t}$, $R_{H,t}$ and $R_{C,0}$, $R_{H,0}$ are the carbon (C) and hydrogen (H) stable isotope ratios at a given point in time (t) and at the beginning of a transformation reaction (0), respectively.

Assuming (i) $R+1\approx1$ and (ii) $\ln\left(\frac{R_t}{R_0}\right)\approx\delta-\delta_0$, equation 4.S9 can be simplified to

$$\Lambda\cdot\Delta_C=\Delta_H \qquad \text{eq. 4.S10}$$

with $\Delta = \delta_t - \delta_0$. Thus, Λ is also given by the slope of the plot of carbon vs. hydrogen isotope discrimination (Δ_C, Δ_H). Δ_C and Δ_H were calculated for each culture based on measured enrichment factors and hypothetical concentration changes using a simplified Rayleigh equation:

$$\Delta \approx \delta_t - \delta_0 \approx \varepsilon \cdot \ln\left(\frac{C_t}{C_0}\right). \qquad \text{eq. 4.S11}$$

For benzene biodegradation characterized by high hydrogen fractionation assumption (i) is still applicable but assumption (ii) is violated due to shifts in δ_H to values of up to 400‰. As a consequence the relationship between Δ_C and Δ_H is not exactly linear. However, for small changes in $\ln(C_t/C_0)$ equation 4.S10 is still valid and results in values for Λ which are approximating the values calculated by $\varepsilon_H/\varepsilon_C$.

Acknowledgments

This work was supported by the Helmholtz Centre for Environmental Research - UFZ as part of the project "SAFIRA II" and by Isodetect - Company for Isotope Monitoring. Steffi Herrmann was supported by the German Federal Ministry of Education and Research (BEOQUE, grant 02 WN 0651). We acknowledge Matthias Gehre and Ursula Günther for their support in the isotope laboratory of the UFZ, Stephanie Hinke for help during cultivation as well as Gwenaël Imfeld and Martin Elsner for their helpful discussions on calculations.

Note for reviewers

S. Herrmann performed the experimental work and data analysis of the sulfate-reducing mixed culture, and contributed to interpretation and manuscript preparation.

5

Characterization of anaerobic xylene biodegradation by two dimensional isotope fractionation analysis

Steffi Herrmann[1], Carsten Vogt[1], Anko Fischer[1,2], Anke Kuppardt[3], and Hans-Hermann Richnow[1]

[1]Department of Isotope Biogeochemistry, UFZ - Helmholtz Centre for Environmental Research, Leipzig, Germany

[2]Isodetect – A Company for Isotope Monitoring

[3]Department of Environmental Microbiology, UFZ - Helmholtz Centre for Environmental Research, Leipzig, Germany

Submitted to Environmental Microbiology

Abstract

We determined stable carbon and hydrogen isotope fractionation factors for anaerobic degradation of xylene isomers by several pure and mixed cultures. All cultures initiated xylene degradation by the addition of fumarate to a methyl moiety, as is known from the literature or verified by the presence of methyl benzylsuccinates as metabolic intermediates of xylene biodegradation. Additionally, gene probes positively identified the alpha-subunit of benzylsuccinate synthase (*bssA*) in the majority of the cultures. Xylene degradation was always coupled to a significant carbon and hydrogen isotope fractionation. The values of the apparent kinetic isotope effect (AKIE) for carbon and hydrogen indicate that the cleavage of a carbon-hydrogen-bond is an isotopically sensitive step during fumarate addition to xylene isomers. The slopes of the linear regression for hydrogen ($\Delta\delta^2H$) vs. carbon ($\Delta\delta^{13}C$) discrimination ($\Lambda = \Delta\delta^2H/\Delta\delta^{13}C \approx \varepsilon H_{bulk}/\varepsilon C_{bulk}$) ranged from 12 ± 4 to 29 ± 5 and were comparable to Λ values previously determined for anaerobic toluene degradation. This indicates that fumarate addition to xylene isomers might proceed via an analogous mechanism to the well-described addition of fumarate to the methyl moiety of toluene, catalyzed by Bss. The results suggest that combined carbon and hydrogen isotope fractionation analyses can be used to monitor anaerobic xylene degradation at contaminated sites.

5.1 Introduction

Petroleum hydrocarbons are frequent soil and groundwater contaminants (Squillace *et al.* 2004). Numerous studies in the last decade have shown that these compounds can be mineralized under oxic or anoxic conditions by indigenous microorganisms, and monitored natural attenuation (MNA) has become an important remediation strategy for hydrocarbon contaminated sites. A promising remediation monitoring method for identifying, and quantifying biodegradation processes is compound specific stable isotope analysis (CSIA; for an overview see Meckenstock *et al.* (2004a) and Elsner *et al.* (2005)). CSIA make use of the isotopic discrimination upon bond changes that occur in many (bio)chemical reactions. Since abiotic, physical processes are generally not coupled to significant isotope fractionation (Harrington *et al.* 1999; Slater *et al.* 2000; Schüth *et al.* 2003; Hunkeler *et al.* 2004; Squillace *et al.* 2004; Kopinke *et al.* 2005), isotope analysis is highly selective for biotransformation reactions in the environment.

Bond changes of isotopically lighter atoms proceed faster than heavier ones due to lower activation energies required to facilitate the (bio)chemical reaction, leading to an enrichment of heavier isotopomers in the residual phase. This process is known as isotopic fractionation and can be quantified as the kinetic isotope effect (KIE). This KIE can be interpreted using the transition state theory (Northrop 1981). The expression of the KIE in a (bio)chemical reaction depends upon kinetic limitations associated with bond changes and rate limitations of non-fractionating reaction steps preceding the bond cleavage. A single isotope effect determined in a biochemical reaction thus

contains information on the nature of the transition state during the bond change and rate limitations associated with non isotopically sensitive rate-limiting steps. In the field of enzymology, rate-limiting but non-fractionating processes occur during the 'commitment to catalysis' (Northrop 1981; Elsner *et al.* 2005; Nijenhuis *et al.* 2005). The apparent kinetic isotope effect (AKIE) of a biochemical reaction can be much smaller than its underlying KIE. The often observed variability of bulk enrichment factors (ε_{bulk}) and AKIEs for specific contaminant degradation pathways is thought to be caused by the commitment to catalysis (Elsner *et al.* 2005).

In comparison to single element studies, the use of two-dimensional isotope fractionation analysis has been encouraged for the characterization of rate-determining reaction steps of hydrocarbon biodegradation pathways more precisely (Elsner *et al.* 2005; Zwank *et al.* 2005; Fischer *et al.* 2007; Fischer *et al.* 2008). The slope of the linear regression for hydrogen ($\Delta\delta^2H$) vs. carbon ($\Delta\delta^{13}C$) discrimination is known as lambda (Λ_{bulk}) = $\Delta\delta^2H/\Delta\delta^{13}C \approx \varepsilon H_{bulk}/\varepsilon C_{bulk}$ (Fischer *et al.* 2008). Since carbon and hydrogen fractionation are thought to be equally influenced by rate limitations prior to the isotope sensitive bond change (Elsner *et al.* 2005), any rate limitations which mask the KIE are cancelled out; hence, the variable Λ can be seen as a fingerprint of the initial biochemical bond cleavage reaction within a distinct degradation pathway.

Xylenes belong to the most relevant alkylbenzenes in oil (Widdel and Rabus 2001), but less is known about their degradation under anoxic conditions. Several *m*-xylene-mineralizing pure cultures have been described (Dolfing *et al.* 1990; Fries *et al.* 1994; Rabus and Widdel 1995; Harms *et al.* 1999b; Morasch *et al.* 2004b). However, only two strains have been reported for anaerobic *o*-xylene mineralization, both sulphate reducers (Harms *et al.* 1999b; Morasch *et al.* 2004b). No pure culture has been identified that is able to grow on *p*-xylene as sole source of carbon and energy, although a few *p*-xylene-degrading enrichments have been described under different electron accepting conditions (Haner *et al.* 1995; Morasch and Meckenstock 2005; Botton and Parsons 2007; Nakagawa *et al.* 2008). For anaerobic xylene degradation of pure and mixed cultures, carbon enrichment factors for *o*-xylene and *m*-xylene have been previously reported, ranging between -1.1 ‰ and -3.2 ‰ (Wilkes *et al.* 2000; Richnow *et al.* 2003a; Morasch *et al.* 2004a), and indicate that the first step of anaerobic xylene degradation is linked to a primary carbon isotope effect. There is some evidence that degradation of xylene isomers under anoxic conditions is initiated by addition of fumarate to a methyl moiety (Beller 1997; Krieger *et al.* 1999; Morasch *et al.* 2004b; Morasch and Meckenstock 2005), a reaction carried out by the enzyme benzylsuccinate synthase (Bss). This enzyme is known to activate toluene under anoxic conditions in several microbial strains (for an overview see Heider (2007)). Recent fractionation studies with several toluene-degrading bacteria indicated that Bss mediated toluene transformation is always associated with a significant carbon and hydrogen kinetic isotope effect (Meckenstock *et al.* 1999b; Morasch *et al.* 2004a; Tobler *et al.* 2008; Vogt *et al.* 2008). We recently observed that Λ_{bulk} values significantly differed between toluene-degrading sulphate reducers,

nitrate reducers and an anaerobic phototrophic bacterium, indicating the presence of several slightly different Bss reaction mechanisms (Vogt *et al.* 2008).

The aim of this study was to explore the carbon and hydrogen isotope fractionation of Bss initiated degradation pathways for xylene isomers in order to obtain information on reaction mechanisms and to assess the variability of isotope fractionation processes associated with Bss, which might be important for the assessment of anaerobic degradation of xylene and toluene in the environment. For our study, we used mixed cultures enriched from a BTEX-contaminated aquifer able to degrade either *m*-xylene, *o*-xylene, or *p*-xylene under sulphate-reducing conditions. For comparison, we investigated the isotope fractionation of two bacterial strains known to utilize Bss as xylene-activating enzyme, *Azoarcus* strain T and *Desulfosarcina ovata* strain oXyS1. For all cultures, enrichment factors for carbon and hydrogen were determined and the respective Λ values for xylene degradation were calculated and compared to isotope fractionation factors of Bss dependent toluene degradation to retrieve mechanistic information on the initial steps of the biodegradation pathway.

5.2 Experimental procedures

5.2.1 Microorganisms and cultivation conditions

The *m*-xylene-degrading consortium was enriched from anoxic, sulphidic groundwater of a BTEX-contaminated aquifer near Zeitz, Germany (Herrmann *et al.* 2008). The consortia degrading *o*-xylene or *p*-xylene, respectively, were enriched from sand particles from an anoxic, benzene-degrading columns system installed at the same field site (Vogt *et al.* 2007). *Azoarcus* sp. strain T (DSM 9506) and *Desulfosarcina ovata* strain oXyS1 (DSM 13228) were ordered from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Desulfotomaculum* sp. strain OX39 (Morasch *et al.* 2004b), which can use *m*-xylene and *o*-xylene as a growth substrate, was included in the screening study, but unfortunately did not grow in our lab. The sulphate-reducing cultures were cultivated in anoxic mineral salt medium amended with 20 mM sulphate described elsewhere (Vogt *et al.* 2007) using the respective xylene isomer or toluene as a sole growth substrate. *Azoarcus* sp. T was cultivated in DSMZ medium 586 with *m*-xylene as the sole growth substrate and 10 mM nitrate as the electron acceptor. For stock cultures, toluene or xylenes were dissolved in anoxic 2,2,4,4,6,8,8 heptamethylnonane (HMN) to reduce the concentration in the liquid phase (20 µL xylene isomers or toluene/3 mL HMN). 3 mL of xylene isomers- or toluene-spiked HMN was used for 100 mL medium.

5.2.2 Isotope fractionation experiments

Isotope fractionation experiments were carried out in batch cultures. Experiments with xylene or toluene-degrading mixed cultures or *Azoarcus* T were performed in single bottles (modified 500 mL flasks, Schott, Germany) which were regularly sampled. The experiment with *D. ovata* was performed using several replicate bottles (56 or 118 mL serum flasks, Ochs, Germany) which were periodically

sacrificed. In a recent study (Vogt *et al.* 2008), both experimental designs produced similar results and were therefore considered as fully comparable. All bottles were sealed gastight with crimped Teflon-coated butyl rubber stoppers (VWR, Germany) and spiked with pure toluene or xylene by glass syringes to a final concentration of 0.2 to 0.45 mM toluene and xylene, respectively, and subsequently shaken over night for equilibration. Afterwards, each bottle was inoculated with 10% (vol/vol) pre-culture, which had been grown with the respective substrate of the following degradation experiment as a sole source of carbon and energy until the early stationary growth phase. These cultures were incubated statically in the dark at temperatures between 20 and 25 °C.

For chemical analysis, various amounts of culture medium were removed with syringes (toluene/xylene, 1 mL; sulphide, 25-100 µL; stable isotope analysis, 14 mL). Before sampling, syringes were flushed with nitrogen to avoid oxygen contamination in the culture bottles. Removed volume was always balanced by sterile nitrogen to avoid low pressure in the bottles. Samples for isotope analysis were conserved with 0.5 mL of concentrated hydrochloric acid and overlaid with 1 mL of *n*-pentane for extraction of toluene/xylene, using 16 mL vials closed with Teflon-coated screw-caps. In the sacrifice bottle experiments, a single culture was killed at each time point by adding 1 mL of concentrated hydrochloric acid per 50 ml culture volume after samples for sulphide and toluene/xylene analysis had been taken. Subsequently, the whole culture volume was extracted with 3 mL *n*-pentane. Samples were stored at 4°C until analysis. Control experiments were set up by using the same media and the same flasks which were sterilised by autoclaving (121°C, 20 min) three times after gas tight sealing.

5.2.3 Analytical methods

Toluene and xylene concentrations were determined by headspace gas chromatography as described elsewhere (Vogt *et al.* 2008). Sulphide was photometrically analyzed according to Cline (1969) using modifications described by Herrmann *et al.* (2008). Toluene- and xylene-degrading consortia were analyzed for metabolites during the latter phase of the degradation experiment. 50 mL culture liquid were acidified with HCl to pH 1 and extracted with diethyl ether. The organic phase was collected and evaporated under a continuous nitrogen stream. The residual fraction was dissolved in 450 µL pure methanol. Subsequently, 50 µL trimethylchlorosilane was added, and the samples were derivatised for 1.5 h at 65°C in order to transform the carboxylic acids into methyl esters. Afterwards, the samples were again dried under a continuous nitrogen stream, and the residue was dissolved in 200 µL *n*-hexane. For identification of metabolites, a HP 6890 gas chromatograph coupled with a HP 5973 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, USA) was used. Metabolites were separated on a Zebron BPX-5 column (30 m × 0.32 mm × 0.25 mm) (Phenomenex, Torrance, USA) with the following temperature program: 40°C (5 min), 20°C min^{-1} to 150°C, 2°C min^{-1} to 250°C, 20°C min^{-1} to 300°C (2 min). The identity of the substances was confirmed by comparison of mass

spectra with published data (Beller 1997; Krieger *et al.* 1999; Morasch *et al.* 2004b) or by co-elution with reference compounds (benzylsuccinic acid; Sigma-Aldrich, Germany).

5.2.4 Stable isotope analysis

The carbon and hydrogen isotope ratios of toluene and xylene were analyzed by gas chromatograph-isotope ratio mass spectroscopy (GC-IRMS). Aliquots of the *n*-pentane extract of samples from biodegradation experiments were used for isotope analyses. The isotope ratios were expressed in the delta notation ($\delta^{13}C$ and $\delta^{2}H$) in per mil (‰) units according to equation 5.1.

$$\delta^{13}C_{sample} \quad or \quad \delta^{2}H_{sample}\,[‰] = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \cdot 1000 \qquad \text{eq. 5.1}$$

R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$- or $^{2}H/^{1}H$-ratios of the sample and an international standard, respectively. Vienna Pee Dee Belemnite (VPDB) was used as the standard for the analysis of carbon isotope signature, and Vienna Standard Mean Ocean Water (VSMOW) was used as the standard for the detection of hydrogen isotope ratios. All samples were measured in at least 3 replicates. The total analytical uncertainty for compound-specific hydrogen and carbon isotope analysis with respect to both accuracy and reproducibility is ± 5 ‰ and ± 0.5 ‰, respectively.

5.2.5 Quantification of isotope fractionation

For the description of isotope fractionation of biochemical reactions, the Rayleigh equation can be expressed in its most general form Mariotti *et al.* (1981):

$$\frac{R_t}{R_0} = \left(\frac{\frac{C_t}{C_0}}{\frac{R_t + 1}{R_0 + 1}}\right)^{\frac{\varepsilon}{1000}} \qquad \text{eq. 5.2}$$

In most natural systems the assumption that $R+1 \approx 1$ is valid and leads to a simplified version of the Rayleigh equation most commonly used for the assessment of biodegradation processes:

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \qquad \text{eq. 5.3}$$

with R_t, C_t and R_0, C_0 as the stable isotope ratios and concentrations of a compound at a given point in time and at the beginning of a transformation reaction, respectively. The enrichment factor ε [‰] correlates the changes in stable isotope ratios (R_t/R_0) with the changes in the concentrations (C_t/C_0). Using the delta notation, the Rayleigh equation can be expressed as:

$$\frac{(\delta_t + 1000)}{(\delta_0 + 1000)} = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \qquad \text{eq. 5.4}$$

The enrichment factor (ε) was determined from the logarithmic form of the Rayleigh equation,

$$\ln\left(\frac{(\delta_t+1000)}{(\delta_0+1000)}\right)=\frac{\varepsilon}{1000}\times\ln\left(\frac{C_t}{C_0}\right) \qquad \text{eq. 5.5}$$

plotting $\ln(C_t/C_0)$ versus $\ln[(\delta_t+1000)/(\delta_0+1000)]$, obtained from the slope of the linear regression (m) using equation 5.6:

$$m=\frac{\varepsilon}{1000} \qquad \text{eq. 5.6}$$

The error of the enrichment factor is given as a 95% confidence interval (CI) and was calculated as described by Elsner *et al.* (2007).

For a general mechanistic interpretation of degradation pathways, non-reacting positions have to be taken into account. Accordingly, bulk enrichment factors (ε_{bulk}) have to be converted to enrichment factors of the reacting position ($\varepsilon_{reactive\ position}$) using the Rayleigh equation (Elsner *et al.* 2005):

$$\ln\left(\frac{(n/x\times\delta_t+1000)}{(\delta_0+1000)}\right)=\frac{\varepsilon_{reactive\ position}}{1000}\times\ln\left(\frac{C_t}{C_0}\right) \qquad \text{eq. 5.7}$$

where δ_t is the isotope ratio at a given point in time corrected for the reactive position. *n* stands for the number of atoms and *x* stands for the number of reactive atoms within the molecule. For toluene activation by methyl moiety attack, only one carbon atom out of seven is located at the reactive site, hence $n = 7$ and $x = 1$. For hydrogen, 3 out of 8 atoms are located at the methyl group, thus $n = 8$ and $x = 3$. For xylene activation by methyl moiety attack, two carbon atoms out of eight are located at reactive sites, hence $n = 8$ and $x = 2$. For hydrogen, 6 out of 10 atoms are located at the methyl group, thus $n = 10$ and $x = 6$. The error of the enrichment factor of the reacting position is given as a 95% confidence interval (CI) and was calculated as described by Elsner *et al.* (2007).

For calculation of the apparent kinetic isotope effect (AKIE), intramolecular isotopic competition has additionally to be considered by using the following equation (Elsner *et al.* 2005):

$$AKIE=\frac{1}{1+z\times\varepsilon_{reactive\ position}/1000} \qquad \text{eq. 5.8}$$

where *z* is the number of atoms which are in intramolecular competition. For reactions at the methyl group of toluene, the number of *z* is 1 for carbon and 3 for hydrogen. In the case of xylene isomers, the number of *z* is 2 for carbon and 6 for hydrogen. Uncertainties associated with AKIE were estimated by error propagation: error of AKIE = $|\partial AKIE/\partial\varepsilon_{reactive\ position}|$ × error of $\varepsilon_{reactive\ position}$.

The factor Λ_{bulk} expresses the slope of the linear regression for carbon and hydrogen discrimination:

$$\Lambda_{bulk}=\Delta\delta^{2}H_{bulk}/\Delta\delta^{13}C_{bulk} \qquad \text{eq. 5.9}$$

In order to compare Λ values for mechanistic studies of toluene and xylene isomers, we used the factor $\Lambda_{reactive\ position}$ which expresses the slope of the linear regression for discrimination of the reactive positions of hydrogen and carbon:

$$\Lambda_{reactive\ position} = \Delta\delta^{2}H_{reactive\ position} / \Delta\delta^{13}C_{reactive\ position} \qquad \text{eq. 5.10}$$

Abe *et al.* (2009) used AKIE − 1 ratios for comparing different (bio)chemical reactions, which might be the most precise approach in the case of low AKIEs which are principally based on primary isotope effects. High fractionation factors can be caused by both strong primary and secondary isotope effects, leading to AKIE values that are difficult to interpret (Elsner *et al.* 2007). Since we observed high fractionation factors for hydrogen during our study, we did not use the AKIE – 1 approach.

5.2.6 DNA preparation and sequencing of *bssA* gene fragments

DNA was extracted from cultures that had previously degraded toluene or xylenes during a fractionation experiment. 40 mL of cultures were centrifuged for 20 min at 5000 rpm (Centrifuge 5403, Eppendorf, Germany) and 4°C in sterile Falcon tubes. The supernatant was discarded, and the pellet was extracted using a FastDNA®Spin Kit for soil (QBiogene, USA). The yield of extraction was controlled by standard agarose gel electrophoresis and ethidium bromid staining using UV-light for visualization (E.A.S.Y. RH-3, 254 nm, Herolab, Germany). *BssA*-fragments were amplified by PCR using the degenerate primers 7772f and 8546r (Winderl *et al.* 2007) and a PTC DNA-EngineTM Thermocycler (Biozym, Germany). The following PCR program was used: 15 min initial denaturation, 30 cycles of amplification with 50 s at 94°C, 40 s at 55°C, and 50 s at 72°C, and 10 min of terminal extension at 72°C. 50 µL PCR reaction contained: 14.5 µL nuclease-free water, 25 µL HotStar master mix (HotStar-Kit, Qiagen, Germany), 1 µL $MgCl_2$ (25 mM), 2.5 µL DMSO; 0.2 µM of each primer, and 2 µL of template DNA. For the *p*-xylene-degrading culture, a mixture of specific and unspecific amplification products were obtained. Therefore, the appropriately sized amplicons (~773 bp) were excised from a 1% agarose gel (Sigma-Aldrich, Germany) and purified using a MiniElute Gel Extraction Kit (Qiagen, Germany) prior to cloning. PCR products were cloned into *Escherichia coli* DH5α using a PCR Cloning plus Kit (Qiagen, Germany). For each culture, 30 to 100 clones were randomly selected and checked for the correct size by vector-targeted PCR and agarose gel electrophoresis. Amplicons were double-digested with *Alu*I and *Hae*III (New England Biolabs, Germany) and checked for restriction fragment pattern similarities. Sequences of representatives for each operational taxonomic unit were analyzed by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) using BigDye terminator v3.1 chemistry (Applied Biosystems, USA) according to the manufacturer's instructions. All publicly available *bssA* gene sequences and the sequences obtained in this study were translated into amino acid sequences with Bioedit v7.0.3 software (www.mbio.ncsu.edu/BioEdit/bioedit.html) and integrated into an alignment using ClustalW. This alignment was used to calculate a phylogenetic tree using MEGA 4 and the Minimum Evolution method.

5.3 Results

5.3.1 Characteristics of xylene-degrading enrichment cultures

Three planktonic enrichment cultures were established under sulphate-reducing conditions using *o*-xylene, *p*-xylene or *m*-xylene as the sole source of carbon and energy, respectively. The inoculum was anoxic groundwater or coarse sand derived from a BTEX-contaminated site (for detailed site information see Schirmer *et al.* (2006), Fischer *et al.* (2007), and Vogt *et al.* (2007)). The ratio of produced sulphide versus consumed xylene isomer indicated that xylene was mineralized by all enrichment cultures (Figure 5.S1, supporting information) according to the following stoichiometry: $C_8H_{10} + 5.25\ SO_4^{2-} + 2.5\ H^+ + 3\ H_2O \rightarrow 8\ HCO_3^- + 5.25\ H_2S$. The degradation rates observed within the isotope fractionation experiments were different, ranging from 15 to 28 µM d^{-1} for *m*-xylene degradation, from 4 to 9 µM d^{-1} for *o*-xylene degradation, and up to 1 µM d^{-1} for *p*-xylene degradation (Figure 5.S1, supporting information). In all enrichment cultures, methyl benzlysuccinates were detected in the course of xylene degradation (Figure 5.S2, supporting information), demonstrating that the first biochemical step of the degradation pathway for all xylene isomers was initiated by the addition of fumarate to a methyl moiety. In addition, 2-methyl, 3-methyl and 4-methyl benzylsuccinates were tentatively identified by GC-MS analysis in degradation experiments with *o*-, *m*- and *p*-xylene, respectively (Figure 5.S2, supporting information).

The number of different Bss within the mixed cultures were approximated by amplifying and sequencing the gene coding for the alpha-subunit of the enzyme, *bssA*, using primers recently introduced by Winderl and colleagues (2007). The *bssA* sequences were compared to the previously described *bssA* sequences of pure cultures and environmental samples to obtain phylogenetic affiliations (Figure 5.1). In the *o*-xylene-degrading enrichment culture, two different sequence types were detected (marked as 'ox clone' in Figure 5.1), affiliated either to the *bssA* of the Gram-positive sulphate-reducing *Desulfotomaculum sp.* strain OX39, or to the *bssA* of the Gram-negative sulphate-reducing strain TRM1. Only a single sequence type was found in the *p*-xylene-degrading culture (marked as 'px clone' in Figure 5.1), which was not closely associated with any *bssA* of cultured species. We were unable to amplify a *bssA* sequence from the *m*-xylene-degrading culture with the primers used in this study.

5.3.2 Characteristics of toluene- and xylene-degrading pure cultures

D. ovata and *Azoarcus* T have been observed to use xylene and toluene as a growth substrates; *D. ovata* can use *o*-xylene (Harms *et al.* 1999b), and *Azoarcus* T can use *m*-xylene (Dolfing *et al.* 1990) as the sole source of carbon and energy. In the isotope fractionation experiments, *D. ovata* degraded *o*-xylene and toluene at rates of 2 to 6 µM d^{-1}. *Azoarcus* T degraded *m*-xylene at a rate of 80 µM d^{-1} (data not shown).

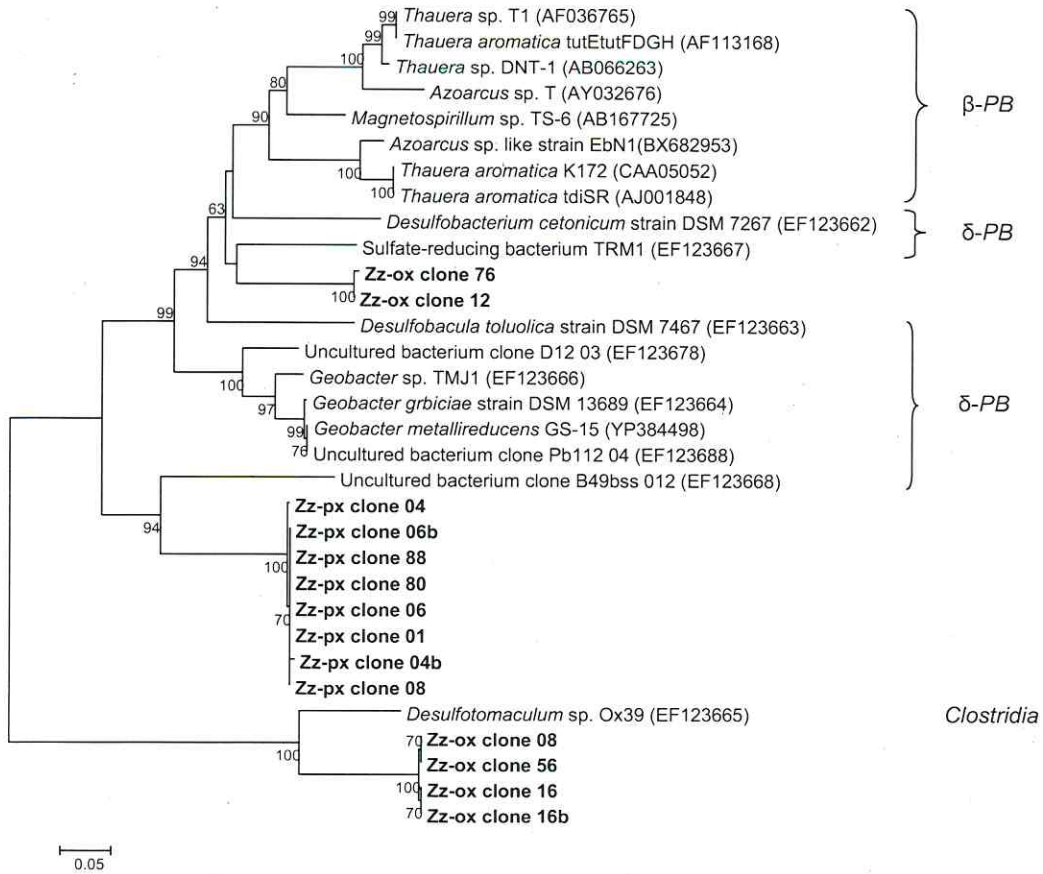


Figure 5.1: Putative *bssA* sequences retrieved from *o*-xylene- or *p*-xylene-degrading enrichment cultures investigated in this study, shown in a phylogenetic tree composed of previously described *bssA* sequences of pure cultures and environmental samples. The *bssA* sequences of environmental samples reported by Winderl *et al.* (2007) are represented by a single sequence for each lineage. *PB* stands for *Proteobacteria*.

Azoarcus T contains a single *bssA* sequence which is closely related to *bssA* sequences from nitrate-reducing *Thauera* and other *Azoarcus* species recently described by Winderl and colleagues (2007) (Figure 5.1). No *bssA* gene sequence was detected in the genome of *D. ovata* using the gene probes in this study.

5.3.3 Carbon and hydrogen isotope fractionation of xylene degradation

Xylene degradation was linked to carbon and hydrogen isotope fractionation within all examined cultures. In sterile control bottles, xylene concentrations as well as carbon and hydrogen isotopic signatures remained stable (data not shown), showing that isotope fractionation was purely based on microbially driven reactions. Substrate concentrations and isotope signatures were highly correlated ($R^2 > 0.84$) with both carbon and hydrogen in all biotic degradation experiments, allowing to calculate

Table 5.1: Bulk and position-specific enrichment factors (ε_{bulk}, $\varepsilon_{reactive\ position}$) for stable carbon and hydrogen fractionation, apparent kinetic isotope effects (AKIE), and bulk and position-specific lambda (Λ_{bulk}, $\Lambda_{reactive\ position}$) values for anaerobic degradation of xylene isomers and toluene by the cultures investigated in this study. For comparison, recently published values for anaerobic toluene degradation are listed. The isotope-sensitive step within all cultures is an addition of fumarate to the methyl moiety of the substrate.

Organism	Terminal electron acceptor	Substrate	Element	ε_{bulk} [‰][a]	$\varepsilon_{reactive\ position}$ [‰][a]	AKIE[a]	$\Lambda_{bulk} = \Delta\delta^2H/\Delta\delta^{13}C \approx \varepsilon H_{bulk}/\varepsilon C_{bulk}$	$\Lambda_{reactive\ position} = \Delta\delta^2H_{rp}/\Delta\delta^{13}C_{rp} \approx \varepsilon H_{rp}/\varepsilon C_{rp}$	Reference
Desulfosarcina ovata	sulphate	*o*-xylene	C	-2.3 ± 0.4	-9 ± 1.7	1.018 ± 0.004	15 ± 4	6 ± 2	This work
			H	-41 ± 9	-66 ± 14	1.65 ± 0.23			
		toluene	C	-2.5 ± 0.5	-17 ± 3	1.017 ± 0.004	41 ± 8	15 ± 3	
			H	-107 ± 23	-236 ± 47	3.42 ± 1.64			
Consortium	sulphate	*o*-xylene	C	-0.7 ± 0.1	-2.8 ± 0.5	1.006 ± 0.001	29 ± 5	12 ± 2	This work
			H	-25 ± 3	-40 ± 4	1.32 ± 0.04			
Consortium	sulphate	*m*-xylene	C	-2.7 ± 0.2	-11 ± 1	1.022 ± 0.002	16 ± 2	7 ± 1	This work
			H	-48 ± 5	-76 ± 8	1.84 ± 0.15			
		toluene	C	-6.7 ± 0.4	-44 ± 3	1.046 ± 0.003	20 ± 2	8 ± 1	
		(cometabolic)	H	-126 ± 9	-262 ± 20	4.73 ± 1.35			
Consortium	sulphate	*p*-xylene	C	-1.2 ± 0.3	-4.8 ± 1.2	1.01 ± 0.002	12 ± 4	5 ± 2	This work
			H	-19 ± 3	-32 ± 5	1.23 ± 0.042			
Desulfosarcina cetonica	sulphate	toluene	C	-2.4 ± 0.1	-17 ± 1	1.017 ± 0.001	30 ± 2	12 ± 2	Vogt *et al.* (2008)
			H	-74 ± 7	-172 ± 17	2:1 ± 0.2			
Strain TRM1	sulphate	toluene	C	-2.0 ± 0.2	-14 ± 1	1.014 ± 0.001	31 ± 11	11 ± 3	Vogt *et al.* (2008)
			H	-66 ± 19	-152 ± 48	1.8 ± 0.5			
Consortium	sulphate	toluene	C	-2.8 ± 0.1	-19 ± 1	1.019 ± 0.001	28 ± 2	11 ± 1	Vogt *et al.* (2008)
			H	-87 ± 6	-224 ± 23	3.1 ± 0.6			
Azoarcus T	nitrate	*m*-xylene	C	-2.3 ± 0.1	-8.9 ± 0.6	1.018 ± 0.001	21 ± 2	9 ± 1	This work
			H	-50 ± 6	-80 ± 9	1.93 ± 0.2			
		toluene	C	-5.7 ± 0.2	-38 ± 2	1.039 ± 0.002	11 ± 3	5 ± 1	Vogt *et al.* (2008)
			H	-78 ± 12	-187 ± 27	2.3 ± 0.4			
Azoarcus EbN1	nitrate	toluene	C	-2.9 ± 0.2	-20 ± 1	1.02 ± 0.001	14 ± 1	5 ± 1	Vogt *et al.* (2008)
			H	-50 ± 7	-122 ± 19	1.6 ± 0.1			
Thauera aromatica	nitrate	toluene	C	-2.7 ± 0.1	-19 ± 1	1.019 ± 0.001	11 ± 5	4 ± 2	Vogt *et al.* (2008)
			H	-35 ± 14	-88 ± 35	1.4 ± 0.2			
Geobacter metallireducens	ferric iron	toluene	C	-1.3 ± 0.1[a] -3.6 ± 0.7[b]	-9.1 -25.2	1.0093 ± 0.0003[b]	25 ± 1[b] 27 ± 1[c]	10[b, d] 10[b, d]	Tobler *et al.* (2008)
			H	-35 ± 0.9[a] -98 ± 3[b]	-93 -261	1.0251 ± 0.0005[c] 1.374 ± 0.013[b] 2.96 ± 0.13[c]			
Blastochloris sulfoviridis	phototrophic	toluene	C	-4 ± 0.5	-28 ± 3	1.029 ± 0.004	4 ± 3	2 ± 1	Vogt *et al.* (2008)
			H	-23 ± 6	-58 ± 14	1.2 ± 0.1			

[a] see section 'experimental procedure' for the calculation, [b] Fe mineral as iron source, [c] ferric iron citrate as iron source, [d] approximated from $\varepsilon_{H,rp}/\varepsilon_{C,rp}$

enrichment factors (ε) (Figures 5.S3 and 5.S4, supporting information) and apparent kinetic isotope effects (AKIEs), using the Rayleigh equation as a basis. In Table 5.1, isotope fractionation data for anaerobic xylene and toluene biodegradation from this study are compared to previously published results. All cultures listed initiate the degradation pathway by fumarate addition to a methyl moiety of the substrate. Bulk enrichment factors (ε_{bulk}) and the corresponding AKIEs for carbon ranged from -0.7 ± 0.1 and 1.006 ± 0.001 for *o*-xylene degradation to -2.7 ± 0.2 and 1.022 ± 0.002 for *m*-xylene degradation by different enrichment cultures, respectively. For hydrogen, the ε_{bulk} and AKIEs were generally higher, ranging from -19 ± 3 and 1.23 ± 0.04 for *p*-xylene degradation by the enrichment culture to -50 ± 6 and 1.93 ± 0.2 for *m*-xylene degradation by *Azoarcus* T, respectively (Table 5.1).

5.3.4 Carbon and hydrogen isotope fractionation of toluene degradation

Isotope fractionation of toluene degradation was determined for *D. ovata* and the *m*-xylene-degrading enrichment culture; the latter transformed toluene cometabolically when growing on *m*-xylene (Figure 5.S5, supporting information). The values for carbon and hydrogen were significantly different for *D. ovata* and the enrichment culture. The cometabolic toluene degradation by the enrichment culture produced the highest ε_{bulk} and AKIE for carbon and hydrogen observed so far for anoxic toluene degradation: -6.7 ± 0.4 and 1.046 ± 0.003, and -126 ± 9 and 4.73 ± 1.35, respectively. These factors are, however, still in the range previously observed for toluene degradation pathways initiated by the fumarate addition mechanism (Table 5.1).

5.3.5 Two dimensional isotope fractionation analyses

We calculated Λ values for bulk enrichment factors (Λ_{bulk}) as well as for enrichment factors at reactive positions in the molecule ($\Lambda_{reactive\ position}$); the former allows comparison of factors for a specific substrate, the latter makes comparison of toluene and xylene isotope fractionation possible. $\Delta\delta^2H$ and $\Delta\delta^{13}C$ values were calculated by subtracting the isotopic value at time t (δ_t) from the initial isotopic value (δ_o) and subsequently plotting these values in a dual parameter plot (Figure 5.2 for Λ_{bulk} and Figure 5.S6 of the supporting information for $\Lambda_{reactive\ position}$). For all degradation experiments described in this study, the carbon and hydrogen discrimination ($\Delta\delta^{13}C$, $\Delta\delta^2H$) showed a strong linear effect with correlation coefficients (R^2) always higher than 0.78 (Figure 5.S3 and 5.S4, supporting information). Λ_{bulk} values ranged between 12 ± 4 for *p*-xylene degradation and 29 ± 5 for *o*-xylene degradation by the different enrichment cultures (Table 5.1). The corresponding $\Lambda_{reactive\ position}$ values were 5 ± 2 for *p*-xylene degradation and 12 ± 2 for *o*-xylene degradation. Λ_{bulk} values for toluene degradation were 41 ± 8 for *D. ovata* and 20 ± 2 for the *m*-xylene-degrading enrichment culture, leading to $\Lambda_{reactive\ position}$ values of 15 ± 3 for *D. ovata* and 8 ± 1 for the enrichment culture, respectively (Table 5.1). For *D. ovata*, *Azoarcus* T and the *m*-xylene-degrading enrichment culture, $\Lambda_{reactive\ position}$ values for both toluene and xylene degradation showed no general trend. *D. ovata* revealed significantly larger $\Lambda_{reactive\ position}$ values for toluene compared to *o*-xylene. *Azoarcus* T showed an opposite trend: $\Lambda_{reactive\ position}$ values of toluene degradation were significantly larger than $\Lambda_{reactive\ position}$ values of *m*-xylene degradation. The enrichment culture produced similar $\Lambda_{reactive\ position}$ values for *m*-xylene and cometabolic toluene degradation.

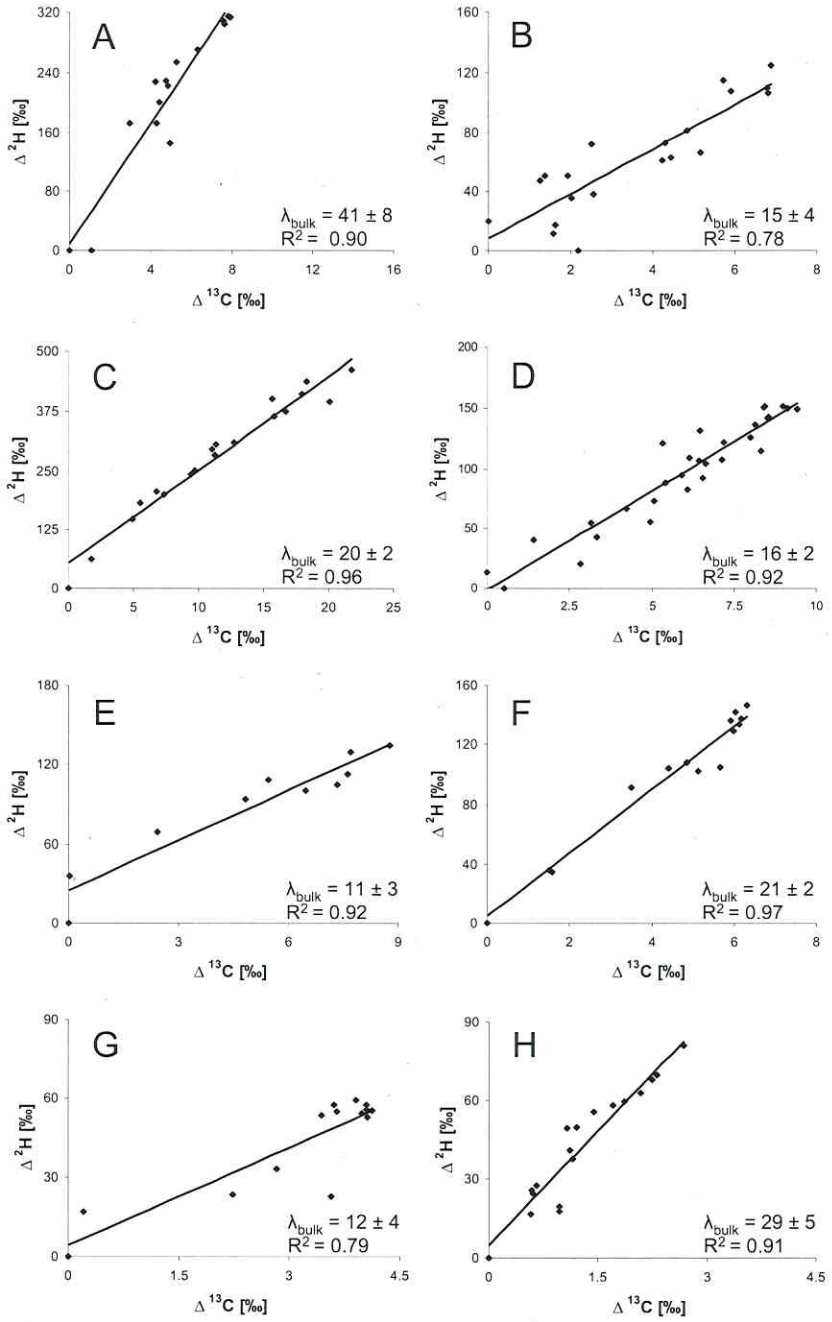


Figure 5.2: A plot of $\Delta\delta^2H_{bulk}$ vs. $\Delta\delta^{13}C_{bulk}$ for each culture and substrate (xylene or toluene) examined in this study. The slope of the regression curves gives the Λ_{bulk} values. (A) *D. ovata*, degradation of toluene; (B) *D. ovata*, degradation of *o*-xylene; (C) *m*-xylene enrichment culture, degradation of toluene; (D) *m*-xylene enrichment culture, degradation of *m*-xylene; (E) *Azoarcus* T, degradation of toluene (data taken from Vogt *et al.* (2008); (F) *Azoarcus* T, degradation of *m*-xylene; (G) *p*-xylene-degrading enrichment culture, degradation of *p*-xylene; (H) *o*-xylene-degrading enrichment culture, degradation of *o*-xylene.

5.4 Discussion

In this study, we have shown that biodegradation of xylene isomers under anoxic conditions were generally coupled to stable carbon and hydrogen isotope fractionation. As demonstrated by literature values or experimental data obtained in this study, all pure and mixed cultures in this study activated the different xylene isomers by the addition of fumarate to a methyl moiety. For methylated aromatics, this reaction is thought to generally be catalyzed by the enzyme benzylsuccinate synthase (Bss). As shown for the Bss of the nitrate reducers *Thauera aromatica* and *Azoarcus* sp. T, the reaction mechanism of fumarate addition to a methyl moiety consists of several single steps which are summarized in Figure 5.3. Bss contains a relatively stable glycyl radical under anaerobic conditions (Krieger *et al.* 2001). It has been proposed that the glycyl radical abstracts the first hydrogen atom from a neighbouring cysteine residue, forming a reactive thiyl radical (Leuthner *et al.* 1998; Krieger *et al.* 2001). The thiyl radical subsequently abstracts another hydrogen atom from the methyl moiety, leading to a benzyl radical intermediate. The benzyl radical attacks the double bond of fumarate, forming a carbon-carbon bond (Beller and Spormann 1998). Finally, the benzylsuccinyl radical is quenched and a hydrogen atom is transferred from the cysteine yielding the end product benzylsuccinate (Figure 5.3). The rate-determining step is thought to be the addition of the benzyl radical to fumarate, which is exothermic and irreversible (Himo 2002; Li and Marsh 2006b). It was postulated that hydrogen abstraction from the methyl moiety of toluene is an isotope sensitive step within the reaction sequence, although the reaction is not fully rate-determining (Li and Marsh 2006b). A second primary isotope sensitive step might be the formation of the carbon–carbon bond during benzylsuccinyl radical formation.

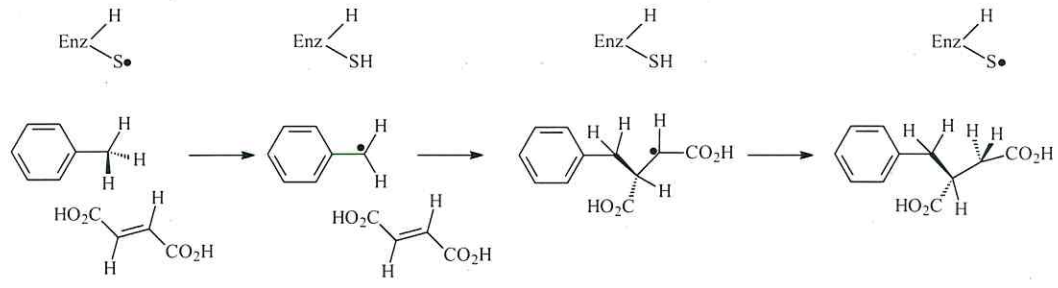


Figure 5.3: Proposed reaction mechanism for benzylsuccinate formation from toluene and fumarate catalysed by Bss (modified from Li and Marsh (2006a)).

In our study, we determined different Λ_{bulk} values for Bss initiated anaerobic xylene degradation in the examined bacterial cultures (Table 5.1). This indicates that xylene activation by Bss proceeds by slightly different reaction mechanisms, as also recently observed by 2D-CSIA for toluene (Vogt *et al.* 2008). Slight variations of potential energy barriers associated with the enzyme or substrate structure might affect the transitions states and the kinetics of isotope sensitive reaction steps and, thus, may

lead to larger variation in hydrogen and carbon fractionation pattern of toluene and xylenes in a formal nearly identical biochemical reaction. Future studies examining the structures of Bss are needed to support this hypothesis.

The variability of Λ_{bulk} values for Bss initiated methylated aromatics activation goes along with the recently observed sequence diversity of *bss*. Winderl *et al.* (2007) developed primers for the detection of the gene coding for the alpha-subunit of Bss (*bssA*), which successfully amplifies the gene in a wide range of anaerobic toluene degraders. By using those primers, the range of *bssA* sequences have been expanded beyond those from cultivated species, and divergent sequences were identified in environmental samples taken from contaminated aquifers (Winderl *et al.* 2007). In our study, the tested xylene-degrading organisms also displayed a broad *bssA* sequence diversity (Figure 5.1), as identified using the primers of Winderl *et al.* (2007). Notably, within two cultures (the *m*-xylene-degrading enrichment culture and *D. ovata*), no *bssA* sequence could be detected, indicating that the diversity of Bss or related fumarate-adding enzymes is broader than currently antipicated. The correlation of *bss* sequence data and isotope fractionation is a topic for future studies. A correlation between enzyme amino acid sequence data and isotope fractionation have been recently observed for the glutathione-S transferase dependent dehalogenation of dichloromethane (Nikolausz *et al.* 2006).

In our study, we determined $\Lambda_{reactive\ position}$ values for both xylene and toluene using three cultures. These values allow us to examine whether a single Bss may convert different substrates similarly. A model organism for these investigations is *Azoarcus* T. The strain likely contains only a single Bss (Winderl *et al.* 2007), which is thought to catalyze the initial activation reaction of both toluene and *m*-xylene, respectively (Krieger *et al.* 1999). In our study, *Azoarcus* T produced two significantly different $\Lambda_{reactive\ position}$ values for toluene and *m*-xylene, indicating that the reaction mechanism, *e.g.* the transition state of the reaction, of Bss was slightly different for toluene and *m*-xylene.

For *D. ovata*, the $\Lambda_{reactive\ position}$ value for toluene degradation was significantly larger than the value of *o*-xylene degradation, as opposed to *Azoarcus* T. Upon toluene transformation, hydrogen isotope fractionation was more pronounced compared to *o*-xylene. The $AKIE_C$s for toluene and *o*-xylene were similar, indicating no differences in the extent of commitment to catalysis, in contrast to the results for *Azoarcus* T. Since no *bssA* gene could be identified in *D. ovata*, we can only speculate whether toluene and *o*-xylene were transformed by the same Bss enzyme or by different (iso)enzymes.

The *m*-xylene-degrading enrichment culture is unique in that it consumes toluene cometabolically along with *m*-xylene, and may, indicate that only a single species was actively expressing a degradation pathway specialized for *m*-xylene. To our knowledge, this is the first report of a culture which can use *m*-xylene as a growth substrate, and lacks the ability to use toluene; usually, xylene-degrading anaerobic cultures can also use toluene as a sole source of carbon and energy (Widdel and Rabus 2001). For this culture, $\Lambda_{reactive\ position}$ values for *m*-xylene and toluene biodegradation were similar, indicating that both compounds were activated by a Bss which used a similar reaction

mechanism. Since no *bssA* gene could be amplified using gene probes, we could not verify whether a single Bss or multiple Bss types were present. The fact that toluene was cometabolically transformed in the course of *m*-xylene degradation indicates, however, that both compounds were transformed by a single Bss enzyme expressed in the *m*-xylene-degrading organism within the enrichment culture. Notably, the carbon and hydrogen AKIEs for toluene degradation were significantly higher than for *m*-xylene, indicating that the isotopically sensitive step within *m*-xylene transformation was partly masked, as also observed for *m*-xylene degradation of *Azoarcus* T. Toluene and xylenes are thought to cross bacterial cell walls and membranes mainly by diffusion; since those compounds have similar structures and physical properties, any rate limitations caused by the transport processes might be in the same order of magnitude for toluene and xylene. Thus, it is more reasonable to conclude that the masking of isotope fractionation was caused by substrate-enzyme interactions. The strong isotope fractionation for toluene might be caused by a low affinity of Bss for toluene, leading to lower toluene transformation rates observed during the course of the degradation experiment.

In summary, our results suggest that two dimensional isotope fractionation analyses are a valuable tool for identifying and monitoring anaerobic biodegradation of xylene isomers. Λ_{bulk} values ranging from 10 to 30 associated with a moderate to strong hydrogen isotope fractionation may be used as a strong indicator that anaerobic xylene biodegradation initiated by fumarate addition is occurring in anoxic zones of contaminated aquifers and anaerobic enrichment cultures. However, due to the observed variability of carbon and hydrogen enrichment factors in our experiments, care should be taken when selecting an appropriate enrichment factor for quantifying xylene biodegradation at contaminated field sites using the Rayleigh equation approach (Meckenstock *et al.* 2004a). In order to perform a conservative assessment for xylene biodegradation, an enrichment factor (for hydrogen or carbon) representing the highest known isotope fractionation from all published values should be used for rate quantification. Λ_{bulk} values may be used to characterise anaerobic degradation pathways for alkylated benzenes in field studies, as suggested before (Vogt *et al.* 2008). Furthermore, the observed variability of $\Lambda_{reactive\ position}$ values for toluene and xylene isomers suggests that the transition state of the Bss reaction during the transformation of aromatic compounds is variable and may be indicative of slightly different reaction mechanisms. Generally, $\Lambda_{reactive\ position}$ values are valuable tools to classify Bss reactions. Combining $\Lambda_{reactive\ position}$ values and molecular information about Bss enzyme structures may be a promising approach in future studies to further our understanding about the C – H bond cleavage of alkyl moieties during degradation of aromatic compounds. The results from our study strongly suggest that fumarate addition to alkylated benzene derivates are always related to carbon and hydrogen fractionation and enable CSIA approaches to be used for the assessment of *in situ* degradation of those compounds in anoxic environments.

5.5 Supporting information

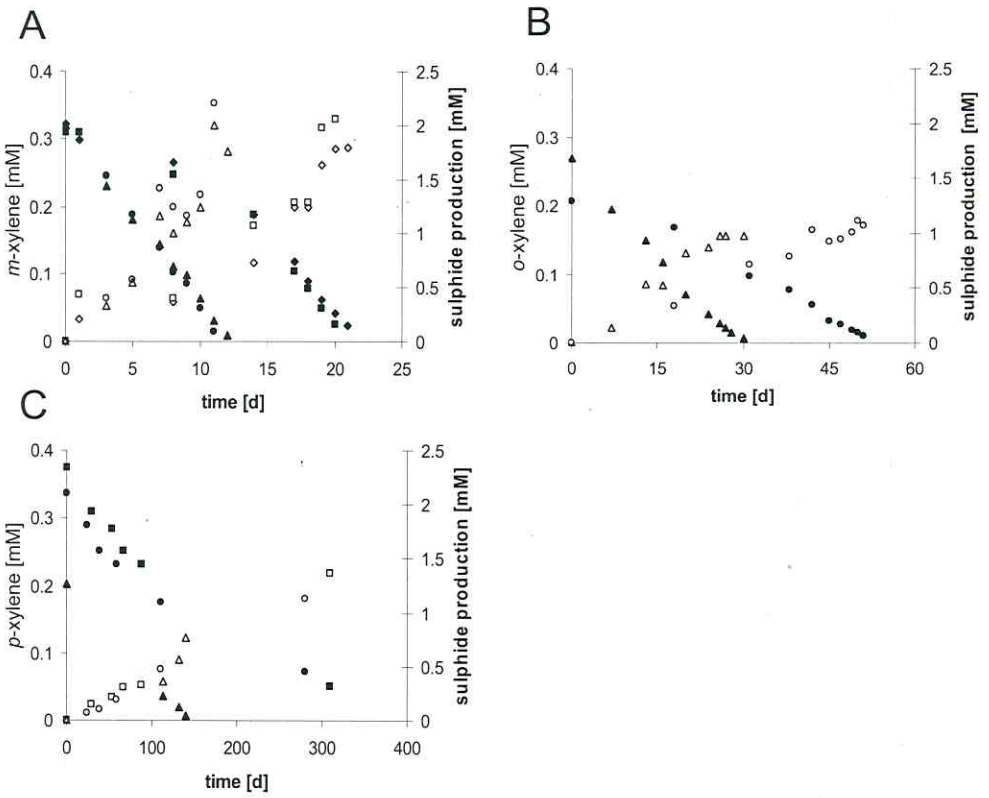


Figure 5.S1: Time courses of xylene degradation and sulphide production within the three examined sulphate-reducing enrichment cultures. (A) Degradation of *m*-xylene (4 replicate bottles); (B) degradation of *o*-xylene (2 replicate bottles); degradation of *p*-xylene (3 replicate bottles). Samples from those experimental bottles were used for isotope fractionation analysis. Degradation rates (k) were calculated by $k = \Delta c_{xylene}/d$, where Δc_{xylene} is the difference between initial and final xylene concentration and d the degradation duration in days.

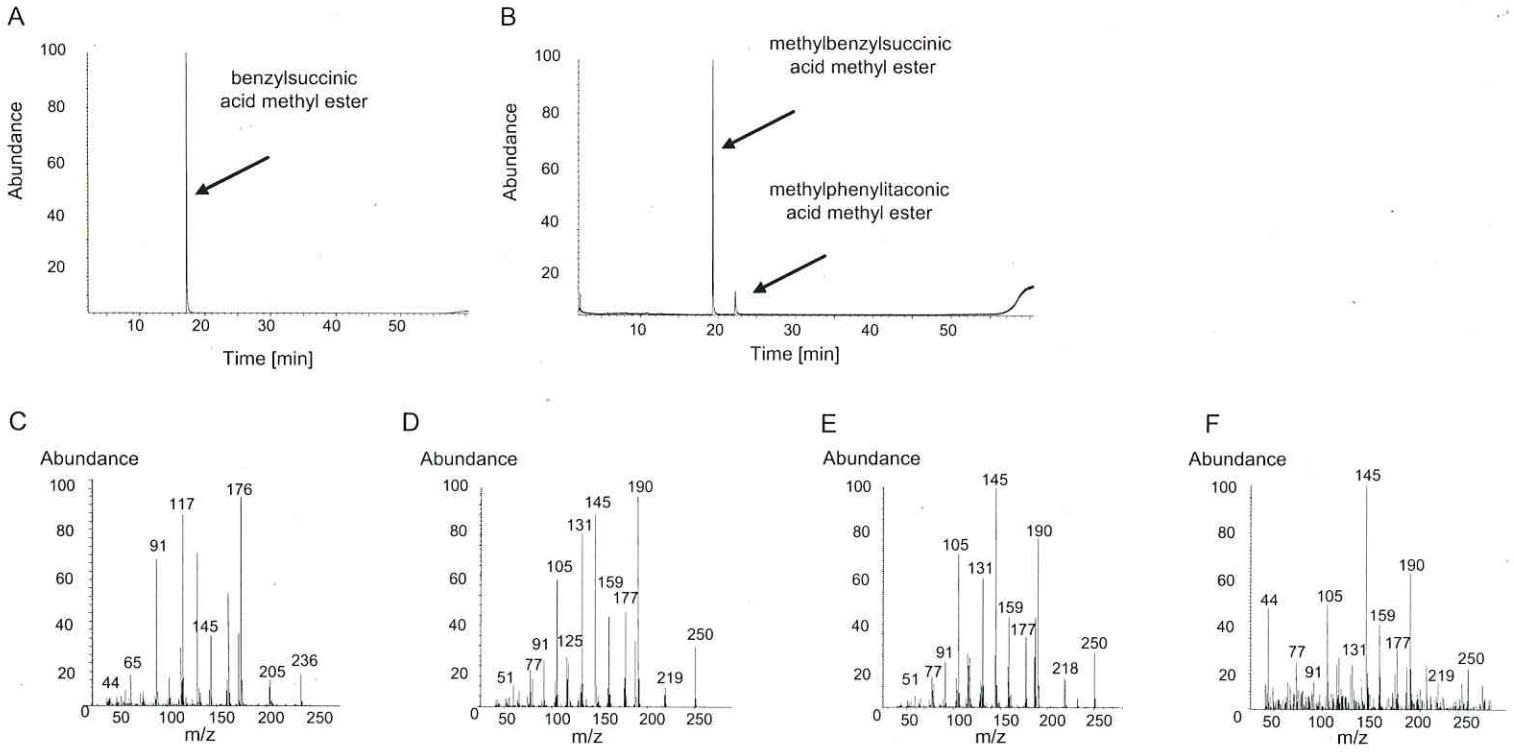


Figure 5.S2: Chromatograms of A) benzylsuccinic acid detected in toluene-degrading cultures, B) methyl benzylsuccinic acid detected in all xylene-degrading enrichment cultures and methyl phenylitaconic acid detected in *m*-xylene and *p*-xylene-degrading enrichment cultures. Shown are the methyl esters of the compounds after derivatisation. Mass-spectrograms gained from GC-MS analysis are shown in C-F for C) benzylsuccinic acid methyl ester (BSME), D) 3-methyl benzylsuccinic acid methyl ester within *m*-xylene-degrading culture, E) 2-methyl benzylsuccinic acid methyl ester within *o*-xylene-degrading culture, and F) 4-methyl benzylsuccinic acid methyl ester within *p*-xylene-degrading culture identified by comparison with co-injected standards (BSME) or published mass-spectrograms (Beller 1997; Krieger *et al.* 1999; Morasch and Meckenstock 2005).

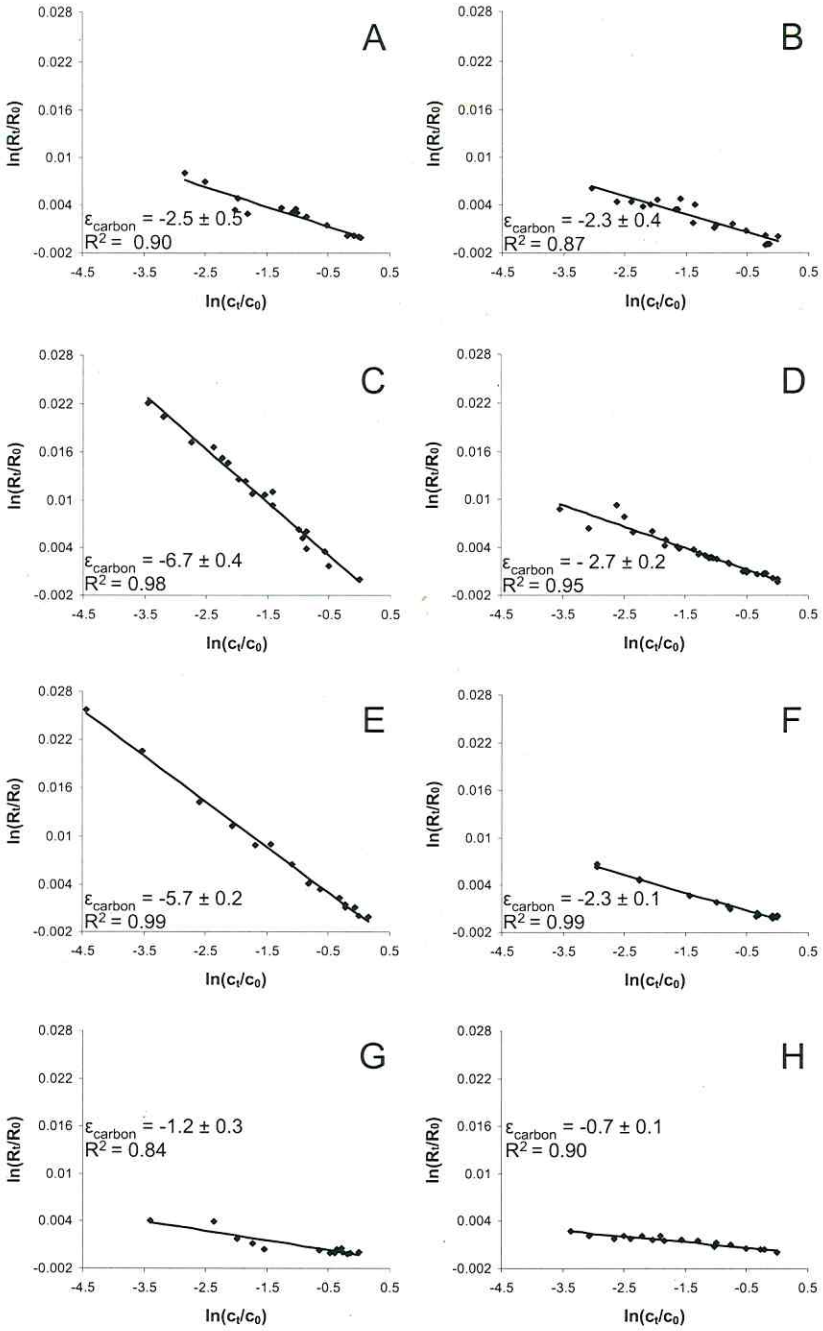


Figure 5.S3: Double logarithmic plot (according to the Rayleigh equation) of the carbon isotopic composition versus the residual concentration of xylene or toluene (bulk enrichment factors, $\varepsilon_{bulk\ carbon}$). The lines correspond to a linear regression model. (A) *D. ovata*, degradation of toluene; (B) *D. ovata*, degradation of *o*-xylene; (C) *m*-xylene enrichment culture, degradation of toluene; (D) *m*-xylene enrichment culture, degradation of *m*-xylene; (E) *Azoarcus* T, degradation of toluene (data taken from Vogt *et al.* (2008)); (F) *Azoarcus* T, degradation of *m*-xylene; (G) *p*-xylene-degrading enrichment culture, degradation of *p*-xylene; (H) *o*-xylene-degrading enrichment culture, degradation of *o*-xylene.

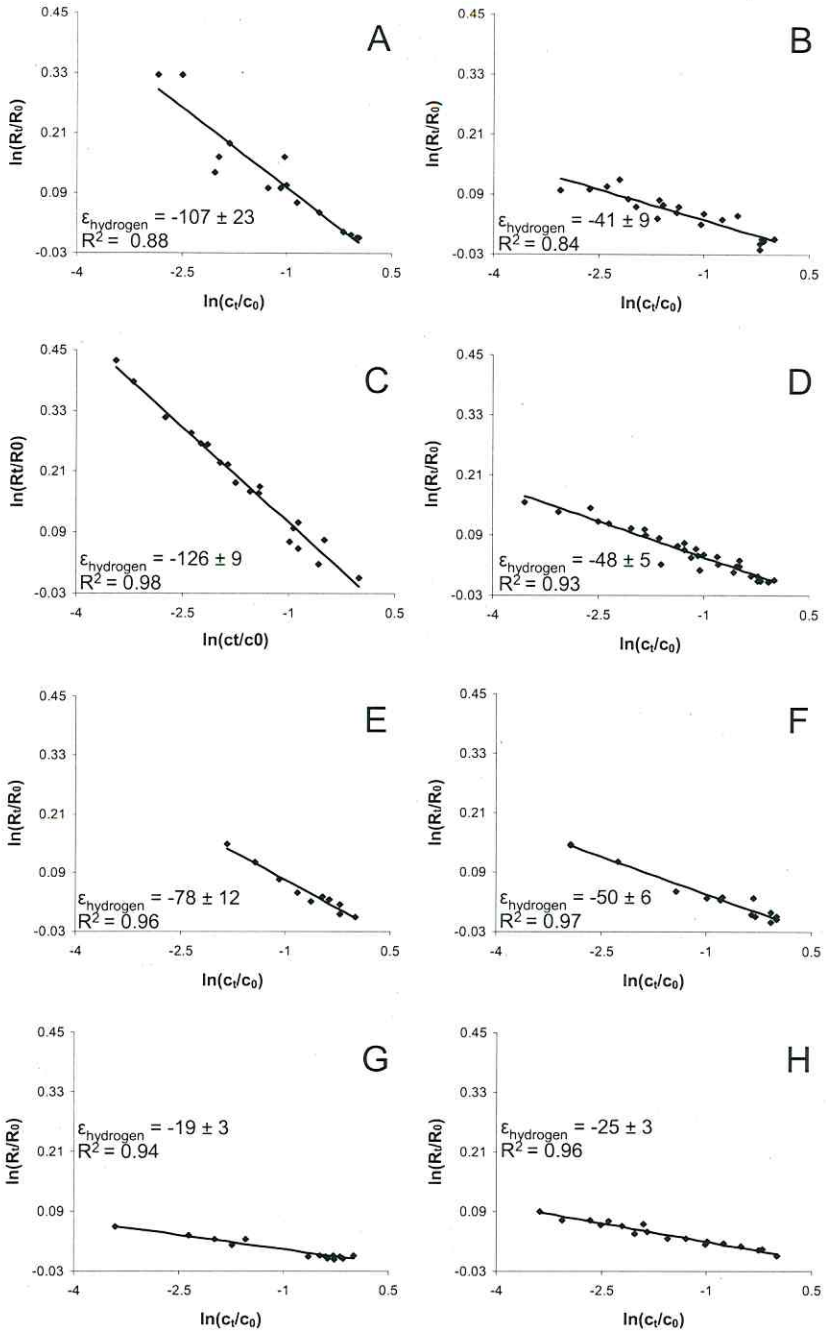


Figure 5.S4: Double logarithmic plot (according to the Rayleigh equation) of the hydrogen isotopic composition versus the residual concentration of xylene or toluene (bulk enrichment factors, $\varepsilon_{bulk\ hydrogen}$). The lines correspond to a linear regression model. (A) *D. ovata*, degradation of toluene; (B) *D. ovata*, degradation of *o*-xylene; (C) *m*-xylene enrichment culture, degradation of toluene; (D) *m*-xylene enrichment culture, degradation of *m*-xylene; (E) *Azoarcus* T, degradation of toluene (data taken from Vogt *et al.* (2008)); (F) *Azoarcus* T, degradation of *m*-xylene; (G) *p*-xylene-degrading enrichment culture, degradation of *p*-xylene; (H) *o*-xylene-degrading enrichment culture, degradation of *o*-xylene.

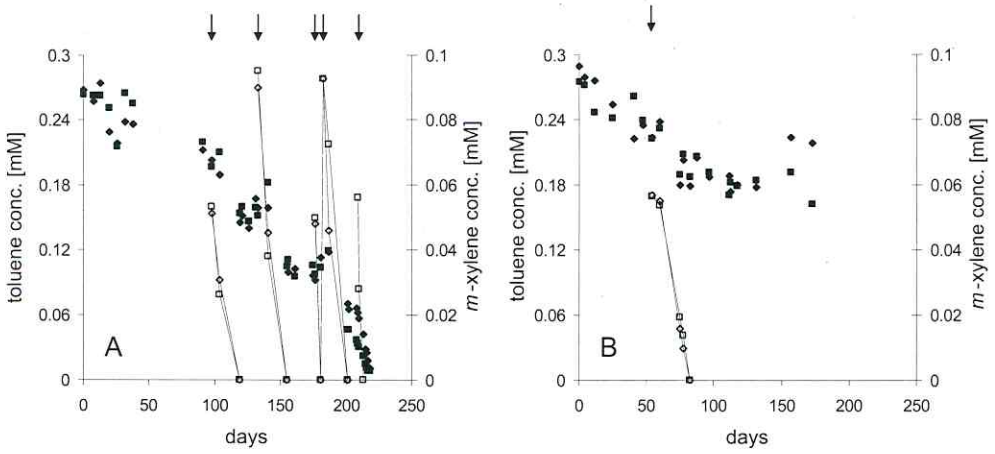


Figure 5.S5: Cometabolic degradation of toluene by the *m*-xylene-degrading enrichment culture. Toluene concentrations within two parallel bottles (black symbols) after (A) frequent addition (white symbols) and (B) one-time addition of *m*-xylene (white symbols). *m*-Xylene addition is indicated by arrows. For reasons of clarity symbols for *m*-xylene concentrations are connected by lines.

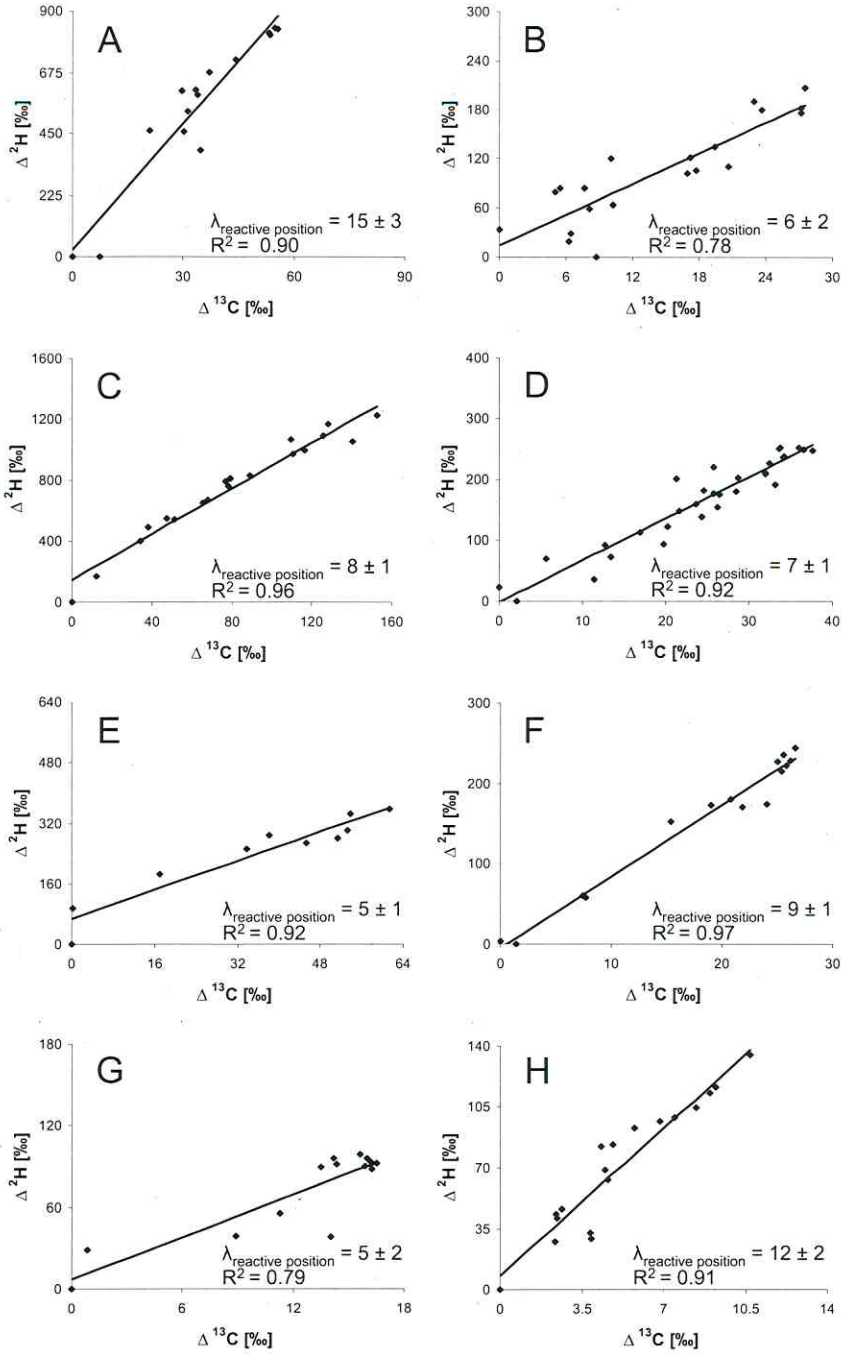


Figure 5.S6: Plot of $\Delta\delta^2H_{reactive\ position}$ vs. $\Delta\delta^{13}C_{reactive\ position}$ for each culture and substrate (xylene or toluene) examined in this study. The slope of regression curve gives the $\Lambda_{reactive\ position}$ values. (A) *D. ovata*, degradation of toluene; (B) *D. ovata*, degradation of *o*-xylene; (C) *m*-xylene enrichment culture, degradation of toluene; (D) *m*-xylene enrichment culture, degradation of *m*-xylene; (E) *Azoarcus* T, degradation of toluene (data taken from Vogt *et al.* (2008)); (F) *Azoarcus* T, degradation of *m*-xylene; (G) *p*-xylene-degrading enrichment culture, degradation of *p*-xylene; (H) *o*-xylene-degrading enrichment culture, degradation of *o*-xylene.

Acknowledgements

We thank Matthias Gehre and Ursula Günther for assistance in our isotope laboratory, Sabine Kleinsteuber and Antonis Chatzinotas for assistance in construction of the *bssA* phylogenetic tree, Stefanie Hinke and Benjamin Remy for excellent technical assistance, and Brandon Emory Morris for proof-reading of the manuscript. Steffi Herrmann thanks the Federal Ministry for Education and Research (BMBF) for funding within the project BEOQUE, grant number 02 WN 0651.

Note for reviewers

S. Herrmann performed or supervised all experimental work for this manuscript. B. Remy contributed with cultivation and sampling of *D. ovata* and *Azoarcus* T. A. Kuppardt optimised the PCR-conditions for *bssA*-amplification, which were applied by S. Herrmann for analyses of the *bssA* diversity. S. Herrmann performed data analysis. S. Herrmann, C. Vogt, A. Fischer and H.-H. Richnow contributed to interpretation and manuscript preparation.

6

BEOQUE

'Bewertung und Optimierung von Quellensanierungs-Ansätzen auf NA und ENA in der Abstromfahne des Standortes Hydrierwerk Zeitz'

– Qualitative and quantitative proof of biodegradation in a benzene-contaminated aquifer in the course of an enhanced natural attenuation approach –

This chapter summarises the main results of the UFZ study (TP1) which was part of the BEOQUE project funded by the German Federal Ministry of Education and Research (BMBF). Project aims, development, experimental set-ups and parts of the results and the discussion are translations of project reports. Some aspects of this chapter, in particular results of MPN-analysis and Bactrap-experiments, are intended to be published.

6.1 Introduction

In situ-air-sparging has been developed since the 1980s as a physical-biological method either to reduce pollution sources and plumes or as a barrier to avoid plume migration (Bass *et al.* 2000; Hall *et al.* 2000; Leeson *et al.* 2002). Target pollutants are volatile chlorinated or petroleum derived hydrocarbons, and low-molecular polyaromatic hydrocarbons within groundwater (saturated aquifer zones) as well as capillary water and soil (unsaturated aquifer zones). Pollutants, either dissolved, adsorbed to aquifer material or trapped within pore volume, are removed from groundwater by introducing air with high pressure into or beneath the contaminated aquifer zone. These pollutants then move upwards along gas filled pores of the aquifer to the vadose zone. During this vesicular or canal-like air-rise the following mechanisms lead to a reduction of contaminant in the aquifer:

1. water dissolved volatile organic compounds exchange from aqueous into gas phase within the contact area water/strip-air (contamination stripping)
2. non aqueous phase liquids (NAPL), either occurring at the capillary fringe or entrapped in and adsorbed to aquifer material of the saturated zone, volatilise and move to the vadose zone
3. air injection causes an oxygen transfer into groundwater, which stimulates microbial growth and microbial degradation of pollutants (Suthersan 1997)

So far, air-sparging-studies mainly investigated the two first processes mentioned above.

The BEOQUE project was aimed at the evaluation of remediation approaches, air-sparging and sulphate addition, regarding their influence on 'natural attenuation' (NA) processes at a BTEX-contaminated site close to the city Zeitz, Germany. Table 6.1 lists institutes and companies that were involved in the project and the respective objectives.

This chapter of the PhD thesis focuses on an air-sparging-trial, which was conducted in the source zone of the test-site, and on the objectives 'high-resolution monitoring', 'quantification of biodegradation', 'characterisation of structure and function of indigenous microbial biocenosis' and 'application of reactive/conservative tracers'.

Thereby, a tracertest was performed to investigate the groundwater flow and direction. Hydrochemical parameters were monitored to observe changes due to air-injection. Benzene concentrations were investigated to estimate the extent of benzene removal. And several methods (MPN-analysis, compound-specific isotope analyis, and Bactrap-experiments) were applied to observe the effects of air-sparging on indigenous microbiota and to quantify the percentage of benzene biodegraded. Finally,

the main objective was to evaluate air-sparging as a remediation technique to 'enhance natural attenuation' (ENA) processes with regard to benzene removal.

Table 6.1: Partners and objectives of the project (modified from report "project proposal (23.05.2005)").

Institution	Objectives
GICON[a]	project management site characterisation characterisation of effects of contamination source remediations construction of groundwater models (streaming and transport models) incl. geological structure models of the water saturated and unsaturated aquifer legwork for optimised model and optimisation participation in application of results to other test sites (mainly outside of Saxony-Anhalt)
MDSE[b]	optimised model and optimisation organisation of operations at the test site project executing organisator and initiator for realisation of source remediation
LAF[c]/ CDM[d]	balancing of pollution removal due to air-sparging characterisation of effects of contamination source remedial actions application of results to other test sites (mainly Saxony-Anhalt), provision of recommendations
UFZ[e]	high-resolution monitoring (UFZ, GWR) **quantification of biodegradation (UFZ, IBGC)** **characterisation of structure and function of indigenous microbial biocenosis (UFZ, IBGC)** **application of reactive and conservative tracers (UFZ, IBGC)**
CAU Kiel[f]	geochemical characterisation regarding redox zones and electron acceptor balances groundwater models (streaming and transport models) incl. geological structure models of water the saturated and unsaturated aquifer modelling of scenarios as legwork for economic evaluation of contamination source remediation conception and realisation of column test in the course of ENA downstream of the contamination plume by sulphate addition
ZSG[g]	organisation of operations at the test site participation in the development of the remediation concept

[a] Großmann Ingenieur Consult GmbH Dresden; [b] Mitteldeutsche Sanierungs- und Entsorgungsgesellschaft mbH, Bitterfeld; [c] Landesanstalt für Altlastenfreistellung, Magdeburg; [d] CDM Consult GmbH, Leipzig; [e] Helmholtz-centre for environmental research, Leipzig, GWR department of Groundwater Remediation, IBGC department of Isotope Biogeochemistry; [f] Christian-Albrechts-Universität zu Kiel; [g] Zeitzer Standortgesellschaft mbH, Alttröglitz; bold-faced objectives were tasks of the department of Isotope Biogeochemistry (UFZ).

6.2 Site, project and methods information

6.2.1 Characterisation of the test site

The test site is located in the area of a former hydrogenation plant and benzene factory close to the city Zeitz (Germany) and is characterised by a highly benzene-contaminated aquifer. The hydrogenation plant was built in 1939 for exploitation of fuels, lubrication oil, benzene and paraffins on the basis of brown coal hydrogenation, but was destroyed almost completely during the Second World War. In 1963 a new benzene factory (distillation of crude oil) was commissioned with an area of about 100×200 m^2. This factory, consisting of a production area (Distex-area), a pump station, a tank top and a rail loading area, produced about 777 000 tons of benzene until closing in 1990 (Ruske *et al.* 2001). During the production process and probably also during deconstruction, products entered the surrounding soil and groundwater. Damages are poorly documented and give no clear evidence for reasons of the detected extent of contamination.

The hydrogeological profile of the test site consists of an upper aquifer with a depth of 5 to 15 m (GWL 18/50) and a lower aquifer with a depth of 5 to 19 m (GWL 61) (Ruske *et al.* 2001). Both aquifers are separated by an aquiclude of clay and silt (5-20 m) which is partially penetrated by a brown coal layer. Due to discontinuities of the lignite-clay layer both aquifers are hydrogeologically connected. Concentrations of the main pollutant benzene reach values up to the saturation limit (1.8 g L^{-1}) within the upper aquifer at the contamination source directly beneath the former Distex-area. The contamination plume has a length of at least 600 m and a width of 400 m (ÖGP*, unpublished data). The lower aquifer attains benzene concentrations up to 150 mg L^{-1} (Gödeke 2004). Pollutants like toluene (max. 50 mg L^{-1}), ethylbenzene (max. 3 mg L^{-1}) and xylenes (max. 5 mg L^{-1}) bear secondary meaning. Both aquifers are mainly anoxic; only at some sampling points of the upper aquifer low concentrations of oxygen were detected (< 1 mg L^{-1}) (Fischer 2004; Gödeke 2004). The main electron acceptor is sulphate with concentrations of more than 1000 mg L^{-1} at the edges of the contamination plume, assumedly formed geogenically (Gödeke 2004). Nitrate and iron(III)-ions are mainly below the detection limits (Wachter *et al.* 2001; Fischer 2004). Natural attenuation processes proceeding in the upper aquifer have been intensively investigated during the last few years (Vieth *et al.* 2005; Fischer *et al.* 2006; Schirmer *et al.* 2006; Stelzer *et al.* 2006; Alfreider and Vogt 2007; Fischer *et al.* 2007). Detailed information on the test site is provided by Vieth *et al.* (2001), Fischer *et al.* (2004), Gödeke *et al.* (2006), and Schirmer *et al.* (2006).

* ÖGP Öffentliches Großprojekt Zeitz

6.2.2 Characterisation of test fields

6.2.2.1 Test field SB1

By the end of 2004, a test field (SB1/TF1) located downstream of the former tank top was installed in order to investigate the potential of air-sparging to enhance natural attenuation (ENA). SB1/TF1 included one air injection well, one soil vapour extraction well and several groundwater monitoring wells. After a certain period of constant and exclusive soil vapour extraction, compressed air was injected to the groundwater with intervals of 10 min injection and 80 min injection stop. Injection rates were 6 $m^3 h^{-1}$. Gas emissions of volatile contaminants by stripping were removed via soil vapour extraction.

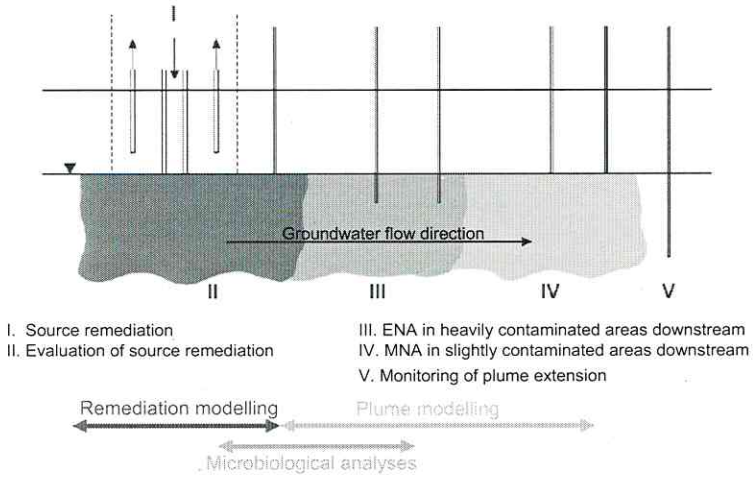


Figure 6.1: Conceptual scheme showing the area affected by air-sparging as proposed at the beginning of the project (modified from report "project proposal (23.05.2005)")

At the beginning of the air-sparging-trial, it was assumed that injected air-oxygen would spread with the groundwater. Figure 6.1 summarises processes (zones) which were assumed to occur due to the air injection. Microbiological analyses were planned to be performed within zones II and III. But hydrological and chemical analyses showed that a single injection well is not sufficient for the formation of zone III, especially the zone which represents the oxidised/aerated area downstream of the air-sparging well. This was probably due to high contamination concentrations directly around the air-sparging well, possibly in the form of 'light non-aqueous phase liquids' (LNAPL), which caused an immediate consumption of injected air-oxygen. Furthermore, since mid 2006 a decreasing injection rate was noticed, caused by undefined clogging effects within the area directly around the air injection well. Free dissolved oxygen (DO) was only detected in one monitoring well located directly next to the injection well, thus the formation of zone II was also doubted. For those reasons the decision was made to build up a new test field (SB2) with a larger extent. All tasks considering microbiological and hydrogeological investigations should be carried out on this field.

6.2.2.2 Test field SB2

The new test field SB2 contained nine air injection wells (EBr), four soil vapour extraction wells (ABr), 19 groundwater monitoring wells (GW), nine ram pumps (RP), and nine soil vapour monitoring wells (BL) which were installed within the ecological large-scale project (ÖGP).

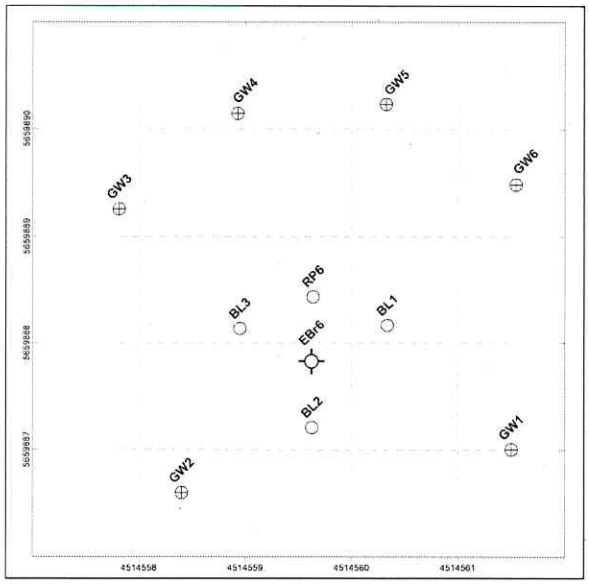


Figure 6.2: Well net configuration of test field TF2 at SB 2 (modified from report "Großmann (26.02.2008)").

Table 6.2: Depths of monitoring well screens of the test field TF2 based on top ground surface (TGS) and groundwater potentials (HN).

Monitoring well	Well screens		Well screens	
	Upper limit [muTGS]	Lower limit [muTGS]	Upper limit [mHN]	Lower limit [mHN]
BL1	7.00^{+}	7.50^{+}	141.75^{+}	141.25^{+}
BL2	7.00^{+}	7.50^{+}	141.75^{+}	141.25^{+}
BL3	7.00^{+}	7.50^{+}	141.75^{+}	141.25^{+}
GW1	5.40^{+}	6.40^{+}	143.35^{+}	142.35^{+}
GW2	5.40^{+}	6.40^{+}	143.35^{+}	142.35^{+}
GW3	6.00^{+}	7.00^{+}	142.75^{+}	141.75^{+}
GW4	6.00^{+}	7.00^{+}	142.75^{+}	141.75^{+}
GW5	6.50^{+}	7.50^{+}	142.25^{+}	141.25^{+}
GW6	6.30^{+}	7.30^{+}	142.45^{+}	141.45^{+}
EBr6	11.20^{-}	12.20^{-}	137.68^{-}	136.68^{-}
RP6	9.00^{-}	12.00^{-}	139.77^{-}	136.77^{-}
GW1	8.25^{-}	12.25^{-}	140.35^{-}	136.35^{-}
GW2	8.15^{-}	12.15^{-}	140.49^{-}	136.49^{-}
GW3	8.15^{-}	12.15^{-}	140.58^{-}	136.58^{-}
GW4	8.20^{-}	12.20^{-}	140.51^{-}	136.51^{-}
GW5	8.25^{-}	12.25^{-}	140.51^{-}	136.51^{-}
GW6	8.25^{-}	12.25^{-}	140.45^{-}	136.45^{-}

$^{+}$ well screen is situated in the unsaturated aquifer zone, $^{-}$ well screen is situated in the saturated aquifer zone

SB2 was started up on 17/03/2007. Initially, soil vapour extraction (SVE) was exclusively performed to reduce the contamination of the unsaturated aquifer zones from about 80 g BTEX per m^3 soil vapour to values lower than 4 g m^{-3}. Air-sparging started on 18/10/2007 for the entire SB2. All nine injection wells were implemented stepwise until January 2008. Microbiological and hydrogeochemical investigations were carried out by project partners of the UFZ at injection well EBr6, which was manually disconnected and therefore independent from operations of the other air injection wells.

Around EBr6, seven groundwater monitoring wells (GW1 – 6, RP6) and nine soil vapour monitoring wells (BL1 – 3, GW1 – 6) were constructed and summarised as TF2 (Figure 6.2). The monitoring wells were equipped with well screens of about 3 m depth within the saturated aquifer zone and/or 50 cm to 1 m depth within the unsaturated aquifer zone (Table 6.2). The groundwater table had an annual average value of 140.91 ± 0.26 mHN.

At the beginning of December 2007, a continuous air-sparging-test (5.5 m^3 h^{-1}) was performed at TF2 for 48 hours. On 13/12/2007 EBr6 was connected to operations of the other air-sparging wells of SB2. Initially, volume fluxes of air injection were 6 m^3 h^{-1} (13/12/07 – 09/01/08), later 10 m^3 h^{-1} (09/01/08 – 25/06/08). The air-sparging-trial ended at the 25/06/08. See Figure 6.3 for a schematic overview on injection volumes over the period of investigation.

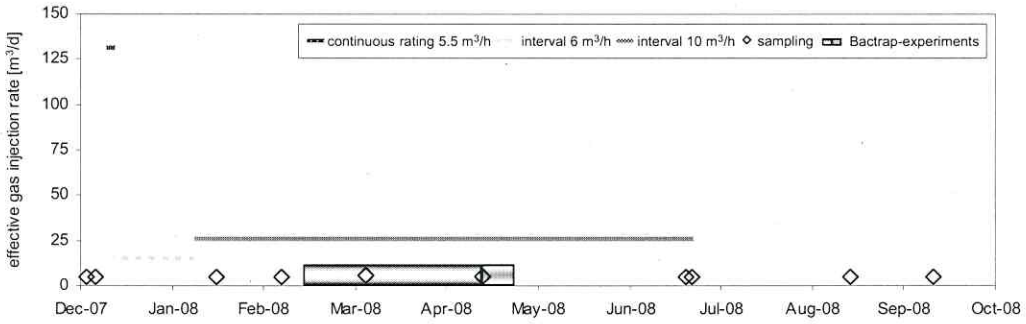


Figure 6.3: Schematic illustration of the effective injection volumes at test field (TF2) from December 2007 to September 2008; sampling dates are indicated by diamonds; duration of Bactrap-experiments by bars on the x-axis, both incubation versions were exposed at the 14/02/08, incubation ended at the 14/04/08 for GW3-Bactraps and 25/04/08 for sediment-Bactraps (modified from report "Großmann (10.09.2008)").

6.2.3 Tracertest

Before air-sparging was started at the newly established test field (TF2), a tracertest was performed with conservative tracers uranin and bromide in order to determine groundwater flow direction and velocity. This test was done in co-operation with Kai Müller (UFZ, GWR).

Fifty litres of groundwater was pumped by a 2"-subsurface motor pump (MP1, GRUNDFOS®) from the well screen area of the centrally located monitoring well RP6. Groundwater was collected in a PE-barrel and mixed with 50 mg L^{-1} uranin and 2 g L^{-1} potassium bromide. Subsequently, the tracers were infiltrated back into RP6 with the lowest possible pressure increase. To keep the tracer concentration

as constant as possible during the injection period, the mixture was led via a circular flow over the storage tank with an injection rate of 2 L min^{-1} and a delivery rate of 1 L min^{-1}.

Tracer extension was monitored by groundwater sample analyses and online-monitoring of RP6 and GW1 – 6 (Figure 6.2). Groundwater sampling was performed with a foot valve pump.

Uranin concentration was analysed by fluorescence spectroscopy. A multiple-channel fibre-optic fluorometer (self-developed device of the University of Stuttgart*) was used for online-measurements of uranin concentrations. Therefore, one fibre-optic sensor was constantly deployed within the well screen of RP6 to monitor tracer injection and temporal development of uranin concentrations. Another sensor was used for *in situ* measurements within the other groundwater monitoring wells to monitor the spatial development of uranin concentrations. Additionally, groundwater samples were analysed with a mobile fluorescence-spectrometer Hitachi F-4500 (λ_{ex} = 490 nm, λ_{em} = 515 nm, detection limit 1 µg L^{-1}) as a control for online-measurements and for uranin concentrations below the online-detection limit.

Bromide was analysed by a bromide electrode BR 501 (WTW) according to manufacturer's instructions.

6.2.4 Sampling

At different times before (t0), during (t1 – 6), and after the air-sparging-trial (t7 – 8), groundwater and soil vapour samples were taken in order to analyse (bio)geochemical parameters listed in Table 6.3. For groundwater sampling, a Grundfos™ MP1 submersible pump was used. Before sampling, a certain pump volume which was dependent on the properties of the monitoring well, was removed according to LAWA guidelines (1998-2008). Afterwards the nature of the water was assessed by measuring on-site parameters, *i.e.* dissolved oxygen, electrical conductivity, pH, redox potential and temperature.

Table 6.3: Examined parameters at different points in time before, during and after air-sparging (modified from report Großmann (10.09.2008))

Time point date	t0 03/12/07	t1 06/12/07	t2 16/01/08	t3 07/02/08	t4 06/03/08	t5 15/04/08	t6 23/06/08	t7 18/08/08	t8 15/09/08
AS on	-	+	+	+	+	+	+	-	-
benzene	+	+	+	+	+	+	+	+	+
anions and cations, TIC	+	+	+	+	+	+	+	+	+
GW-benzene-IS	+	+	+	+	+	+	-	-	-
MPN	+	+	+	+	+	+	-	-	-
BL-CO_2-IS	+	-	+	+	-	-	-	-	
GW-CO_2-IS	+	-	-	+	+	-	-	-	-
CO_2/O_2 [%]	+	20/12/07	+	+	+	+	20/05/08	09/06/08	23/07/08
on-site pm	+	+	+	+	+	+	+	+	+

AS (air-sparging), benzene (concentration), MPN (most probable number – bacterial counts), BL (soil vapour), GW (groundwater), IS (isotope signature), pm (parameters), TIC (total inorganic carbon); bold-faced parameters were analysed by the department of IBGC, the other ones by Kay Müller (UFZ, GWR).

* Research faculty for subsurface remediation (VEGAS) at the institute of hydraulic engineering (Dr. Barczewski)

Sampling started when constant values of those parameters were observed. Soil vapour samples from unsaturated aquifer zones of soil vapour monitoring wells (BL/GW) were taken and analysed by an ATEX-landfill gas monitor GA94. Sampling was performed in co-operation with the Department GWR (Kai Müller).

6.2.5 Hydrochemical parameters

Samples for the detection of benzene concentration were filled on-site into GC-vials which were gas-tightly sealed and stored till further processing at 4°C. Concentrations were detected by an automated headspace-gas chromatograph (Varian GC 3800, PALO ALTO) equipped with a CP SIL 5 CB capillary column (25 m × 0.12 mm ID × 0.12 µm FD; Varian, Germany) and a flame ionisation detector. The chromatographic conditions were as follows: injector temperature, 250°C (split 1:50); detector temperature, 260°C; and an oven temperature program consisting of 70°C for 2 min, followed by an increase at a rate of 10°C min^{-1} up to 90°C and then at a rate of 60°C min^{-1} until 220°C. Helium (1 mL min^{-1}) was used as carrier gas. Aliquots of chilled samples were diluted within 20-mL-autosampler-vials for headspace-analyses (Hewlett-Packard®) with 1.6 mM H_2SO_4 by a ratio of 1:10 or 1:20 and a final volume of 10 mL. The samples were incubated for 30 min at 70°C in an agitator (rotation regime, 250 rpm for 5 s and no rotation for 2 s) prior to analysis, and 1 mL of each sample's headspace was injected. For calibration, diluted standards of benzene prepared from stock solutions were treated in the same way as the samples. The stock solutions were prepared in pure methanol.

Samples of inorganic parameters (main an- and cations, TIC) were filtrated on-site (0.45 µm, cellulose-acetate-filter) and stored within glass vials. Samples for cation-analysis were conserved with a few drips of concentrated nitric acid (final pH < 2). All samples were stored and transported at 4°C in the dark until further analyses by project partners of the Institute of Geosciences at the Christian-Albrechts-University of Kiel. Cations were analysed by inductively coupled plasma - optical emission spectrometry (ICP-OES) using a Vista AX (Varian, Australia). Anions were analysed by ion chromatography (IC) using a DX-500 (Dionex) with an AS9-HC separation column (4 × 250 mm), a AG9-HC precolumn (4 × 50 mm), and a NG1 protection column (4 × 25 mm), 9 mM Na_2CO_3 as eluent (pumprate 1.2 mL min^{-1}), an injection volume of 25 µL, a suppressor ASRS Ultra II (4 mm), and a CD20 conductivity detector. Total inorganic carbon (TIC) was detected by analysing bound carbon with a Multi N/C 2000 (Analytik Jena, Germany).

Measurements of on-site parameters (dissolved oxygen concentrations, pH, electrical conductivity, and reduction potential) were performed by Kai Müller using a flow-through cell. pH-values were measured by means of an electrode SenTix 41 (WTW; Germany) with an integrated temperature sensor. At each sampling day, a 2-point-calibration was performed with standard buffer solutions of pH 4 and pH 7. Electrical conductivity was analysed with a measuring cell TetraCon 325 (WTW, Germany). A redox electrode SenTix ORP (WTW, Germany) was used to measure the redox potential. The electrode was calibrated once per sampling day by a set of control buffers (228 mV at 20°C). An

electrode OxiCal®-SL CellOx 325 (WTW, Germany) was used to determine oxygen concentrations. Calibration was conducted several times per sampling day within a calibration jar with humid compartment air.

Concentrations of oxygen and carbon dioxide of soil vapour samples were detected with an ATEX -landfill gas monitor GA94 according to manufacturer's guidelines (GWR, Kai Müller).

6.2.6 Isotope chemical parameters

50 mL-cultivation flasks were filled to the top with groundwater and immediately sealed with crimped Teflon-coated butyl rubber stoppers. In the laboratory, the bottles were opened, fixed with 1 mL concentrated hydrochloric acid and overlaid with 3 mL *n*-pentane as extracting agent, sealed again with Teflon-coated stoppers and stored upside down at 4°C till further processing (UFZ, IBGC).

The carbon and hydrogen isotope signatures of benzene were analysed by gas chromatograph-combustion-isotope ratio mass spectroscopy (GC-C-IRMS) according to Fischer *et al.* (2007). Aliquots of the *n*-pentane extract were used. The isotope ratios are expressed in delta notation ($\delta^{13}C$ and $\delta^{2}H$) and in per mil (‰) units according to equation 6.1,

$$\delta^{13}C_{sample} \quad or \quad \delta^{2}H_{sample}\,[‰] = \left(\frac{R_{sample} - R_{\text{standard}}}{R_{\text{standard}}}\right) \cdot 1000 \qquad \text{eq. 6.1}$$

with R_{sample} and $R_{standard}$ as $^{13}C/^{12}C$- or $^{2}H/^{1}H$-ratios of the sample and an international standard. Vienna Pee Dee Belemnite (V-PDB) was used as the standard for the analysis of carbon isotope ratios, and Vienna Standard Mean Ocean Water (VSMOW) was used as standard for the detection of hydrogen isotope ratios (Gonfiantini *et al.* 1995; Coplen *et al.* 2006).

The remaining substrate fraction $f_t = C_t/C_0$ was calculated using the isotope fractionation factor (α) obtained in laboratory experiments (eq. 6.3). The percentage of biodegradation of the residual substrate fraction (Bf_t) was calculated with equation 6.4 according to Richnow *et al.* (2003a).

$$R_t / R_0 = (\delta_t + 1000)/(\delta_0 + 1000) \qquad \text{eq. 6.2}$$

$$f_t = (R_t / R_0)^{(1/1/\alpha - 1))} \qquad \text{eq. 6.3}$$

$$Bf_t[\%] = (1 - f_t) \times 1000 \qquad \text{eq. 6.4}$$

Prior to the detection of isotope ratios of soil vapour carbon dioxide, air samples were aspirated by an ATEX-landfill gas monitor GA94 and fixed by introducing aspirated air to alkaline, bi-distilled water (10 pellets of sodium hydroxide per 100 mL water). In preparation for analysis, 2 mL of the fluid was transferred to a 10 mL-GC-vial, gastightly sealed, fixed with concentrated hydrochloric acid, and heated to 60°C shortly before analysis. Analysis was performed by a GC-C-IRMS equipped with a gas chromatograph (6890 Series, Agilent Technology, Palo Alto, USA), a conflow III interface (ThermoFinnigan), and a MAT 252 mass spectrometer (ThermoFinnigan). CO_2 was isothermally separated at 40°C via a poraplot Q column (25 × 0.32 mm ID, 1 µm film; Chrompack). Helium was

used as carrier gas with a flow rate of 2.0 mL min^{-1}. Headspace samples (50 – 100 µL) were injected into the GC-IRMS-system with split ratios of 1:10 to 1:50. The carbon isotope signature was expressed in delta notation (‰) relatively to Vienna Pee Dee Belemnite Standard (V-PDB). Groundwater samples were prepared and analysed analogously.

6.2.7 Determination of MPN

For the quantification of most probable numbers (MPN) of aerobic microorganisms and aerobic benzene degraders, groundwater was filled into sterilised 50 mL-flasks (Duran) which were gastightly closed with Teflon coated screw caps. Samples were stored at 4°C until further processing. MPN cultures were prepared on the day of sampling. Therefore, dilution series (10^{-1} to 10^{-8}) were made in media for cultivation of aerobic microorganisms (R2A medium according to Reasoner and Geldreich (1985)) or benzene degrading aerobic microorganisms (DSMZ medium 457). Medium 457 was modified according to Trüper and Pfennig (1992) by using trace element solution SL-10 (1 mL L^{-1}) and vitamin solution (5 mL L^{-1}) according to Pfennig *et al.* (1965). Dilution series were performed in ten parallels within 96-well-plates (Whatman) which were incubated in gastight jars (Oxoid) at room temperatur and in the dark. Benzene was added to medium 457 via gas phase by applying 200 µL to a filter which was added to the jar shortly before closing. Plates were frequently observed during growth; most probable numbers were quantified after two (aerobic bacteria) and six weeks (benzene degraders).

6.2.8 Bactrap-experiments

6.2.8.1 Set-up and loading of Bactraps

In order to detect metabolisation of benzene induced by the air-sparging-approach different Bactrap-experiments were performed. Bactraps are perforated devices which are filled with Bio-Sep®-bio-catalyser-beads (K.L. Sublette, Tulsa, USA). The spherical beads with a diameter of 2 to 3 mm consist of powdered activated carbon (PAC) incorporated in an aramid polymer matrix (Nomex®). The beads have a density of about 0.16 g cm^{-3} and an internal porosity of 74%. The average pore diameter of the outer matrix is about 1.9 µm, including some macro pores (> 20 µm) (White *et al.* 2003). These surface properties provide excellent attachment possibilities for groundwater bacteria. Due to a high adsorption capacity (> 600 m^2 g^{-1}) beads can be loaded with a great variety and a high amount of substances (*e.g.* hydrocarbons). Incubated in groundwater, hydrocarbons desorb and may function as carbon source for microorganisms. Using ^{13}C-labelled substances label-incorporation into biomass-molecules proves biodegradation (Geyer *et al.* 2005; Kästner *et al.* 2006; Stelzer *et al.* 2006).

Bio-Sep® beads were heated for 4 hours at 300°C to remove organic residues and filled into perforated Teflon tubes with a diameter of 0.5 cm (0.5 g Bio-Sep® beads (~50 pellets) / tube). The tubes were plugged at both ends with glass wool to keep beads inside the tubes (Figure 6.4). The filled tubes were autoclaved at 121°C for sterilisation and rehydration of the beads. Bactraps were loaded with $^{13}C_6$-

labelled (> 98 atom%, Campro Scientific, The Netherlands) or unlabelled benzene (100 mg benzene / g Bio-Sep® beads), respectively, via gas phase under reduced pressure according to Geyer *et al.* (2005). Therefore, Bactraps and benzene were placed into small stained steel containers which were evacuated at 60 mbar. After incubation for 24 hours, the vacuum was released by filling nitrogen into the containers. The Bactraps were stored in separate bottles filled with anoxic, sterilised distilled water differentiated by loading type until aquifer positioning. Unloaded, but analogously pretreated beads functioned as controls. For each loading type four Bactrap parallels were set up.

Figure 6.4: Bactraps with Bio-Sep® beads. Activated carbon pellets are filled into perforated Teflon tubes, sealed with glass wool, and loaded with a substrate under vacuum. Prepared Bactraps are fixed with cable clip to wires or plastic ropes and are subsequently lowered into a monitoring well. Groundwater microorganisms enter through holes of the Teflon tube and attach to Bio-Sep® beads.

6.2.8.2 Bactrap-set-up versions

Bactrap-experiments were performed using two different experimental set-ups. With the first set-up, Bactraps were incubated in a groundwater monitoring well (GW3) of TF2 (Figure 6.2). Of each loading type (unlabelled benzene, $^{13}C_6$-benzene or no loading) two Bactraps were fixed with cable clip to a steel tether of a packer system at the same height (Figure 6.5A and Figure 6.13) and incubated freely suspended within the water column of the monitoring well for 60 days at a depth ~9 m beneath top ground surface.

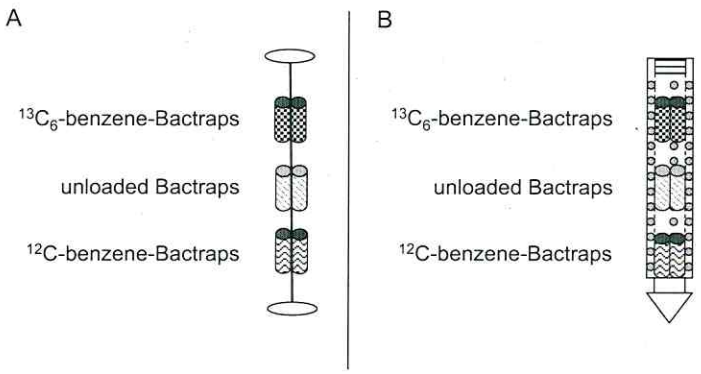


Figure 6.5: Schematic alignment of Bactraps for the experiment within A) groundwater monitoring well and B) sediment. Six Bactraps were used for each incubation version ($^{13}C_6$-benzene, unlabelled benzene and unloaded (^{12}C); each in two parallels). Sediment-Bactraps were placed in a modified Geoprobe-sampler-sheath SP16 with ram cone; hollow spaces between Bactrap-versions were filled with glass wool to separate unequal loaded Bactraps (modified from report "Vogt *et al.* (17.10.2008)").

Within the second experiment, Bactraps were incubated directly in the aquifer-sediment of TF2 with a distance of ~1 m to GW3. Bactraps were inserted into the aquifer with a lance specially designed for this experiment. The lance based on a modified Geoprobe-Sampler-Sheath SP-16. The sheath was shortened, perforated (to allow contact with circumjacent groundwater), and equipped with a stainless steal ram cone (Figure 6.6A and B). Bactraps were separately stored within the lance depending on loading type (Figure 6.5B and Figure 6.6B and C). Of each differently loaded Bactrap (unlabelled benzene, $^{13}C_6$-benzene or no loading) two parallels were used. Glass wool was taken to separate different loading types within the lance spatially (Figure 6.6C). The lance was connected by a screw thread to drill pipes, which are commonly used to drill small wells into the aquifer. The lance was placed ~9 m beneath the top ground surface. After an incubation of 71 days, it was recovered and Bactraps were stored differentiated by loading type in 15 mL-falcon-tubes at -40°C until further processing.

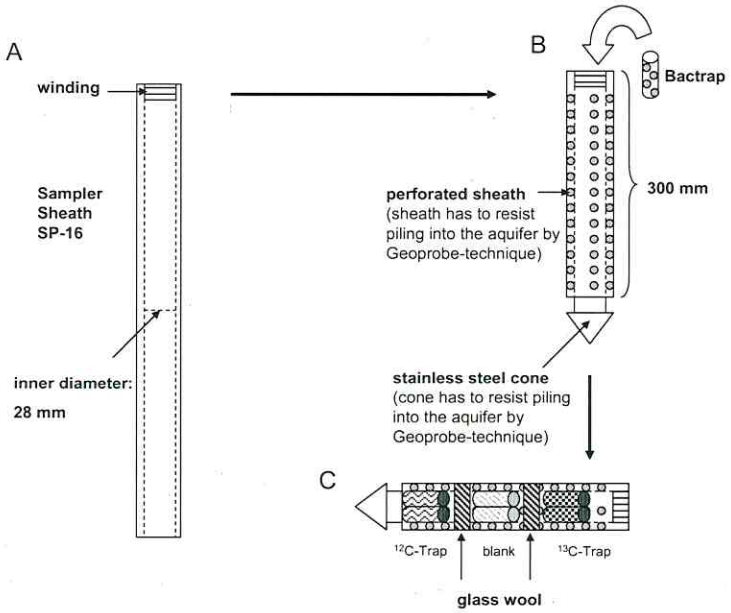


Figure 6.6: Scheme of design development of the lance which was used to incubate Bactraps in the aquifer-sediment. A) modification of Geoprobe Sampler Sheath SP-16; B) model of the lance; C) positioning of Bactraps within the lance (modified from report "Vogt *et al.* (08.02 2007)").

6.2.8.3 Extraction, derivatisation, analysis, and quantification of fatty acids

The Bio-Sep® beads were removed from Teflon tubes. Fatty acids of settled and grown biomass were extracted according to Bligh and Dyer (1959) using a mixture of dichloromethane, methanol, and water (1:2:1). The dichloromethane phase, containing the fatty acids, was separated and evaporated to dryness. Subsequently, fatty acids were derivatised using a mild alkaline methylation according to Guckert *et al.* (1985). Afterwards, completely dried samples were dissolved in 100 µL *n*-hexane

containing a methylated standard of heneicosanoic acid (21:0) with a concentration of 20.06 ng μL^{-1}. Formed fatty acid methyl esters (FAME) were analysed and identified by gas chromatography-mass spectrometry (GC-MS) according to Härtig (2008). Quantification was performed by peak area comparison:

$$c_{FAME} = \left(\frac{Area_{FAME}}{Area_{21:0}} \right) \times c_{21:0} \times V_{21:0} / (g\,bead) \qquad \text{eq. 6.5}$$

where c_{FAME} is the concentration [ng g^{-1} beads] of fatty acid methyl ester, $Area_{FAME}$ and $Area_{21:0}$ are peak areas of fatty acid methyl esters, $c_{21:0}$ is the concentration of 21:0 methyl ester within *n*-hexane (20.06 ng μL^{-1}), and $V_{21:0}$ is the volume of the standard (100 µL) (Findlay 2004). Calculated concentrations for each fatty acid methyl ester were recalculated for fatty acids with equation 6.6

$$c_{FA} = M_{FA} / M_{FAME} \times c_{FAME} \qquad \text{eq. 6.6}$$

where M_{FA} and M_{FAME} are molar masses of fatty acids and fatty acid methyl esters, respectively. The fatty acids are designated in the form of *i*/*a*A:BωCc/t where A is the number of carbon atoms, B is the number of double bonds and C is the distance of the closest double bond from the aliphatic end of the molecule (ω-nomenclature). The letters c and t stand for *cis* and *trans* configuration describing the position the carbon atoms on either side of the double bond adopt. The prefixes *i* (*iso*) and *a* (*anteiso*) refer to methyl branching. Concentrations of carbon (c_C) within fatty acids [ng g^{-1} beads] were calculated by:

$$c_C = \frac{c_{FA} \times N \times M_C}{M_{FA}} \qquad \text{eq. 6.7}$$

where N is the number of carbon atoms within the fatty acid and M_C is the molar mass of carbon.

Carbon isotope ratios of fatty acid methyl esters were detected by gas chromatography-combustion-isotope ratio mass spectroscopy (GC-C-IRMS) explained in detail by Stelzer *et al.* (2006). FAME were identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Sigma-Aldrich, Germany). The carbon isotope ratio of fatty acids is reported in δ-notation (per mil) relatively to the Vienna Pee Dee Belemnite standard (V-PDB) with known isotopic composition. During methylation of fatty acids (FA) to fatty acid methyl esters, an additional carbon atom is incorporated into the product, hence the original isotope signature of all fatty acids is changed constantly. For that reason, measured $\delta^{13}C$ values of FAME were corrected by equation 6.8 (Abraham 1998) to verify the isotope signatures of fatty acids ($\delta^{13}C_{FA}$).

$$\delta^{13}C_{FA}[‰] = \frac{(N+1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}}{N} \qquad \text{eq. 6.8}$$

$\delta^{13}C_{FAME}$ is the isotope signature of fatty acid methyl esters, and $\delta^{13}C_{MeOH}$ is the isotope signature of methanol which was used for methylation (-38.15‰).

Absolute amounts of ^{13}C-label incorporated (c_{13C}) were calculated from the product of biomarker carbon concentration (c_C) and the increase in the fraction ^{13}C after labelling (F_t^{13}) relative to the control (F_c^{13}); since the unlabelled controls showed also an unnatural small ^{13}C-label, the increase was calculated relative to the BAME standard (F_{BAMEst}^{13}):

$$c_{13C} = (F_t^{13} - F_c^{13}) \times c_C \qquad \text{eq. 6.9}$$

The fraction ^{13}C can be calculated from $^{13}C/^{12}C$-ratios (R) as:

$$F^{13} = R/(R+1) \qquad \text{eq. 6.10}$$

And R was calculated from the $\delta^{13}C$ ratios using the reverse of equation 6.1 (Boschker 2004).

$$R_{sample} = (\delta^{13}C_{sample}/1000 + 1) \times R_{standard} \qquad \text{eq. 6.11}$$

6.3 Results

6.3.1 Exploration of groundwater flow direction and velocity of TF2

Before air-sparging started, a field experiment was performed to elucidate TF2 concerning groundwater flow direction and velocity using the conservative tracers uranin and bromide. The tracer containing groundwater was infiltrated into a centrally located monitoring well (RP6). Temporal development of uranin concentrations within RP6 was monitored online. Additionally, groundwater samples of RP6 and radially positioned monitoring wells (GW1 – 6) were analysed for uranin and bromide concentrations. Although a tracer decrease was observed within RP6 (Figure 6.7), no significant signal was detected within GW1 – 6 during the entire period of examination (data not shown). Groundwater direction and velocity could not be verified.

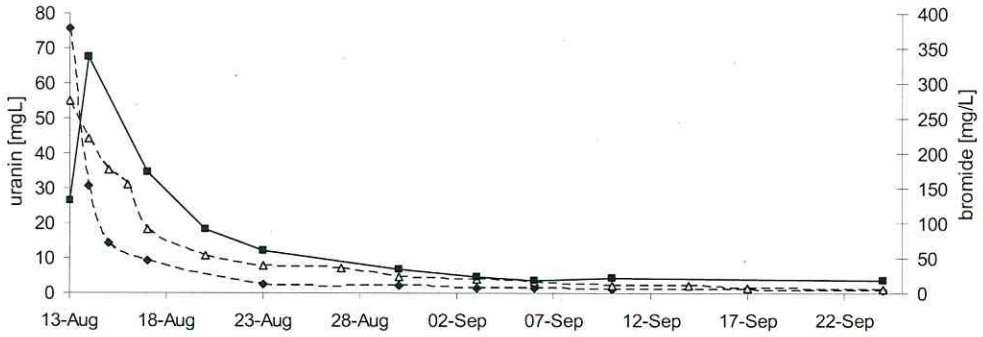


Figure 6.7: Temporal development of tracers bromide (■, mg L^{-1}, right axis) and uranin (online monitoring (△) and groundwater measurements (◆); left axis) within RP6 from 13/08/07 to 24/09/07 (modified from report "Großmann (26.02.2008)").

However, qualitative evidence on very slow velocities was confirmed, which had been postulated previously due to several measurement campaigns for groundwater level determination of monitoring wells within TF2 with the result of very low groundwater level gradients. Groundwater flow velocities were estimated at a few centimetres per day. A groundwater level measurement campaign on 05/11/2007 concerning the entire SB 2 characterised the aquifer as inhomogeneous with respect to streaming properties, mainly showing areas with low gradients, but also some parts with higher gradients (Figure 6.S1 of the supporting information). The area in which TF2 was located showed no change of the water table. Thus, an identification of flow paths on TF2 was not possible.

The last attempt to evaluate groundwater flow direction and velocity was carried out by a gastracer-test shortly before air-sparging-finish. Thereby, a tracer gas (SF_6) was added to the compressed air shortly before injection. The spreading of SF_6 was observed within soil vapour and groundwater of all BL and GW. Results of this test were: Gas spreading was relatively low for areas located to the southeast of EBr6 (~5 m) and relatively high for areas located to the northwest of EBr6 (detected ~8 m). The spreading was influenced by preferential flow beneath a local aquitard (layer with low permeability) within the saturated zone of the aquifer to NNW-direction. O_2-transfer rates from injected gas phase to aqueous phase were detected to be very low and not quantifiable.

6.3.2 Hydrochemical groundwater analyses

Hydrochemical groundwater analyses included measurements of benzene, on-site parameters, major cat- and anions, and total inorganic carbon (TIC). Before starting to inject air, benzene concentrations in groundwater samples of TF2 ranged from 200 to 350 mg L^{-1}.

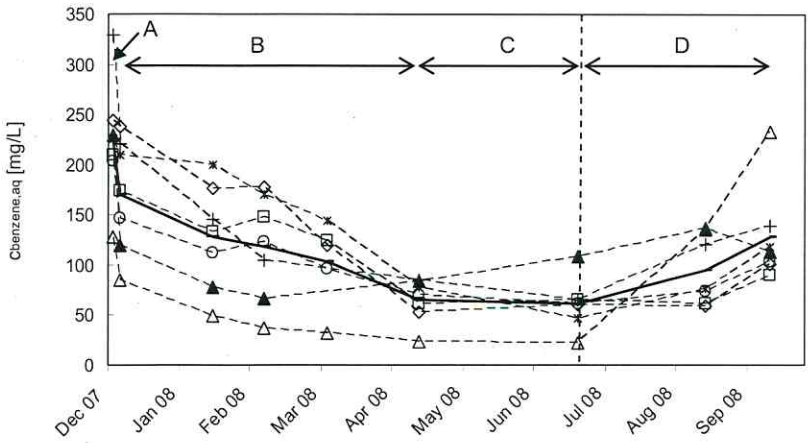


Figure 6.8: Temporal development of benzene concentration before (t0), during (t1 – t6), and after (18/08/08 and 15/09/08) injecting air with symbols of groundwater wells GW1 ◇, GW2 □, GW3 ▲, GW4 △, GW5 ∗, GW6 ○, RP6 +, an the arithmetic mean of all wells —; vertical dashed line indicates air-sparging-stop at the 25/06/08, A to D describe periods with different benzene removal rates (modified from reports "Großmann (10.09.2008)" and "Müller (10.12.08)").

Benzene concentrations decreased within all groundwater monitoring wells linearly or exponentially once air-sparging started. Initially high extraction rates (t0 to t1, phase A, Figure 6.8) are probably due to a high chemical gradient between groundwater and injected air. After t5 (phase B) concentrations (arithmetic mean of all monitoring wells) remained almost constant for 5 weeks at a value of ~60 mg L^{-1} (phase C). As soon as air-sparging stopped (25/06/08), benzene concentrations increased (phase D).

Before air injection, the groundwater of TF2 samples was anoxic, pH neutral, and showed an electrical conductivity of ~1700 µS cm^{-1} (25°C) and a redox potential (U_H) of ~ -30 mV (based on standard-hydrogen-electrode).

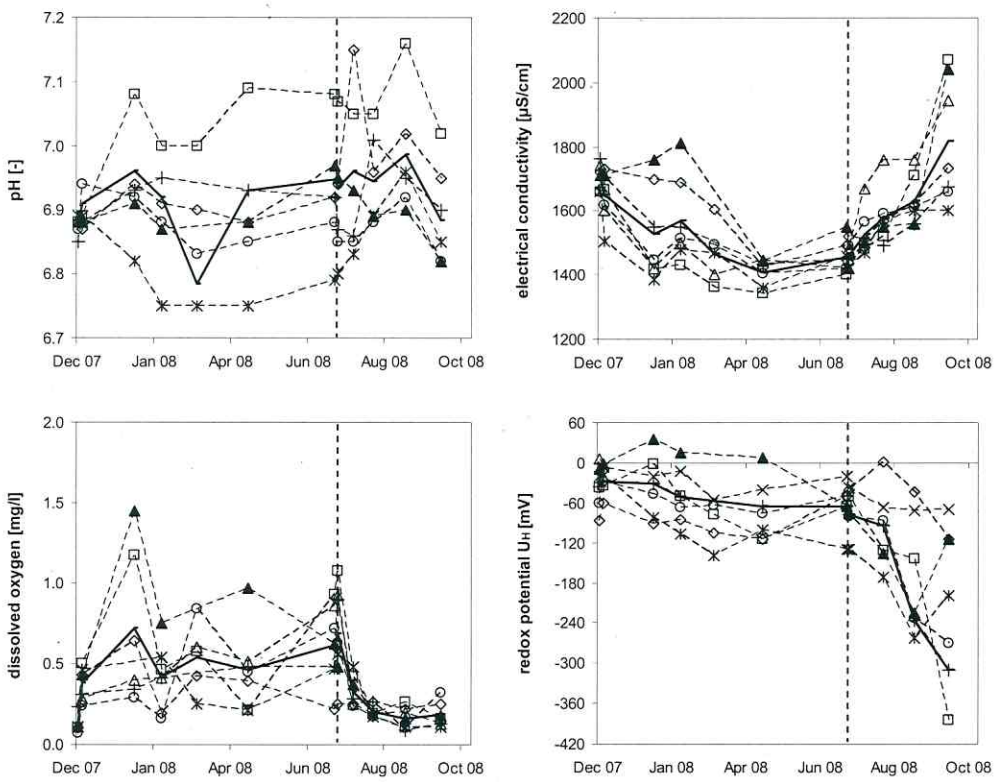


Figure 6.9: Temporal development of pH, electrical conductivity, dissolved oxygen, and redox potential in groundwater samples of TF2 before (t0), during (t1 – t6), and after (t7 – t8, 23/07/08, 18/08/08, and 15/09/08) injecting air with symbols for groundwater wells GW1 ◇, GW2 □, GW3 ▲, GW4 △, GW5 ∗, GW6 ○, RP6 +, arithmetic mean — ; dashed line indicates the stop of air-sparging at the 25/06/08 (modified from report "Großmann (10.09.2008)")

Changes were detected for all parameters (Figure 6.9). The arithmetic mean of pH for all 7 monitoring wells increased by 0.3 units. The electrical conductivity decreased from an average of 1700 to 1400 $\mu S\ cm^{-1}$ during air-sparging and increased as soon as air-sparging was stopped. Dissolved oxygen values of mixed well samples showed only a small increase, indicating that the groundwater was not significantly aerated during air-sparging. As soon as the air-sparging-trial stopped, dissolved oxygen levels decreased in groundwater of all seven monitoring wells.

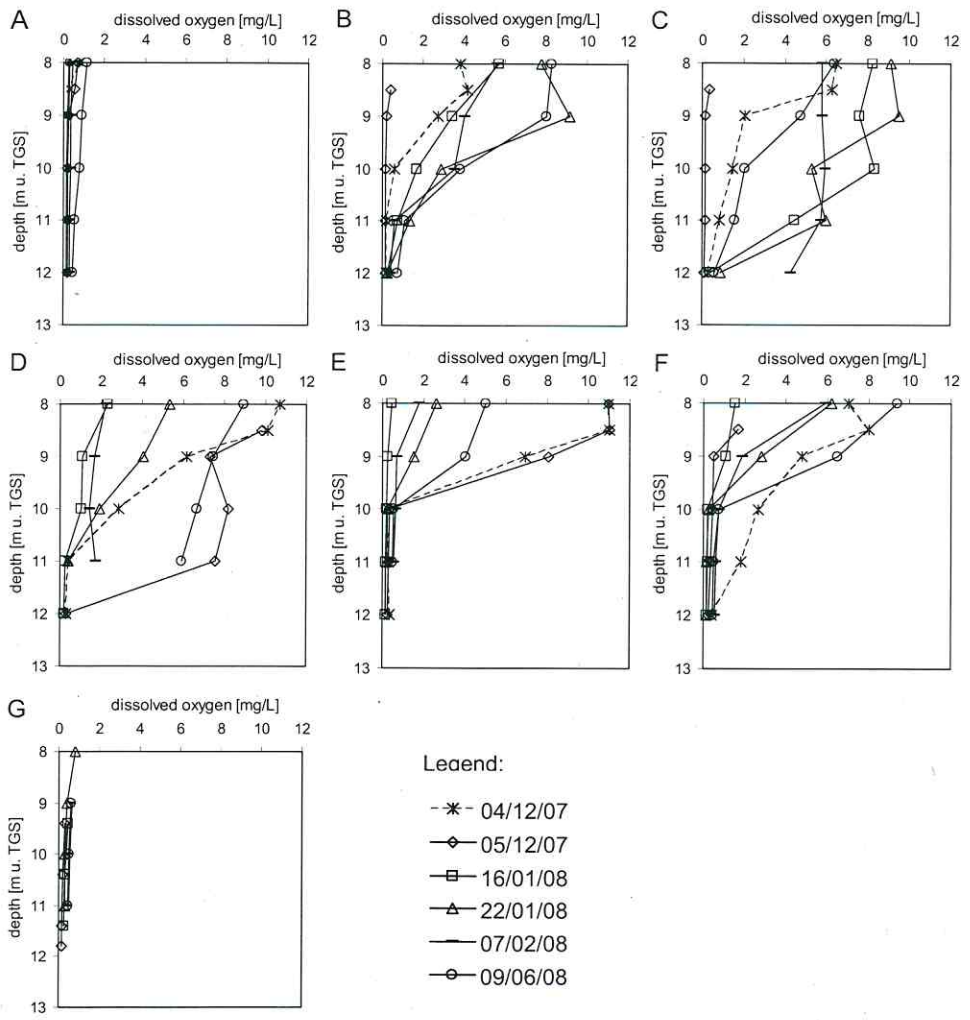


Figure 6.10: Depth profiles for dissolved oxygen [mg/L] within the groundwater monitoring wells A) GW1, B) GW2, C) GW3, D) GW4, E) GW5, F) GW6, and G) RP6 at different times during the air-sparging-trial.

Depth profiles of dissolved oxygen (Figure 6.10) showed for the majority of groundwater monitoring wells (exceptions: GW1 and RP6) and nearly all time points high oxygen concentrations (up to 10 mg L^{-1}) for depths from 8 – 10 m TGS. For GW2, GW5 and GW6 concentrations dropped drastically (< 2mg L^{-1}) for regions below 10 m TGS. High oxygen concentrations within deeper zones were detected only for some sampling time points in GW3 and GW4. Contradictory, redox potentials decreased and sulphide odour increased during the air-sparging-experiment. After stopping air injection, the redox potentials within all monitoring wells dropped immediately to values down to -400 mV.

Based on inorganic parameters, groundwater was classified as calcium-sulphate-hydrocarbonate-type before air-sparging (Furtak and Langguth 1967). Temporal development of major inorganic parameters is illustrated in Figure 6.11 (data acquisition was performed by project partners of the Christian-Albrechts-University of Kiel, Germany).

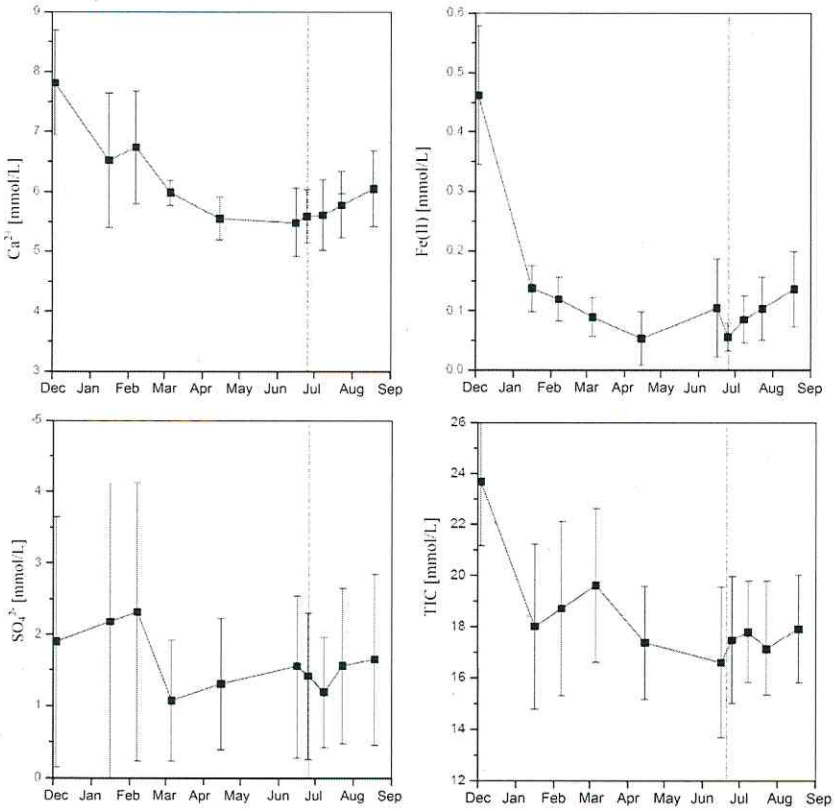


Figure 6.11: Temporal development of inorganic hydrochemical parameters (arithmetic means and standard derivations of groundwater wells GW1 – 6 and RP6) before (t0), during (t1 – t6) and after air-sparging; dashed lines indicate air-sparging-stop (25/06/08) (modified from report "Großmann (10.09.2008)").

During air-sparging, significant changes were detected for mean values of Ca^{2+} concentrations which dropped about 2 mM between t0 and t6, Fe(II) concentrations which dropped about 0.4 mM, and concentrations of the total inorganic carbon (TIC) which decreased about 7 mM. The arithmetic mean of all seven monitoring wells for sulphate concentration also decreased slightly, but was strongly influenced by the heterogeneity of the aquifer. The sulphate distributions varied strongly within different monitoring wells (see high standard deviations within sulphate-graph of Figure 6.11) as well as for different time points of the same well (data not shown). Ca^{2+}, Fe(II), and TIC concentrations started to increase as soon as air-sparging was stopped.

6.3.3 Soil vapour investigations

Prior to air-sparging, continuous soil vapour extraction was performed for a period of 18 days (18/10/07 – 05/11/07) which already resulted in an increase of oxygen [vol%] and a decrease of carbon dioxide concentrations [vol%] shortly after the extraction started. After 23/10/08, arithmetic means of soil vapour monitoring wells BL1 – 3 and GW1 – 6 remained constant at ~12 vol% for oxygen and 6.5 vol% for carbon dioxide (Figure 6.12). As soon as air-sparging was started, oxygen concentrations further increased resulting in arithmetic means for BL1 – 3 and GW1 – 6 ranging from 11 to 16 vol%. CO_2 concentrations further decreased to arithmetic means of 1 to 8 vol%. Cessation of permanent soil vapour extraction before the air-sparging-start as well as cessation of air-sparging at the end of the air-sparging-test resulted in a decrease of oxygen and a corresponding increase of CO_2.

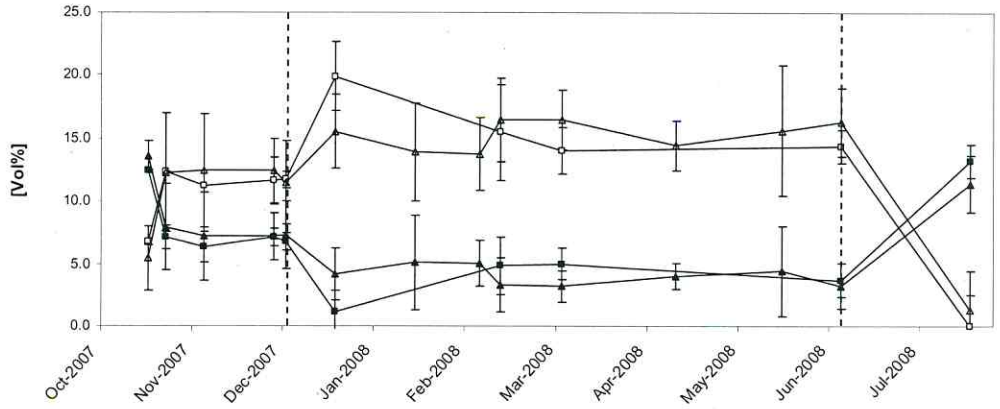


Figure 6.12: Temporal development of soil vapour properties at TF2 before (17/10/07 – 23/11/07, t0), during (t1 – t7), and after (t8) injecting air (oxygen [achromatic symbols] and carbon dioxide concentrations [black symbols]; arithmetic means of BL1 – 3 [quads] and GW1 – 6 [triangles]; dashed lines flank the period of the air-sparging-trial (from 03/12/07 to 25/06/08) (modified from report "Großmann (10.09.2008)").

6.3.4 Isotope chemical parameters

Benzene isotope ratios ($^{13}C/^{12}C$ and $^{2}H/^{1}H$) **of groundwater** were analysed as marker for microbial activity for samples of GW1 – 6 and RP6 before and at different time points during air-sparging. The temporal development is illustrated in Figure 6.S2 of the supporting information (left and middle columns). With decreasing benzene concentration of up to 63 or 82% until t5, $^{13}C/^{12}C$-isotope ratios of benzene slightly decreased (0.2 to 1.5‰), which means no or an inverse isotope fractionation. $^{2}H/^{1}H$-isotope ratios slightly increased in GW1, 2, 4, 5 and RP6 (< 15 ‰). In GW3 and 6 no change in $^{2}H/^{1}H$-isotope ratios were detected during the examination.

The percentage of *in situ* biodegradation, Bf_t [%], was assessed with equation 6.4 using the hydrogen isotope composition of benzene in the groundwater of each monitoring well before air-sparging started (R_0) and at the last sampling point (R_t) (Table 6.4). The isotope fractionation factor ($\alpha = 1.017$ and 1.060) was chosen from literature using the highest value for benzene degradation under aerobic and anaerobic conditions, respectively, to avoid an overestimation of the biodegradation extent (Fischer *et al.* 2008). The uncertainty of the calculations was estimated by adding the standard deviations (1σ) of the isotope values. Bf_t ranged from 11 ± 23% to 51 ± 28% using the isotope fractionation factor for aerobic degradation and from 4 ± 7% to 19 ± 12% for anaerobic degradation, respectively (Table 6.4).

Table 6.4: Initial (δ_0) and final (δ_t) hydrogen isotope signatures of benzene in groundwater of monitoring wells GW1 – 6 and RP6 with calculated percentages of biodegradation Bf_t [%] according to eq. 6.4 using isotope fractionation factors $\alpha = 1.017$ for aerobic and $\alpha = 1.060$ for anaerobic degradation.

	GW1	GW2	GW3	GW4	GW5	GW6	RP6
δ^2H_0 [‰]	-74.37	-70.53	-72.56	-74.46	-78.93	-74.01	-70.92
δ^2H_t [‰]	-65.74	-59.25	-70.68	-65.14	-68.96	-69.65	-60.72
Bf_t [%]; $\alpha = 1.017$	43 ± 24	51 ± 28	11 ± 23	45 ± 9	48 ± 13	25 ± 31	48 ± 28
Bf_t [%]; $\alpha = 1.060$	15 ± 9	19 ± 12	4 ± 7	16 ± 4	17 ± 5	8 ± 10	18 ± 11

Isotope ratios of **soil vapour CO_2** (BL) are listed in Table 6.5 for different time points during air-sparging. For all monitoring wells, carbon isotope signatures between -32.7 and -36.7‰ were observed for time points before injecting air. They were significantly higher than signatures of ambient air (-18 ± 3‰), which was sampled and analysed for control measures. During air-sparging, the carbon isotope signatures of CO_2 slightly decreased to -40.7‰. Comparatively, carbon isotope signatures of groundwater dissolved inorganic carbon (DIC) sampled from corresponding groundwater monitoring wells (GW) showed no significant change over time and space with values ranging from -22.6 to -23.8 ‰ (Table 6.5).

Table 6.5: Carbon isotope signatures [‰] of CO_2 sampled at different times in the unsaturated aquifer zone (BL) and DIC in the saturated aquifer zone (GW) of GW1 to GW6 and RP6; ambient air served as control.

	GW1	GW2	GW3	GW4	GW5	GW6	RP6	ambient air
BL 23/10/07	-35.7±0.1	-35.1±0.2	-34.8±0.1	-36.7±0.2	-34.3±0.3	-35.8±0.2	Nd	-16.1±0.1
BL 03/12/07	-34.6±0.2	-33.7±0.2	-34.7±0.2	-35.6±0.2	-34.2±0.2	-32.7±1.1	Nd	nd
BL 16/01/08	-39.1±0.3	-37.7±0.2	-36.0±0.2	-36.8±0.2	-35.6±0.3	-37.7±0.3	Nd	-20.7±0.3
BL 07/02/08	-40.7±0.3	-40.6±0.2	-39.4±0.3	-39.5±0.2	-39.7±0.1	-40.3±0.2	Nd	-18.1±0.4
GW 03/12/07	-23.0±0.1	-22.6±0.4	-23.2±0.1	-23.4±0.1	-23.7±0.1	-23.6±0.2	-24.1±0.1	nd
GW 07/02/08	-24.0±0.1	-22.3±0.1	-22.8±0.3	-22.3±0.3	-23.0±0.2	-23.2±0.4	-22.4±0.1	-16.2
GW 06/03/08	-23.5±0.1	-23.0±0.2	nd	-23.0±0.2	-23.7±0.1	-23.2±0.2	-22.8±0.1	-16.6±2.0

nd not detected

6.3.5 MPN of aerobic and aerobic benzene-metabolising microorganisms

Groundwater of GW1 – 6 and RP6 was analysed for most probable numbers (MPN) of aerobic microorganisms and aerobic benzene-degraders before and at different times during the air-sparging. The temporal development of MPNs is illustrated in Figure 6.S2 of the supporting information (right column) for all groundwater monitoring wells. GW1 showed constant MPNs for aerobic benzene degraders during the entire monitoring period. In GW2 – 6 and RP6, MPNs for aerobic benzene degraders increased 2 to 3 orders of magnitude within the first six weeks of air-sparging. However, with proceeding air-injection MPN showed constant or even decreasing values. MPNs of aerobic microorganisms, cultivated in complex media, nearly correlated with values of aerobic benzene-metabolising organisms. Figure 6.S2 (right column) also illustrates the ratio between total aerobes and aerobic benzene degraders (dashed lines). Initially, the ratio reached values up to 327, but strongly decreased after the first weeks of air-sparging resulting in maximum values of about 6, indicating that initially a diverse group of aerobes grew using different carbon or energy sources, whereas later organisms mainly grew on benzene.

6.3.6 Bactrap-experiments

Bactraps are colonisation materials which can be used to detect *in situ* microbial degradation of contaminants. These Bactraps consist of activated carbon pellets, so called Bio-Sep® beads, which are loaded with ^{13}C-labelled hydrocarbons, here $^{13}C_6$-labelled benzene. Usually, Bactraps are incubated in the saturated zone of an aquifer via groundwater monitoring wells. Microorganisms then colonise Bactrap pellets. Some of these organisms use the labelled substrate as carbon and/or energy source and incorporate the labelled carbon in their biomass. The detection of ^{13}C-incorporation in biomarker molecules, here fatty acids, proves the existence of active benzene-metabolising microorganisms.

Furthermore, colonisation- and label-patterns of fatty acids can indicate geochemical changes of the aquifer and can be used to quantify biodegradation.

Bactrap-experiments were performed in order to prove (aerobic) microbial degradation of benzene within the aquifer of Zeitz depending on air-sparging. Two different Bactrap-experiments were conducted. One set of Bactraps was incubated in a groundwater monitoring well as used commonly. The other one was placed in the aquifer-sediment by direct-push-technique in order to verify artefacts of monitoring wells.

6.3.6.1 Choice of a monitoring well for Bactrap-incubation

The aim of the Bactrap-experiments was to assess *in situ* benzene degradation within an oxygenated zone of TF2. GW3 was chosen to be best suited for the monitoring-well-Bactrap-experiment. The following points were crucial:

- Within GW3 highest increases of oxygen concentration and redox potential were detected after the first weeks of air-sparging (Figure 6.9; GW3 ▲, t0 – t3).
- MPNs of aerobic benzene-metabolising organisms increased significantly in GW3 after start of air injection (Figure 6.S2, right column).
- Benzene concentrations remained constant in GW3 within the first weeks of air-sparging (Figure 6.S2 left and middle column).

Based on those observations, a constant supply of oxygen and benzene, activity of aerobic benzene-metabolising organisms, and optimal conditions for the Bactrap-experiments were concluded for GW3.

Bactrap-experiments started on the 14/02/08, after air-sparging had been operated already nine weeks. Sediment-Bactraps were deployed in the saturated zone of the aquifer within a distance of ~1 m to the north-east of GW3 with Geoprobe-direct-push-technique.

Figure 6.13: Bactraps briefly after recovery from groundwater well (GW3). Teflon tubes are covered with a rusty layer, possibly iron oxides (from report "Vogt *et al.* (17.10.2008)").

After incubation of 60 (GW3-Bactraps) or 71 days (sediment-Bactraps), Bactraps were recovered from GW3 and the sediment. Bactraps deployed in GW3 were covered with a rusty coating indicating iron-oxidising processes within the groundwater of the monitoring well (Figure 6.13). Bio-Sep® beads of Bactraps deployed within the sediment were mixed with aquifer material which entered the lance and Teflon tubes of Bactraps during deployment or recovery of the lance.

6.3.6.2 Fatty acid analysis

In order to detect metabolisation of $^{13}C_6$-benzene, fatty acids of biomass accumulated on Bactraps were analysed for ^{13}C-incorporation by means of GC-C-IRMS. More than 10 different fatty acids were extracted and identified (Table 6.6) from differently loaded (unlabelled benzene, $^{13}C_6$-benzene or no loaded traps) Bactraps incubated in GW3. Hexadecenoic acid (16:1ω7c), hexadecanoic acid (16:0), and octadecenoic acid (18:1ω7c) occurred dominantly and made up 16 to 32% of all detected fatty acids. The proportions of remaining fatty acids were less than 7%, respectively (Table 6.8). Fatty acids of Bactraps loaded with $^{13}C_6$-labelled benzene were strongly enriched with ^{13}C; isotope ratios ($\delta^{13}C$) ranged from +8200‰ (18:2ω6c,9c) to +199500‰ (17:0). Mainly occurring fatty acids 16:1ω7c, 16:0 und 18:1ω7c had signatures between +50300 and +82600‰ (this corresponds to 37 to 50 atom percent ^{13}C-carbon).

Table 6.6 demonstrates that fatty acids of unloaded Bactraps and Bactraps loaded with natural benzene were slightly ^{13}C-labelled, although the label extent was much lower than for fatty acids of $^{13}C_6$-benzene loaded Bactraps (more than 100 fold lower). It is probable that Bactraps, which were not loaded with $^{13}C_6$-benzene, were contaminated during incubation by $^{13}C_6$-benzene which desorbed from $^{13}C_6$-loaded Bactraps and diffused to or was transported with groundwater flow to the control-Bactraps. Distances of differently loaded Bactraps were only about 10 cm. Small amounts of labelled benzene were then metabolised by organisms attached to control-Bactraps which resulted in a small ^{13}C-label of fatty acids. Metabolisation of unlabelled benzene with isotope signatures of -28 to -30‰ (occurring in groundwater of the test site and used for Bactrap loading) can not lead to a ^{13}C-enrichment of fatty acids to the extent observed for the control-Bactraps.

Table 6.6: Carbon isotope signatures of fatty acids, $\delta^{13}C_{FA}$, in ‰ and (atom%) extracted from Bactraps incubated for 60 days within GW3. A and B are biological parallels. Analyses were performed twice (n = 2) for unloaded and unlabelled-(^{12}C)-benzene-loaded Bactraps, n = 1 for $^{13}C_6$-labelled Bactraps (modified from report "Vogt *et al.* (17.08.2008)").

Fatty acids	$^{13}C_6$-benzene-Bactraps		unloaded Bactraps		^{12}C-benzene- Bactraps	
	A	B	A	B	A	B
14:0	41700 (33)	28900 (25)	76 ± 2 (1.2)	67 ± 4 (1.2)	26 ± 1 (1.1)	42 ± 7 (1.2)
i-15:1ω10c	74600 (47)	u.d.l.	273 ± 6 (1.4)	307 ± 8 (1.4)	126 ± 4 (1.2)	u.d.l.
i-15:0	52000 (38)	50300 (37)	224[a] (1.3)	213 ± 1 (1.3)	98 ± 0.5 (1.2)	117 ± 2 (1.2)
a-15:0	18800 (18)	u.d.l.	97 ± 6 (1.3)	u.d.l.	40[a] (1.1)	u.d.l.
16:1	56500 (40)	65900 (44)	170 ± 1 (1.3)	174 ± 0.2 (1.3)	48 ± 0.6 (1.2)	46 ± 0.8 (1.2)
16:0	68900 (45)	82600 (50)	258 ± 2 (1.4)	232 ± 0.2 (1.4)	70 ± 0.4 (1.2)	106 ± 0.2 (1.2)
17:0	199500 (72)	199400 (72)	310 ± 3 (1.4)	266 ± 6 (1.4)	76 ± 0.3 (1.2)	99 ± 4 (1.2)
18:2ω6c,9c	10600 (12)	8200 (9)	10 ± 9 (1.1)	51 ± 0.9 (1.2)	-7 ± 1 (1.1)	-6 ± 0.2 (1.1)
18:1ω7c	50300 (37)	55100 (39)	106 ± 7 (1.2)	121 ± 5 (1.2)	48 ± 2 (1.2)	42 ± 1 (1.2)
18:0	78600 (48)	u.d.l.	181[a] (1.3)	152 ± 19 (1.3)	158 ± 144 (1.3)	48 ± 1 (1.2)
19:0	156000 (66)	196400 (71)	144 ± 8 (1.3)	136 ± 6 (1.2)	64 ± 7 (1.2)	65 ± 5 (1.2)

[a] detected only for one parallel; u.d.l. = under detection limit

Table 6.7: Carbon isotope signatures of fatty acids, $\delta^{13}C_{FA}$, in ‰ and (atom%) extracted from Bactraps incubated for 71 days in the aquifer-sediment close to GW3. A and B are biological parallels. Analyses were performed twice (n = 2) for all Bactraps (modified from report "Vogt *et al.* (17.08.2008)").

Fatty acids	$^{13}C_6$-benzene-Bactraps		unloaded Bactrap		^{12}C-benzene-Bactraps	
	A	B	A	B	A	B
14:0	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.
i-15:1ω10c	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.
i-15:0	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.
a-15:0	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.
16:1ω7c	u.d.l.	159200[a] (67)	u.d.l.	-7 ± 0.2 (1.2)	1630[a] (3.0)	61 ± 6 (1.3)
16:0	4750 ± 20 (6.2)	7380 ± 74 (8.8)	u.d.l.	188 ± 0.3 (1.4)	63 ± 14 (1.2)	680 ± 40 (2.0)
17:0	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.
18:2ω6c,9c	450 ± 20 (1.7)	5980 ± 535 (7.7)	u.d.l.	-25 ± 0.1 (1.2)	-38 ± 5 (1.2)	-31 ± 0.5 (1.2)
18:1ω7c	11090 ±240 (12)	143400 ± 770 (64)	u.d.l.	-11 ± 0.2 (1.2)	2580 ± 570 (4.0)	57 ± 2 (1.3)
18:0	1330 ± 12 (2.7)	1910 ± 190 (3.4)	u.d.l.	u.d.l.	u.d.l.	u.d.l.
19:0	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.

[a] detected only for one parallel; u.d.l. = under detection limit; from unloaded Bactrap A no fatty acids could be extracted

Table 6.8: Concentrations [ng g^{-1} beads] of fatty acids (c_{FA}) and ^{13}C incorporated into fatty acids (c_{13C}) for $^{13}C_6$-amended Bactraps incubated in GW3 (mean values and standard deviations for both parallels), and incubated in aquifer-sediment for both parallels separately.

Fatty acids	GW3-$^{13}C_6$-Bactraps		Aquifer $^{13}C_6$-Bactrap A		Aquifer $^{13}C_6$-Bactrap B	
	c_{FA} [ng g^{-1} beads]	c_{13C} [ng g^{-1} beads]	c_{FA} [ng g^{-1} beads]	c_{13C} [ng g^{-1} beads]	c_{FA} [ng g^{-1} beads]	c_{13C} [ng g^{-1} beads]
14:0	1790±30	379±70	u.d.l.	u.d.l.	u.d.l.	u.d.l.
i-15:1ω10c	1170[a]	390[a]	u.d.l.	u.d.l.	u.d.l.	u.d.l.
i-15:0	1740±280	460±80	u.d.l.	u.d.l.	u.d.l.	u.d.l.
a-15:0	5380±70	680[a]	u.d.l.	u.d.l.	u.d.l.	u.d.l.
16:1ω7c	21230±2020	6380±200	u.d.l.	u.d.l.	2000	954
16:0	13270±1770	4460±290	1760	66	2500	140
17:0	3590±500	1840±260	u.d.l.	u.d.l.	u.d.l.	u.d.l.
18:2ω6c,9c	2190±150	160±40	920	3.4	1560	74
18:1ω7c	26370±1820	7350±200	1900	157	1900	120
18:0	2790±690	800[a]	180	11	1430	660
19:0	2765±120	1380±20	u.d.l.	u.d.l.	u.d.l.	u.d.l.
Σ [µg/g beads]	82.3±7.4	24.3±1.2	5.7	0.24	9.4	1.9

[a] detected only for one parallel extracted from GW3-$^{13}C_6$-Bactraps; u.d.l. = under detection limit

Far less fatty acids could be extracted from Bactraps incubated in the aquifer-sediment by direct-push-technique. Merely, 16:1ω7c, 16:0, 18:1ω7c and octadecadienoic acid (18:2ω6c,9c) were detected (Table 6.7 and Table 6.8) indicating a much lower attachment or growth rate compared to Bactraps incubated in the monitoring well. Fatty acids of $^{13}C_6$-benzene loaded Bactraps showed a differentiated extent of ^{13}C-enrichment with a span of +450‰ (this corresponds to 1.7 atom percent) to +159000‰ (16:1ω7c, this corresponds to 67 atom percent). Additionally conspicuous is the fact that isotope signatures of the same fatty acids of biological parallels differ partially to a great extent (esp. 18:1ω7c and 18:2ω6c,9c, see Table 6.7). As already observed for Bactraps incubated in GW, fatty acids of control-Bactraps were also enriched with ^{13}C: relatively low in the case of the unloaded Bactraps (maximum +188‰ ≙ 1.4 atom percent); partially relatively high in the case of Bactraps loaded with unlabelled benzene (maximum +2580 ‰ ≙ 4.0 atom percent). Distances of unequally loaded Bactraps within the lance were about 5 cm. Thus, ^{13}C-enrichment of control-Bactraps can be explained by diffusion of ^{13}C-labelled benzene to control-Bactraps similarly to observations for GW3-Bactraps.

Fatty acid methyl esters were quantified after GC-MS analysis by comparison of chromatogram peak areas relative to peak areas of the methyl ester of heneicosanoic acid (21:0) as internal standard. Concentrations of methyl esters were converted to concentrations of fatty acids (C_{FA}) by equation 6.6 which are summarised in Table 6.8 for Bactraps amended with $^{13}C_6$-benzene for both incubation versions. For parallels of $^{13}C_6$-Bactraps incubated in the monitoring well GW3 arithmetic mean values and respective standard deviations were calculated, whereas values for biological parallels incubated in the sediment were calculated separately on account of their great variability. By means of equations 6.8 to 6.11 the concentrations of ^{13}C-carbon (c_{13C}) bound to respective fatty acid were calculated which are also summarised in Table 6.8. Total concentrations ($\sum$) of fatty acids ranged from 6 µg to 82 µg g^{-1} beads (Table 6.8). The total concentrations of ^{13}C incorporated in all fatty acids ranged from 0.24 to 24.3 µg g^{-1} beads (Table 6.8).

6.4 Discussion and conclusions

The following questions should be answered with the present study:

A) How does air-sparing effect hydrochemical parameters?

B) What hydrogeological properties (groundwater flow velocity and direction) are characteristic for the test site? And does this indicate the spreading of the injected air-oxygen?

C) What is the amount of benzene removed during air-sparging?

D) How much of the removed benzene is biodegraded?

The following sections discuss the results concerning the above questions.

6.4.1 Effects of air-sparging on hydrochemical parameters

Air-sparging provides an O_2-supply to the groundwater. It was found that dissolved iron ($Fe(II)_{(aq)}$) as the most sensitive inorganic parameter in terms of redox conditions decreased with an average value of around 0.4 mM for the entire duration of air-sparging. Iron(II) ions are oxidised as soon as they get in contact with oxygen according to the equation

$$Fe^{2+} + O_2 + H_2O \leftrightarrow Fe(OH)_3 \downarrow + 2H^+ . \qquad \text{eq. 6.12}$$

Thereby, Fe(III) ions are formed, which precipitate as hydroxides and were observed for instance as a rusty coating around Bactraps that had been incubated within GW3. During iron(II) oxidation, protons are released which cause a decrease of pH. The average pH decreased about 0.3 units. Precipitation of iron ions causes a decrease of dissolved ions. The electrical conductivity, which is a measure of the content of dissolved salts (ions, electrolytes), decreased by approximately 300 $\mu S\ cm^{-1}$ (average values) during the entire air-sparging-period. This was not only caused by the decrease of Fe(II) ions, but also by a decrease of Ca^{2+} ions. Air-sparging causes, next to an O_2-supply, a decrease of the CO_2-partial pressure within groundwater due to CO_2-losses by stripping effects, indirectly detectable in a decrease of the total inorganic carbon (TIC). The depletion of freely dissolved $CO_{2(aq)}$ unbalances the chemical equilibrium of carbonic acid and calcium carbonate as described by the following equation

$$Ca^{2+} + 2HCO_3^- \leftrightarrow CaCO_3 \downarrow + CO_2 \uparrow \qquad \text{eq. 6.13}$$

since the depletion of a product supports the further formation of products and consequently a decrease of adducts, *i.e.* Ca^{2+} and bicarbonate ions.

Sulphate, as main electron acceptor for the field under non-engineered conditions, showed no increase over the entire period of air-sparging. It had been assumed that sulphide, formed and accumulated during natural attenuation processes prior to air-sparging, was oxidised by injected air-oxygen (chemical autoxidation) to form elementary sulphur and later sulphate according to the following equations

$$2S^{2-} + O_2 + H^+ \leftrightarrow 2S^0 + 2H_2O \qquad \text{eq. 6.14}$$

$$2S^0 + 2H_2O + 3O_2 \leftrightarrow 2\ SO_4^{2-} + 4H^+ . \qquad \text{eq. 6.15}$$

One possible reason for the absence of sulphate production would be the absence of sulphide formation by natural attenuation processes beforehand within the monitored area. Another reason might be the high vapour pressure of hydrogen sulphide (1819 kPa) and a discharge via gas phase by the air-injection flow before autoxidation could take place. This is reasonable, because chemical autoxidation of sulphide requires long reaction times (several days) (Weismann and Lohse 2007). But on the other hand, biological sulphide oxidation by aerobic sulphide oxidisers, *e.g. Thiobacillus* species, is by far faster (Rohwerder *et al.* 2003). It is also imaginable that the amount of dissolved oxygen (DO) was too low for sulphide oxidation ($S^{2-} \rightarrow SO_4^{2-}$ requires 2.5 mg O_2/mg S), or was used for other chemical reactions, *e.g.* iron oxidation or biodegradation of other electron donors. Measured

dissolved oxygen values were relatively low (0.5 mg L^{-1}, integral sampling) over the whole period of air-sparging, showing that aeration was inefficient, since oxygen was rapidly consumed. The same was observed for redox potentials with values between -20 and -80 mV and a decreasing tendency with increasing sparging duration. These redox potentials by far do not represent aerobic conditions (+820 mV), but are at least higher than potentials which were observed after the air-sparging-trial stopped (~ -300 mV) (Wiedemeier *et al.* 1999).

6.4.2 Determination of ground water flow direction and velocity

Results of the tracer-experiment, groundwater level measurement campaigns as well as the gas-tracer-test, revealed very low groundwater velocities of only a few centimetres per day at TF2, which could not be exactly quantified. Furthermore, an exact groundwater direction could not be determined. Gas-tracer-test-results disclosed a very low oxygen transfer rate from gas phase to aqueous phase as well as a preferential flow of gas phase along an aquitard which additionally lowers the oxygen transfer due to the short time of air and water contact. Probably, an oxygen gradient within the saturated aquifer according to Figure 6.1, as presumed at the beginning of the project, did not develop. It is more likely that only a restricted area around the injection well (r ~ 5 m) was actually affected by air-sparging (Figure 6.14).

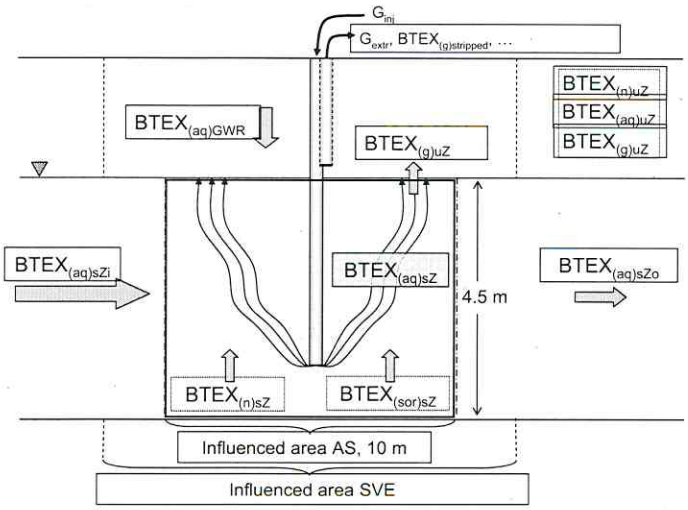


Figure 6.14: Conceptual scheme showing the affected area by air-sparging (AS) (G_{inj} = injected air-stream) and soil vapour extraction (SVE) (G_{extr} = extracted air stream). Additionally cited are phases in which BTEX may occur: (aq) aqueous, (g) gaseous, (n) NAPL, (sor) adsorbed to sediment. sZ is an abbreviation for saturated aquifer zone, uZ for unsaturated aquifer zone, and GWR for natural groundwater replenishment. i stands for inflow, o for outflow. Grey arrows illustrate transfer processes of BTEX into or out of the affected air-sparing area (capture volume = 353 m^3) (modified from report "Müller (10.12.2008)").

6.4.3 Efficiency of air-sparging – calculations based on benzene removal

Looking at Figure 6.14, several processes may cause a reduction of BTEX concentration or mass in a certain volume of a contaminated aquifer. These processes are:

- transport of benzene with groundwater beyond the examined area ($BTEX_{(aq)sZo}$);
- adsorption processes to aquifer material ($BTEX_{(sorb)}$);
- transfer from aqueous phase ($BTEX_{(aq)}$) to gas phase ($BTEX_{(g)}$) by volatilisation dependent on Henry coefficients of the specific compound and compound concentrations within the gas phase;
- microbial degradation

Next to these processes, the BTEX concentration is influenced by processes that increase the amount of BTEX in the examined aquifer volume. These processes are termed as 'rebound processes' and include:

- transport of benzene with groundwater into the examined area (from the contamination source, $BTEX_{(aq)sZi}$);
- compound desorption from aquifer material ($BTEX_{(sorb)}$) and solution of compounds within non aqueous phase liquids (NAPL, $BTEX_{(n)}$) depending on water solubility of the specific compound and the compound concentration within the aqueous phase.

NAPLs were detected at the examined test field and are present on top of the groundwater table and within areas of the capillary fringe. NAPL benzene can dissolve in seepage water and move to the saturated zone of the aquifer ($BTEX_{(aq)GWR}$).

Relevant transfer processes within the saturated zone of aquifers are transition from:

- adsorbed or NAPL phase to aqueous phase or directly to gaseous phase;
- aqueous phase to gas phase;
- aqueous phase to depletion by biodegradation and CO_2, H_2O and biomass formation.

Under natural conditions, processes for BTEX removal and BTEX rebound are usually equilibrated which causes an almost stable BTEX concentration in groundwater. Air-sparging unbalances this equilibrium, since every single process is influenced by the air-injection flow (Figure 6.14). The air-injection flow (G_{inj}) causes an accelerated transfer of water dissolved benzene from aqueous to gaseous phase (benzene stripping). Also NAPL and soil adsorbed benzene is transferred to the gaseous phase due to the high vapour pressure of benzene. Solid phase extraction wells remove vaporised benzene from the unsaturated zone (G_{extr}, $BTEX_{(g)stripped}$). This enables further volatilisation of benzene within the aqueous phase. The decrease of benzene concentration in the aqueous phase enhances processes of NAPL dissolution and desorption from aquifer material. Dependent on the transfer rate of oxygen from gas to aqueous phase, aerobic microbial degradation processes may also be enhanced.

Benzene concentrations of the aqueous phase decreased as soon as air-sparging started. Removal rates were up to 31 mg $(L \times d)^{-1}$ for the first 1.7 days (phase A, Table 6.9, Figure 6.8), probably due to the strong concentration gradient between the injected air and the non-engineered aqueous phase. At a concentration of approximately 170 mg L^{-1}, the rate decreased and was stable for 131 days at a value of 0.84 mg $(L \times d)^{-1}$ (phase B). After 133 days, benzene concentrations decreased only slightly for the rest of the air-sparging period (0.06 mg $(L \times d)^{-1}$), indicating a balance of benzene removal and rebound processes (phase C).

Table 6.9: Mean benzene concentration changes within the aqueous phase of the aquifer ($\Delta c_{Benzene,aq}$) detected during phases A, B, C, and D in the monitoring wells GW1 – 6 and RP6; calculated removal/rebound rates ($\Delta c_{Benzene,aq}$ d^{-1}); estimated total benzene removal including correction for benzene increase by rebound processes ($|\Delta c_{Benzene,aq}$ $d^{-1}|$+rebound); and total masses of removed benzene (Δm) from TF2, calculated for the capture volume (CV_{aq}) of 95 m^3 and the respective durations; $\sum$ sum of benzene mass removed during the entire air-sparging trial (202 days) (modified from report "Müller (10.12.2008)").

Phase	Duration [d]	$\Delta c_{Benzene,aq}$ [mg L^{-1}]	$\Delta c_{Benzene,aq}$ d^{-1} [mg $(L \times d)^{-1}$]	$\lvert\Delta c_{Benzene,aq}$ $d^{-1}\rvert$ +rebound [mg $(L \times d)^{-1}$]	Δm_{CVaq} [kg]
A	1.7	-53	-31	31.7	5.1
B	131	-110	-0.8	1.5	18.7
C	69	-4	-0.06	0.76[ψ]	5.0
D	119	+78	+0.7		
				$\sum$	28.8

[ψ] rate used for estimation of effective air-sparging-duration (see equation 6.16)

As soon as the air-sparging was stopped, the newly established equilibrium again was disturbed, this time by the dominance of rebound processes. Benzene concentrations increased constantly by a rate of 0.66 mg $(L \times d)^{-1}$ for at least 119 days to concentrations up to 139 mg L^{-1} (monitoring ended, phase D). This increase demonstrated that air-sparging for ~200 days was not sufficient to decrease benzene concentrations sustainably.

An estimation of an effective time of performance may be calculated on the basis of the results of benzene removal and rebound processes as well as the following assumptions. As depicted in Figure 6.14, only a small cylinder-like volume was affected by air-sparging. This volume captures (CV) in the saturated zone 353 m^3 according to the following equation:

$$CV = \pi \times r^2 \times h = \pi \times (5\ m)^2 \times 4.5\ m = 353\ m^3 \qquad \text{eq. 6.16}$$

Calculating with a porosity of approximately 0.27, common for coarse and medium grained sediments, the volume of the aqueous phase in the saturated zone is at most 95 m^3 (CV_{aq}). The packing density was estimated at 1.7 t kg^{-1}, thus the sediment volume (CV_{solid}) is about 600 t.

Considering only the measured decrease of benzene, $\Delta c_{Benzene,aq}$, for phases A to C, a total mass of ~16 kg benzene was removed from the aqueous phase during the entire air-sparging-test. Phase C reveals that rebound processes occurred during the whole air-sparging-period. Therefore, the calculated amount of removed benzene has to be corrected by the amount of benzene which increased the concentration by rebound processes. An approximation of rebound processes can be calculated on the basis of the benzene increase within phase D (~0.7 mg $(L \times d)^{-1}$, Table 6.9). Then the corrected mass of the benzene removal is 28.8 kg (∑, Table 6.9) during the entire air-sparging period (202 days).

The overall benzene mass within the capture volume can be estimated by the following assumptions:

Assumption 1: The benzene concentration in the aqueous phase is ~250 mg L^{-1} under non-engineered conditions and therefore contributes approximately 24 kg to the overall benzene mass ($= CV_{aq} \times 250\ mg\ benzene / 1\ L\ groundwater$).

Assumption 2: Within the aquifer ~100 mg benzene are adsorbed to 1 kg of soil. Soil analyses resulted in an arithmetic average of ~10 mg kg^{-1} (date no shown), but these analyses often underestimate benzene masses due to its high volatility*. Benzene adsorbed to soil particles then contributes ~60 kg ($= CV_{solid} \times 100\ mg\ benzene / 1\ kg\ soil$) to the overall benzene mass.

Assumption 3: ~0.1% of the pore volume is filled with NAPL. Thus, NAPL ($\rho_{benzene} = 0.88$ g cm^{-3}) contributes ~84 kg ($= CV_{aq} \times 0.1\% \times 0.88\ g / cm^3$) to the overall benzene mass.

Assumption 4: ~2.5 g kg^{-1} benzene is adsorbed to the brown coal layer, which was found to occur below air-injection and monitoing wells. Assuming that the first 0.2 m of the layer contain the main part of the benzene which may affect the aqueous phase ($CV_{coal} = \pi \times (5\ m)^2 \times 0.2\ m = 15.7\ m^3$) and assuming a brown coal density of 1000 g m^{-3}, ~40 kg benzene ($= CV_{coal} \times 1000\ g / m^3 \times 2.5\ g / kg$) will be released from the brown coal layer.

Assumption 5: Benzene is removed by a constant rate of 70 g d^{-1}[ψ]. This rate conforms to the rate of phase C where removal and rebound processes are equilibrated (Figure 6.14, Table 6.9).

Summarising assumptions 1 to 4, approximately 208 kg benzene would have to be removed within the capture volume. Calculating with assumption 5 the air-sparging would need to be performed for another period of ~7 years (see equation 6.17) to remove benzene of TF2 sustainably:

$$t = (m_{t0} - \Delta m_{AS}) / R_m = (208\ kg - 28.8\ kg) / (70 g / L) = 2560\ d \qquad \text{eq. 6.17}$$

* Kai Müller (UFZ-GWR), personal communication, 12/2008

However, the used calculation model only provides an estimation. Stated assumptions can vary, more likely with a tendency to higher values. For instance, a calculation with 0.5% of NAPL within the pore volume rather than 0.1% results in another period of 20 years. Thus, air-sparging is probably not an eligible remediation technique at this site. To make a more precise forecast, more complex models have to be used that consider additional aspects such as the observed preferential flow or the air-sparging activity of the other eight injection wells of SB2. This was beyond the scope of this PhD project, but might be done by other project partners within the BEOQUE project.

6.4.4 The role of microorganisms

Different experiments and analyses were performed to determine whether air-sparging enhanced microbial activity, especially concerning microbial degradation of the main contaminant benzene, and to elucidate how much of the removed benzene was aerobically biodegraded. The results of the different analyses and the information gained are discussed in the following paragraphs.

6.4.4.1 MPN analysis

The detection of most probable numbers (MPN) was used as a screening method for viable cell counts of microorganisms able to degrade benzene under oxic conditions and aerobes in general present in the groundwater of TF2 depending on air-sparging. An increase of the MPN for aerobic benzene-metabolising microorganisms indicates an induced aerobic *in situ* benzene degradation. In a study of Vogt *et al.* (2004), increased MPNs for aerobic chlorobenzene-metabolising microorganisms were observed in an *in situ* reactor percolated with chlorobenzene-contaminated groundwater and hydrogen peroxide, indicating aerobic *in situ* degradation of chlorobenzene as a result of hydrogen peroxide addition. The results of the present study indicate a rapid induction of aerobic benzene degradation. Low ratios between aerobes in general and aerobic benzene degraders suggest that organisms mainly grew on benzene. Stagnation/decrease of MPNs with on-going air-sparging indicates some kind of limitation, *e.g.* concerning the nutrient, electron donor or acceptor supply. Since hydrogeological investigations characterised groundwater flow velocities of the aquifer at TF2 as very low, all of those parameters might be limited due to a faster consumption than subsequent delivery. Dissolved oxygen concentrations were very low generally (~0.5 mg L^{-1}), despite air-injection, which is a strong indication that the bioavailability of oxygen is the limiting factor. However, MPN stagnation also indicates that at least a certain amount of oxygen had to be present to keep viable cell numbers at this level.

Limitations due to toxic effects of high benzene concentrations, as documented in various studies (Gibson *et al.* 1970; Shirai 1987; Yarmoff *et al.* 1988; Sikkema *et al.* 1995; Shim *et al.* 2005), also can not be ruled out. Shim *et al.* (2005) investigated a coculture of *Pseudomonas* strains which was able to grow aerobically on BTEX compounds as sole source of carbon and energy. Cell growth was completely inhibited at benzene concentrations of 500 mg L^{-1}. Benzene concentrations within the present study were found at a level up to 350 mg L^{-1} for the aqueous phase. But these analyses on the

one hand are only snap-shots and on the other hand capture only concentrations of water dissolved benzene. Benzene that is adsorbed to sediments or occur as NAPL may increase benzene concentrations under spatial differentiation up to the solubility limit (~1.8 g L^{-1}) and may cause toxic effects on the microbial flora.

6.4.4.2 Isotope fractionation analyses

Isotope composition of benzene in groundwater samples showed a slight enrichment of deuterium during air-sparging, but no or an inverse isotope effect for carbon. Hunkeler *et al.* (2001), Mancini *et al.* (2003), and Fischer *et al.* (2008) investigated isotope fractionation of benzene degradation under different redox conditions, with different pure and mixed cultures which used different degradation initiating enzymes. Fischer *et al.* (2008) analysed those data within a two-dimensional compound-specific isotope (2D-CSIA)-approach and could clearly separate aerobic and anaerobic degradation. Whereas $^{13}C/^{12}C$-isotope fractionation was observed for all investigated cultures (enrichment factors ranged from -0.7‰ to -4.3‰), $^{2}H/^{1}H$-isotope fractionation was observed only for anaerobic cultures and for aerobic organisms which used monooxygenases as initiating enzymes for benzene degradation. Generally, fractionation was stronger for anaerobic conditions with enrichment factors ranging from -29‰ to -35‰ for nitrate reducers and -59‰ to -79‰ for strict anaerobes, *i.e.* sulphate reducers and methanogens. Aerobic degradation catalysed by monooxygenases showed enrichment factors from -11‰ to -17‰. Whereas aerobic degradation catalysed by dioxygenases indicated no deuterium enrichment. Regarding to Fischer *et al.* (2008), the hydrogen isotope shift detected in the present study indicated benzene degradation either by monooxygenation or an anaerobic pathway. No (or inverse) carbon isotope effects seem to contravene this suggestion. No carbon isotope effects may indicate aerobic degradation catalysed by dioxygenases, which were characterised by lowest carbon isotope effects (IE). Inverse carbon isotope effects have never been observed during biodegradation processes of benzene (Hunkeler *et al.* 2001; Mancini *et al.* 2003; Fischer *et al.* 2008; Mancini *et al.* 2008).

Contrary results of hydrogen and carbon isotope on the one hand may be explained by the detection limit of stable carbon isotope effects. Analysis of carbon isotope signatures are indeed more sensitive than analysis of hydrogen isotope ratios since the natural abundance of deuterium is approximatly 50 times lower than the one of ^{13}C (Hoefs 1997); this results in higher analytical uncertainties of hydrogen analyses and necessitates higher concentrations of the examined compound (Meier-Augenstein 1999; Hunkeler *et al.* 2001). But hydrogen isotope effects are usually about two orders of magnitude higher than carbon isotope effects (Hilkert *et al.* 1999). These properties make $^{2}H/^{1}H$-isotope analysis most appropriate to detect small biodegradation activities which might not be detected by carbon isotope analysis.

On the other hand inverse carbon isotope effects may indicate other than biotic processes. Stable isotope effects resulting from abiotic processes, *e.g.* sorption, dissolution, dispersion, and volatilisation, are generally considered insignificant (Meckenstock *et al.* 2004a). But several studies

reported strong fractionation of stable isotopes due to progressive volatilisation (Baertschi and Kuhn 1957; Grootes *et al.* 1969; Harrington *et al.* 1999; Huang *et al.* 1999; Poulson and Drever 1999; Wang and Huang 2001; Wang and Huang 2003; Bouchard *et al.* 2008). Baertschi *et al.* (1953) found that distillation of carbon tetrachloride, chloroform, methanol, and benzene, in all four cases leads to an enrichment of ^{13}C in the distillate (vapour phase), whereas ^{37}Cl and ^{18}O are enriched in the liquid phase (normal IE). A similar phenomenon was observed for carbon and oxygen isotope fractionation between carbon dioxide liquid and vapour (Grootes *et al.* 1969). Harrington *et al.* (1999) and Huang *et al.* (1999) observed a depletion of ^{13}C isotopes within the residual phase of benzene, toluene, and ethylbenzene, and TCE and DCE, respectively due to progressive volatilisation (inverse IE). The same effect was shown for deuterium during vaporisation of *n*-alkanes or a VOC-mixture by Wang and Huang (2001; 2003) and trichloroethylene by Poulson and Drever (1999). Theoretical calculations of the vapour pressure ratios of these species in general predict that molecules bearing the light isotopes are slightly more volatile (Urey *et al.* 1932). The presence of heavy isotopes in the molecule reduces its zero-point energy (E_0) resulting in a decrease of its vapour pressure. This is particularly significant for isotopomers differing by 2 daltons, like ^{16}O vs. ^{18}O (Kuder *et al.* 2009). For isotope species differing by 1 dalton, *e.g.* ^{12}C vs. ^{13}C, the effect of the zero-point energy may be overshadowed by intermolecular forces. The relative enrichment of ^{13}C observed in the vapour phase was explained by having the smaller binding energy in the liquid phase for the ^{13}C-bearing molecules (Baertschi and Kuhn 1957; Grootes *et al.* 1969). Wang and Huang (2003) noted that significant isotope fractionation due to vaporisation will occur only in natural systems where a high percentage of contaminant mass has been lost to the vapour phase and the vapour phase is constantly removed from the system. This is given in the present study, thus the detected isotope effect is likely to be influenced by vaporisation effects and not only representative for biological processes.

All mentioned studies, except for Kuder *et al.* (2009), reported for both carbon and hydrogen an inverse isotope effect during vaporisation of each examined compound containing the elements. Kuder *et al.* (2009) investigated effects of volatilisation on carbon and hydrogen isotope ratios of MTBE and observed normal and inverse isotope effects for different volatilisation scenarios. The scenarios were: (i) passive NAPL-volatilisation, (ii) passive volatilisation of an aqueous solution, (iii) soil vapour extraction, and (iv) air-sparging. During passive volatilisation (i and ii), ^{13}C-carbon enriched within the residual phase for both NAPL and aqueous solution, ^{2}H enriched within the aqueous solution but depleted within NAPL. The depletion of ^{2}H during NAPL-volatilisation was attributed to the higher vapour pressure of the heavy isotope. The contrary hydrogen isotope effect during volatilisation of the dilute solution was explained by the preferential binding of water to ^{2}H-MTBE via hydrogen bondings. The enrichment of ^{13}C was explained by the dominance of vapour diffusion processes. The diffusive mass flux is larger for molecules with light isotopes (Bouchard *et al.* 2008). During soil vapour extraction (iii), residual MTBE was initially depleted in both heavy isotopes, but shifted to a normal isotope effect for both isotopes after MTBE mass was reduced to low concentrations. Initial inverse

IEs were suggested to be caused by a predominance of molecular exchange (higher vapour pressure of heavy isotopomers). The later reversal of IE direction was suggested to be caused by a switch to liquid-diffusion by supply of MTBE. In the air-sparging experiment (iv) the residual MTBE became progressively enriched in ^{2}H with larger effect for the more dilute solution. This was explained both by liquid diffusion analogous to late stage of solid phase extraction and by lower vapour pressure of ^{2}H due to hydrogen bonding analogous to passive volatilisation. In the case of carbon only a very small decrease of $\delta^{13}C$ was observed during air-sparging.

Assuming MTBE as a good model compound for BTEX, it is likely that isotope signatures of both carbon and hydrogen of benzene analysed in the present study are influenced by abiotic processes next to biotic processes, since solid phase extraction as well as air-sparging were performed and passive volatilisation occurred.

Thus, the quantification of biodegradation by means of isotope fractionation according to several field studies, *e.g.* Mancini *et al.* (2002), Griebler *et al.* (2004b) or Vieth *et al.* (2005), has to be handled with caution when air-sparging is applied. Moreover, a combination of low isotope fractionation factors and a small degree of degradation is associated with a high degree of uncertainty (Meckenstock *et al.* 2004a). Significant values for degradation pathways with low fractionation factors are only detectable if biodegradation is higher than 90%. Vice versa, significant values for lower extents of degradation are only detectable, if the dominant degradation pathway is linked to a high isotope fractionation factor.

Soil vapour was characterised by very high CO_2 concentrations (4.5 to 10.1%), which cannot have been produced abiotically (Battersby 1997). Detected $\delta^{13}C$ values of carbon dioxide were strongly ^{13}C-depleted, ranging from -32.7 to -40.7‰. Generally, soil gas CO_2 is isotopically similar to the carbon source it is derived from (Deines 1980; Rabenhorst *et al.* 1984). Due to preferential degradation of molecules with light isotopes (Clark and Fritz 1997; Hoefs 1997), stable isotope ratios of metabolic end products can be shifted (or fractionated) relative to the initial $\delta^{13}C$ values of the substrate material (Landmeyer *et al.* 1996; Conrad *et al.* 1997). However, these effects are generally small (< 6‰) (Blair *et al.* 1985; Conrad *et al.* 1997). Several studies used this interrelation to prove the microbial degradation of petroleum hydrocarbon, since those are ^{13}C-depleted ($\delta^{13}C \sim -28‰$) and produce isotopically lighter soil gas CO_2 or groundwater DIC compared to CO_2 and DIC that occur in uncontaminated areas (Suchomel *et al.* 1990; Aggarwal and Hinchee 1991; Baedecker *et al.* 1993). $\delta^{13}C$ values between -20 and 0‰ are representative for CO_2 or DIC of pristine aquifers (Clark and Fritz 1997; Hoefs 1997). Thus, $\delta^{13}C$ values of this study strongly indicate that soil gas CO_2 was produced by microbial degradation of benzene, in both cases before air-sparging started (from -32.7 to -36.7‰) and during the period of air-injection (from -35.6 to -40.7‰). The ^{13}C-depletion of soil gas CO_2 with increasing duration of air-sparging might be indicative for an accelerated benzene degradation which only would be conceivable under oxic conditions. Correlation between the soil

vapour CO_2 increase and the O_2 depletion after air-sparging was stopped supports the presumption of aerobic microbial benzene degradation.

Relatively heavy $\delta^{13}C$ values of dissolved inorganic carbon (-22.6 to -23.8‰) indicate that a source of isotopically heavy carbon has contributed to the overall carbon budget of the groundwater. This is very likely due to mixing with uncontaminated groundwater from upstream areas (Fischer *et al.* 2004).

6.4.4.3 Bactrap-experiments

The fatty acids profiles of all Bactraps suggest that the microbial communities attached to Bio-Sep® beads were composed primarily of bacteria, since fatty acids in the range of C14 – C19 are characteristic for bacteria (Kates 1964; Shaw 1974). Dominant fatty acids were hexadecenoic acid (16:1ω7c), hexadecanoic acid (16:0), and octadecenoic acid (18:1ω7c). Weber *et al.* (1994) demonstrated that fatty acid profiles of different *Pseudomonas putida* strains, known to be capable of degrading BTEX compounds aerobically, are also dominated by 16:1c, 16:0 and 18:1c fatty acids. Hexadecanoic acid occurs in all bacteria. However, high abundances of monounsaturated fatty acids are indicative for aerobic bacteria (Guckert *et al.* 1985; Findlay *et al.* 1990; Bossio and Scow 1998). Particularly 18:1ω7c was found to be associated with aerobic and facultative aerobic organisms (Parkes and Taylor 1983; Bossio and Scow 1998). In contrast, fatty acids known as biomarkers for anaerobic organisms, in particular sulphate reducers, such as 10Me16:0, *i*17:1ω7c, 17:1ω6 were not detected on Bactraps (Scheuerbrandt and Bloch 1962; Taylor and Parkes 1983; Edlung *et al.* 1985; Parkes and Calder 1985; Dowling *et al.* 1986). This indicates growth of aerobic or facultative aerobic organisms.

^{13}C-benzene-amended Bactraps showed significant incorporation of the ^{13}C-label into all identified fatty acids for both incubation versions. This undoubtedly verifies the *in situ* biodegradation of the ^{13}C-labelled compound. Natural isotope ratios of carbon were reported for non-labelled microbial fatty acids in aquifer microcosms (-28.9 ± 2.8‰) (Pelz *et al.* 2001b), for fatty acids produced during growth on a range of different non-labelled carbon sources (Abraham *et al.* 1998), and for growth on benzene (with a natural $^{13}C/^{12}C$-ratio of -26.6 ± 0.2‰) (Mancini *et al.* 2003). Positive $\delta^{13}C$ values in microbial fatty acids were detectable only if assimilation of labelled carbon occurred from a source artificially enriched in ^{13}C.

Within Bactraps incubated in groundwater well GW3, the carbon enrichment ranged from +8200 to +199500‰ (9-72 atom%); within Bactraps incubated in the aquifer-sediment enrichment ranged from +450 to +159000‰ (1.7 to 67 atom%). Such a high label can only be explained by intensive growth of ^{13}C-benzene assimilating bacteria on the Bactraps. A control experiment (data not shown) with Bactraps incubated in the anoxic zone (19 m below TGS) of a monitoring well not influenced by air-sparging yielded fatty acid patterns with carbon isotope signatures of at most +56‰, mainly fatty acid isotope signatures were close to natural ^{13}C-abundance (-30‰). Metabolisation of benzene with sulphate, as main electron acceptor at the test site Zeitz, was shown to be very slow and resulted in

low growth yields (Vogt *et al.* 2007; Herrmann *et al.* 2008). Stelzer *et al.* (2006) investigated fatty acid isotope patterns by means of comparable Bactrap-experiments at a groundwater well of the test field Zeitz under natural attenuation conditions. The well showed similar geochemical conditions to GW3, but was located outside of the contamination centre, within the contamination plume. The authors incubated $^{13}C_6$-benzene amended Bactraps within different depths of the well ranging from 9.40 m to 16.70 m. Bactraps incubated in regions close to water table (9.05 m) showed highest isotope signatures within extracted fatty acids (up to +468‰). The authors suggested a more intensive transformation of ^{13}C-benzene within those regions due to thermodynamically more favourable electron acceptors, *e.g.* nitrate, manganese, iron or even oxygen, conceivably stemming from the gaseous phase of the capillary fringe. Within deeper zones (4 m below water table), that were stated as strictly anoxic, isotope signatures with highest values of +174‰ were detected. Geyer *et al.* (2005), who developed the Bactrap-approach with Bio-Sep® beads and isotopic analysis of fatty acids of attached microorganisms, performed the first Bactrap-experiment also at the test site Zeitz within a well close to the one of Stelzer *et al.* (2006) within a depth of about 4 m below the groundwater table. Extracted fatty acids showed isotope signatures up to +13400‰.

Highest isotope signatures measured within the present study were 15 fold to 425 fold higher for Bactraps incubated in GW3 and 12 fold to 340 fold higher for Bactraps incubated in the aquifer-sediment compared to those detected under non-engineered conditions within the studies of Geyer *et al.* (2005) and Stelzer *et al.* (2006), respectively. Thus, organisms analysed in the present study degraded ^{13}C-benzene much more effectively than those examined by Geyer *et al.* (2005) and Stelzer *et al.* (2006). This is strongly indicative of aerobic conditions.

Both incubation versions showed a very diverse extent of ^{13}C-labelling within different fatty acids of the same sample. These observations were also made by Geyer *et al.* (2005) and Stelzer *et al.* (2006) who even detected fatty acids of $^{13}C_6$-amended Bactraps with signatures close to natural ^{13}C-abundance. They explained the variability of ^{13}C-label within different fatty acids by the presence of a mixed microbial community, where only some members assimilated $^{13}C_6$-benzene. Fatty acids with low or no isotopic label indicated organisms which used preferentially other carbon sources like non-labelled benzene (background concentration), other organic substrates from the aquifer, or CO_2. Since only isotope ratios higher than +450‰ were detected within fatty acids of the present study, it is likely that all microorganisms, which were attached to the beads, transformed benzene at least to a small extent or used metabolites or products formed during aerobic ^{13}C-benzene degradation.

Bactraps incubated within the aquifer-sediment had generally lower amounts as well as a lower diversity of fatty acids compared to GW3-Bactraps (Table 6.8) which is certainly due to a lower amount of attached microorganisms. The higher variability of ^{13}C-labelling might result from a lower abundance of $^{13}C_6$-benzene-metabolising organisms within the attached microbial community and, vice versa, a higher abundance of organisms preferentially using other carbon sources than the $^{13}C_6$-labelled benzene. The variability of fatty acid ^{13}C-label within sediment-Bactraps of parallel-

experiments might be caused by a spatially limited growth of $^{13}C_6$-benzene-metabolising bacteria: maybe, beads were only partially accessible for oxygen, and benzene was only degraded at 'hot spots'. This could be an explanation for the observation that only fatty acids of $^{13}C_6$-benzene loaded Bactrap 'B' were highly ^{13}C-enriched (Table 6.7). A restricted oxygen supply had been expected, since uniform aeration of the aquifer is unlikely due to its heterogeneity. It is likely that only zones with a high hydraulic permeability were aerated continuously. Presumably, sediment material surrounding the lance was compressed due to the piling process which lowered the oxygen supply of the Bactraps. Accordingly, lance holes, as well as spaces around Bactraps, were filled with aquifer-sediment after recovery; even Bio-Sep® beads of Bactraps were mixed with aquifer material. These conditions could roughly reflect aquifer *in situ* conditions.

The amount of bioavailable oxygen is unknown, since it is not clear what oxygen concentrations are necessary to cause a fatty acid ^{13}C-labelling to the extent that was observed. It is possible that hypoxic conditions (DO < 2 mg L^{-1}) or a frequent supply with low amounts of oxygen may also cause such a labelling. On the one hand, there are several reports on the requirement for a minimum of dissolved oxygen in the range of 1.0 to 1.5 mg L^{-1} to initiate biodegradation of monoaromatic hydrocarbons and in particular benzene, which has been shown to be the most recalcitrant BTEX compound at low DO concentrations (Chiang *et al.* 1989; Wilson and Bouwer 1997). Chiang *et al.* (1989) reported little or no biodegradation of hydrocarbons with DO concentrations below 0.5 mg L^{-1}. Olsen *et al.* (1995) showed that eight different bacterial strains isolated for growth on benzene under aerobic conditions were unable to degrade benzene under oxygen limited conditions. On the other hand, investigations of Yerushalmi *et al.* (2001) demonstrated that traces of dissolved oxygen (0.05 mg L^{-1}) enabled benzene degradation and formation of phenol, catechol and benzoate as intermediates. In later work the authors showed that the mass ratio between the transformed benzene and the consumed oxygen increased with the decrease of initial DO concentration, reaching a value of 2.8:1, considerably higher compared to the theoretical value of 1:3.08, obtained for complete aerobic oxidation of benzene according to equation 6.18 (Wiedemeier *et al.* 1995; Yerushalmi *et al.* 2002)

$$C_6H_6 + 7.5\,O_2 \rightarrow 6\,CO_2 + 3\,H_2O \qquad \text{eq. 6.18}$$

Thus, the present results reveal no direct elucidation on the efficiency of air-sparging regarding the transfer of oxygen from gaseous phase to aqueous phase and allow no direct conclusions with respect to the bioavailability of oxygen. Nevertheless, they reveal at least qualitatively an enhancement of biodegradation by air-sparging compared to natural attenuation conditions.

The concentrations and isotope compositions of individual fatty acids were used to calculate the amount of ^{13}C converted from the contaminant according to Kästner *et al.* (2006). On Bactraps incubated in GW3 about 24.3 µg ^{13}C per g beads was bound in the fatty acid fraction (Table 6.8), thus about 26.3 µg ^{13}C-labelled benzene was converted per g beads, since benzene molecules consist of

carbon to an extent of 92.2%. For Bactraps incubated in the sediment, concentrations were calculated for both ^{13}C-labelled Bactrap parallels separately. On parallel B (with higher ^{13}C-label-incorporation) about 2.1 µg ^{13}C-labelled benzene was found to be converted per g beads, in the case of the parallel A (with lower incorporation) only 0.3 µg ^{13}C-labelled benzene was converted per g beads. Considering that fatty acids account for up to 10% of the total biomass carbon, the overall amount of labelled carbon bound within the biomass is 10 times higher than the amount calculated with fatty acids (Gottschalk 1986). Considering furthermore that approximatly 50% of benzene is dissimilated and 50% is used for biosynthesis of biomass under aerobic conditions as demonstrated for *Pseudomonas aeruginosa* by Kim *et al.* (2003), the total productive metabolisation can be estimated as 530 µg benzene per g beads for Bactraps incubated in GW3, 42 µg benzene per g beads for Bactrap parallel B, and 5 µg benzene per g beads for Bactrap parallel A incubated in the sediment over incubation periods of 60 or 71 days and with bead loadings of 100 mg $^{13}C_6$-benzene per gram Bio-Sep® beads.

6.4.4.4 Conclusion – quantification of benzene degraded by microorganisms on TF2

Results of MPN proved the presence of viable benzene degrading aerobic microorganisms. Isotope fractionation analyses indicated microbial degradation of benzene initialised by monooxygenases or by anaerobic organisms and allowed only an uncertain estimation of the extent of biodegradation. Thereby, these data only give information on biodegradation that has to occur to alter the isotope composition of benzene within the examined monitoring wells. They are not quantitative in terms of the contaminants concentrations.

By means of fatty acid stable isotope labelling in the course of Bactrap-experiments, it was possible to estimate the amount of benzene degraded by microorganisms per gram Bio-Sep® beads. This estimation enables further calculations to quantify the total amount of benzene degraded within TF2 by assuming homogeneous properties concerning oxygen and microorganisms distribution within the entire capture volume CV_{solid}.

Assuming a compactness of packing of 1.7 t m^{-3}, approximately 600 t sediment is captured. Former analysis comparing fatty acids of biomass grown on Bio-Sep® beads and sediment material revealed a 40 times higher cell density on Bio-Sep® beads probably due to the large inner surface (Bombach and Stelzer, unpublished data). Taking this into account, 8 kg, 0.6 kg or 0.08 kg of benzene were degraded by microorganisms within the CV and the period of Bactrap-incubation calculating with results of Bactraps incubated in GW3, or directly in the aquifer-sediment for the Bactrap parallels B and A, respectively. Calculated for 200 days of air-sparging duration, 26 kg, 1.8 kg, or 0.2 kg benzene were degraded, which account for 92% (GW3-Bactrap), 6% (sediment-Bactrap parallel B), or 0.8% (sediment-Bactrap parallel A) of the total amount of benzene (28.8 kg, Table 6.9) removed during the air-sparging trial within the capture volume.

Comparing all results, quantification by means of the Bactrap-experiment with GW3-incubation certainly overestimates the extent of microbial influences on benzene removal. This might be due to a better oxygen supply within monitoring wells compared to actual aquifer conditions.

More reliable are values gained from experiments with sediment-Bactraps. Two different results for parallel-Bactraps additionally support that sediment-Bactraps are more suited for reflecting aquifer conditions, since there must have been parts of the aquifer with higher and parts with lower concentrations of oxygen, carbon sources and/or nutrients due to spatial restrictions. Thus, a value between 0.8 and 6% as an index for the biodegraded percentage of the totally removed benzene should be a good estimation for the examined field under air-sparging conditions. Although these values are also very likely subjected to errors due to aspects which might cause an overestimation, *e.g.* the improvement of microbial growth conditions by Bio-Sep® beads, *i.e.* activated carbon provides both a constant supply and low concentrations of the toxic compound benzene. On the other hand, fatty acid extraction from beads and derivatisation efficiency are aspects which might cause an underestimation of biodegradation. Experiments which quantified the mass ratio of fatty acids revealed that only ~20% are captured with the used methods (Stelzer and Bombach, unpublished data).

6.5 Supporting information

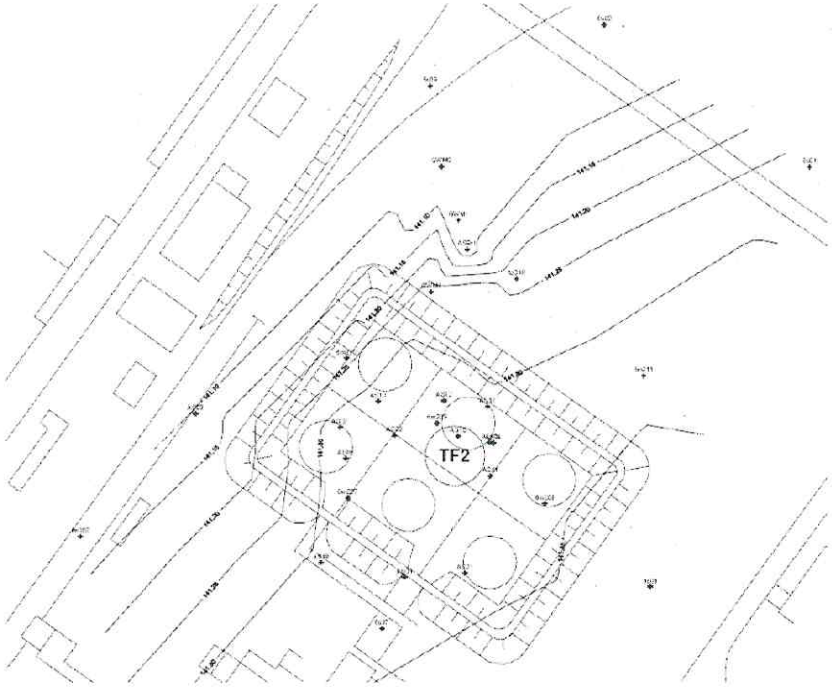


Figure 6.S1: Scheme of hydroisohypses of SB2 of groundwater table measurement campaign on 05/11/2007 (from report "Großmann (26.02.2008)").

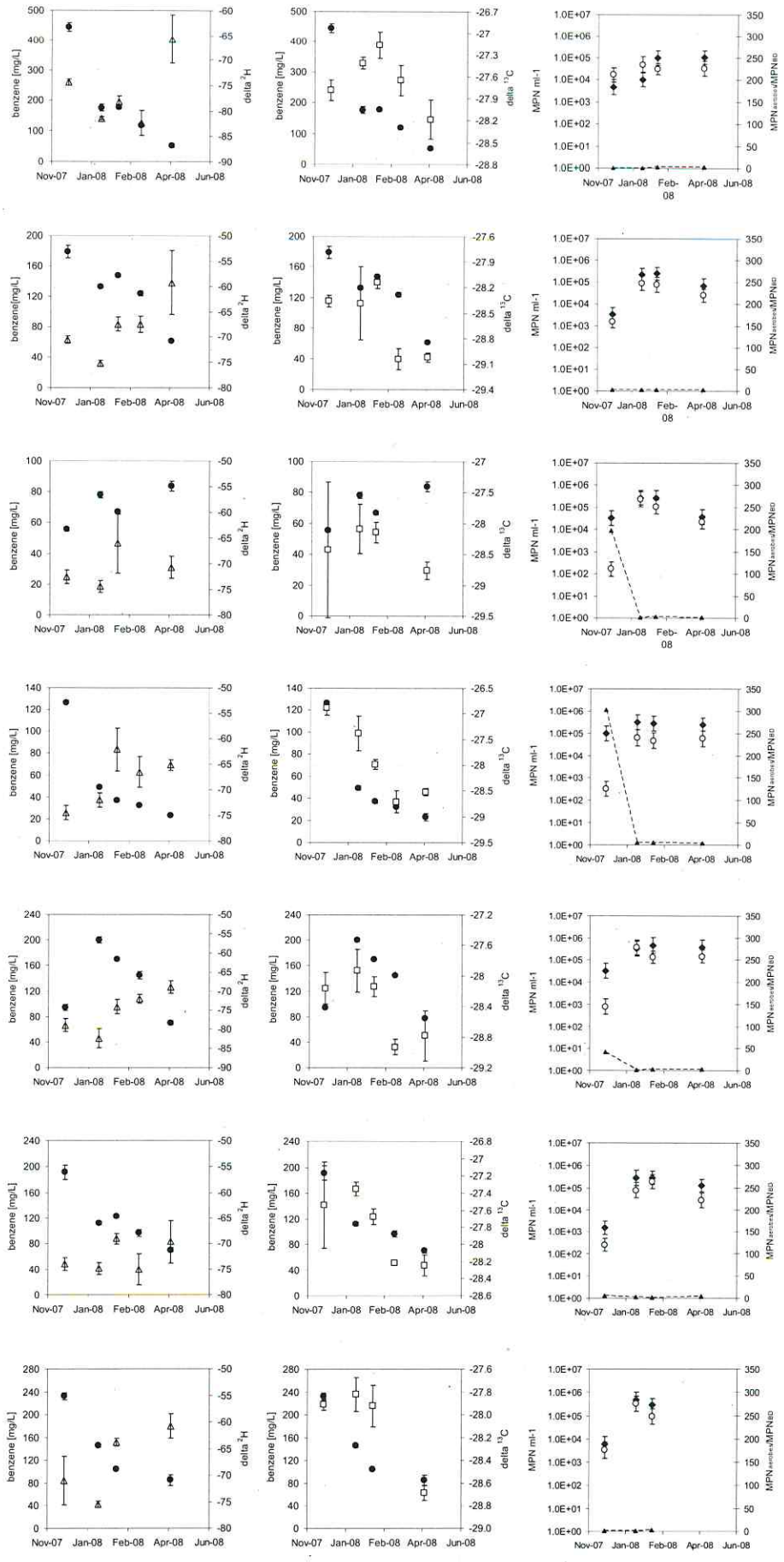

benzene [mg/L]
delta ^{2}H
delta ^{13}C
MPN ml-1
MPN aerobes/MPN ae
Nov-07
Jan-08
Feb-08
Apr-08
Jun-08

Figure 6.S2: isotope ratios for hydrogen (left column) and carbon (middle column) for GW1 – 6 and RP6; right column: MPNs for aerobes (black symbols) and aerobic benzene degraders (BD, achromatic symbols); symbols combined by dashed lines illustrate ratios of aerobes and aerobic benzene degraders (right axis).

Cited reports

Project proposal (23.05.2005): Projektantrag WNTNA02022304 zum BMBF-Vorhaben Bewertung und Optimierung von Quellensanierungs-Ansätzen im Hinblick auf NA und ENA in der Abstromfahne des Standortes Hydrierwerk Zeitz (BEOQUE) – Teilprojekt 1: Sanierungsuntersuchungen und Implementierung sowie Projektmanagement. Vorhabensbeschreibung Projekt ÖGP Zeitz.

Vogt, C., Herrmann, S., Richnow, H.H., Müller, K. (08.02.2007): Kurzbericht – Prototyp einer Lanze zur Versenkung von Bactraps im Aquifersediment.

Großmann, J. (28.02.2007): Zwischenbericht zum Verbundvorhaben BEOQUE – Teilprojekt TP1 Sanierungsuntersuchung, Implementierung und Projektmanagement.

Vogt, C., Herrmann, S., Richnow, H.H. (23.03.2007): Bericht – Allgemeines Konzept für einen Tracerversuch zur Untersuchung der Hydrodynamik auf dem TF2.

Vogt, C., Herrmann, S., Richnow, H.H., Müller, K. (20.07.2007): Bericht – Angepasstes Konzept für einen Tracerversuch zur Untersuchung der Hydrodynamik auf dem TF2.

Großmann, J. (26.02.2008): Zwischenbericht zum Verbundvorhaben BEOQUE – Teilprojekt TP1 Sanierungsuntersuchung, Implementierung und Projektmanagement.

Vogt, C., Herrmann, S., Richnow, H.H. Vortrag zum 14. Arbeitstreffen, Magdeburg (30.05.2008): Ergebnisse der Isotopenuntersuchungen.

Vogt, C., Herrmann, S., Richnow, H.H. Vortrag zum 14. Arbeitstreffen, Magdeburg (30.05.2008): Ergebnisse der MPN-Untersuchungen.

Vogt, C., Herrmann, S., Richnow, H.H. Vortrag zum 14. Arbeitstreffen, Magdeburg (30.05.2008): Bactrap- Untersuchungen, Zielstellung des Bactrap-Experiments – Nachweis der *in situ*-Metabolisierung von Benzol nach erfolgter bzw. vorangeschrittener Aerobisierung.

Großmann, J. (10.09.2008): Zwischenbericht zum Verbundvorhaben BEOQUE – Teilprojekt TP1 Sanierungsuntersuchung, Implementierung und Projektmanagement.

Vogt, C., Herrmann, S., Müller, K., Richnow, H.H. (17.10.2008): Bericht – Ergebnisse des Bactrap-Feldexperiments auf dem TF2.

Müller, K. Vortrag zum 15. Arbeitstreffen, GICON, Dresden (10.12.2008): TP1 Konzeptmodell/Bilanzierung Schadstoffaustrag.

Notes for reviewers

S. Herrmann performed the experimental work of microbiological, isotope geochemical and tracer analyses with support from C. Vogt, S. Hinke, and K. Müller. Data analysis of microbiological and isotope geochemical analysis was performed by S. Herrmann and C. Vogt. K. Müller performed groundwater and soil vapour sampling, gastracer test, analyses of on-site parameters and BTEX concentrations, and data analysis and interpretation of hydrogeochemical parameters and the tracer tests. Cations, anions and TIC were analysed by project-partners of the Christian-Albrechts-University of Kiel. S. Herrmann prepared the manuscript under supervision of C. Vogt and H.H. Richnow.

7

Summary and final conclusions

7.1 Anaerobic benzene degradation

Anaerobic BTEX degradation has been studied since the mid 1980s. For some alkylbenzene compounds, detailed information on the anaerobic degradation pathway has been achieved to date (*e.g.* for toluene) (Heider 2007; Foght 2008). However, information on the mechanisms of the anaerobic degradation of benzene and the organisms that are involved in this process is still very limited (see introduction in **chapter 1**). This is mainly due to the lack of available pure or enrichment cultures that can degrade benzene anaerobically and the slow growth of microorganisms in the available enrichment cultures. The motivation for the studies described in chapter 2 to 6 was to gain more insight into not yet understood aspects of BTEX degradation.

Chapter 2 describes a new method by which a benzene-degrading sulphate-reducing microbial consortium was established in the laboratory. Enrichments were initiated by exposing *in situ*-microcosms filled with different solids (sand, lava, and Amberlite XAD-7) for 67 days in the sulphidic part of a groundwater monitoring well downstream of the source zone of a benzene-contaminated aquifer. These *in situ*-incubated enrichments were further incubated in the laboratory. In control microcosms without filling material, benzene was initially degraded, but the benzene-degrading capacity could not be sustained. These results suggest that it is favourable to use solids for the *in situ* enrichment of anaerobic benzene-degrading bacteria, a strategy that might be generally useful for the cultivation of bacteria considered to be hardly or not cultivable. Subject of future research will be to obtain planktonic cultures which facilitate the isolation of responsible organisms.

Although isolates capable of mineralising benzene under denitrifying and chlorate reducing conditions have been obtained (Coates *et al.* 2001b; Kasai *et al.* 2006; Weelink *et al.* 2008), no pure cultures able to degrade benzene under iron- or sulphate-reducing conditions have been isolated so far. The current understanding of the process of benzene degradation under strictly anoxic conditions is purely based on enriched microbial consortia.

Chapter 3 describes how DNA-based stable isotope probing (SIP) and subsequent terminal restriction fragment length polymorphism (T-RFLP) was used to distinguish the active microorganism(s) in a benzene-degrading sulphidogenic consortium gained from the same BTEX-contaminated aquifer described in Chapter 2. Stable isotope incorporation after consumption of ^{13}C-labelled benzene was detected for two phylotypes within the consortium: a phylotype affiliated to the family *Peptococcaceae* (genus *Cryptanaerobacter/Pelotomaculum*), and a phylotype belonging to the ε-*Proteobacteria*. In contrast, DNA of organisms affiliated to the δ-*Proteobacteria* were not enriched in ^{13}C. These results excluded a single sulphate reducer to be the responsible benzene degrader as observed in other sulphate-reducing benzene-degrading consortia (Phelps *et al.* 1998; Musat and Widdel 2008; Oka *et al.* 2008; Laban *et al.* 2009). Rather, the results supported the assumption of a syntrophy of organisms within the consortium as postulated by Kleinsteuber *et al.* (2008). The *Cryptanaerobacter/Pelotomaculum* phylotype is thought to be a key player within the anaerobic

benzene degradation of this consortium; it is suggested that it initially attacks benzene and ferments it to acetate according to equation 7.1:

$$\text{Benzene} + 6\ H_2O \rightarrow 3\ \text{Acetate} + 3\ H^+ + 3\ H_2 \qquad \Delta G^{0'} = +72.3\ \text{kJ mol}^{-1} \qquad \text{eq. 7.1}$$

The formation of acetate was assumed since small amounts of ^{13}C-labelled methane were produced during benzene mineralization and archaea belonging to the family *Methanosaetaceae* were detected known to use acetate as sole carbon and energy source (Chapter 3). Equation 7.1 is thermodynamically unfeasible under standard conditions but becomes exergonic in the case of low hydrogen and acetate concentrations after consumption by other organisms according to equations 7.2 and 7.3:

$$3\ H_2 + 0.75\ SO_4^{2-} + 1.125\ H^+ \rightarrow 3\ H_2O + 0.375\ HS^- + 0.375\ H_2S \qquad \Delta G^{0'} = -116.3\ \text{kJ mol}^{-1} \qquad \text{eq. 7.2}$$

$$\text{Acetate} + SO_4^{2-} + 0.5\ H^+ \rightarrow 2\ HCO_3^- + 0.5\ HS^- + 0.5\ H_2S \qquad \Delta G^{0'} = -47\ \text{kJ mol}^{-1} \qquad \text{eq. 7.3}$$

Since DNA of several sulphate reducers belonging to the δ-*Proteobacteria* showed no ^{13}C-incorporation these organisms probably mainly use H_2 as the electron donor. The role of the ε-proteobacterium is not clear. Due to the ^{13}C-incorporation it possibly uses acetate or even benzene as carbon source. Subject of future studies will be to elucidate which specific roles the consortial members execute.

7.2 Elucidating degradation pathways by means of compound specific isotope analysis

In the last years compound specific stable isotope analysis has become a valuable tool for the assessment of biodegradation of groundwater contaminants (Meckenstock *et al.* 2004a; Schmidt *et al.* 2004; Elsner *et al.* 2005). By combined laboratory and field studies, *in situ* biodegradation of several contaminants was demonstrated qualitatively and in some cases also quantitatively. Furthermore, two-dimensional isotope fractionation factors can be related to the initial reaction of a respective biochemical degradation pathway.

Chapter 4 describes how carbon and hydrogen isotope effects were combined by a two-dimensional approach (2D-CSIA) during aerobic and anaerobic benzene degradation in enrichment and pure cultures in order to decipher the initial reaction of the respective biodegradation pathway. Correlations of hydrogen and carbon isotope effects either derived from the slope of hydrogen vs. carbon discriminations or by calculating the ratio of hydrogen to carbon enrichment factors ($\Lambda = \Delta_H/\Delta_C \approx \varepsilon_H/\varepsilon_C$) yielded values that were unique for distinct benzene degradation pathways. Specific Λ values were < 2 for dihydroxylation and between 3 and 11 for monohydroxylation as initial benzene-attacking mechanism. Under anaerobic conditions Λ values were about 16 for degradation under nitrate-reducing conditions and > 22 for degradation under sulphate-reducing and methanogenic conditions. The high variability in Λ values for monohydroxylation as well as for anaerobic benzene

degradation suggests that in both cases more than a single reaction mechanism for benzene activation exists. Future tasks will be to elucidate the unknown pathways under different redox conditions and to find out what causes the variability of isotope effects.

Vogt *et al.* (2008) studied aerobic and anaerobic degradation pathways for toluene by means of 2D-CSIA, which resulted also in Λ value variations for different initial toluene-attacking enzymatic steps. Monohydroxylation of the methyl group resulted in high Λ values ($\Lambda = 53 \pm 5$) and assumed ring-dihydroxylation in low values of $\Lambda = 2 \pm 2$. Λ values for organisms attacking toluene under anoxic conditions were significantly different and ranged from $\Lambda = 4 \pm 3$ for a phototrophic organism to $\Lambda = 11 - 14$ for nitrate-reducing organisms and $\Lambda = 28 - 31$ for sulphate-reducing organisms, although all organisms activated toluene by the same mechanism, *i.e.* fumarate addition to the methyl moiety catalysed by benzylsuccinate synthase (Bss). This indicates that the reaction mechanism of Bss is slightly different in these organisms.

In order to obtain information on the Bss reaction of xylene-degrading bacteria, associated isotope effects were analysed by 2D-CSIA which is described in **chapter 5.** Different pure and mixed cultures able to degrade *m*-xylene, *o*-xylene, or *p*-xylene under sulphate-reducing or nitrate-reducing conditions were investigated. Based on metabolite studies and literature data, all examined cultures activated the xylene isomers by the addition of fumarate to a methyl moiety. Biodegradation of xylene isomers was generally coupled to a significant carbon and hydrogen isotope fractionation. Λ_{bulk} values differed significantly for the analysed cultures and ranged between 12 ± 4 and 29 ± 5. These results supported the assumption of Vogt *et al.* (2008) that slightly different mechanisms exist for the benzlysuccinate synthase catalysed reactions. This might be due to slightly different benzyl succinate synthases as indicated by different Bss encoding gene sequences reported by Winderl *et al.* (2007) and broadened by this thesis. Another possible reason might be a variable contribution of different isotope sensitive reaction steps within the overall compound activating reaction sequence. Recently, it was shown that the reaction mechanism of Bss consists of several single steps including different C – H and C – C bond changes (Leuthner *et al.* 1998; Krieger *et al.* 2001; Himo 2002; Li and Marsh 2006b). It was postulated that hydrogen abstraction from the methyl moiety of a benzyl radical intermediate is an isotope sensitive step within the reaction sequence, although the reaction is not fully rate-determining. A second primary isotope sensitive step might be the formation of the carbon–carbon bond during benzylsuccinyl radical formation, which is thought to be an irreversible and rate-limiting step. It remains to be elucidated whether the hypothetic transition states of the complex Bss reaction mechanism affect isotope fractionation.

7.3 Evaluation of air-sparging as a strategy to enhance natural attenuation

In **chapter 6**, an air-sparging trial located at the source zone of the contaminated aquifer close to Zeitz is described. The air-sparging was monitored to demonstrate its efficiency to enhance natural attenuation concerning the removal of the main contaminant benzene and also to detect the part microorganisms have in this process. Monitoring of removed benzene concentrations revealed that air-sparging is not sufficient under the given hydrogeochemical conditions of the aquifer. Aeration of groundwater was limited due to several reasons such as preferential flow of injected air along impermeable strata or geochemical oxygen sinks (*e.g.* reduced iron and sulphur species). Additionally, groundwater flow velocities were very low (a few cm d^{-1}) preventing an effective transport of potentially dissolved oxygen. Presumably, only a small area around the air-injection well was affected by air-sparging. Calculations revealed that at least another period of seven years would be necessary to remove benzene from the affected area sustainably. Several methods were used for monitoring biodegradation. MPN and isotope fractionation analyses gave evidence for microbial degradation, but allowed no quantification. By the use of Bactraps, it was possible to calculate the extent of biodegradation, though with large uncertainty. Bactraps or bio-traps are small beads of activated carbon (Bio-Sep® beads) which can be amended with $^{13}C_6$-labelled hydrocarbons and exposed to groundwater monitoring wells (White *et al.* 2003; Peacock *et al.* 2004; Geyer *et al.* 2005; Kästner *et al.* 2006; Stelzer *et al.* 2006). By detecting ^{13}C-label incorporation into fatty acids of microorganisms grown on Bio-Sep® beads, *in situ* biodegradation of the hydrocarbon is proved. During the air-sparging period, Bactraps loaded with $^{13}C_6$-labelled benzene were exposed either to a monitoring well or directly to the aquifer-sediment by direct-push technique. ^{13}C-label incorporation was detected in all analysed fatty acids. Calculations using results of the sediment-Bactrap-experiment indicated that 0.8 to 6% of the overall removed benzene was biodegraded.

References

Abe, Y., Aravena, R., Zopfi, J., Shouakar-Stash, O., Cox, E., Roberts, J. D. and Hunkeler, D. (2009): Carbon and chlorine isotope fractionation during aerobic oxidation and reductive dechlorination of vinyl chloride and *cis*-1,2-dichloroethene. Environ Sci Technol. 43: 101-107.

Abraham, W. R., Hesse, C. and Pelz, O. (1998): Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Appl Environ Microbiol. 64: 4202-4209.

Acton, D. W. and Barker, J. F. (1992): *In situ* biodegradation potential of aromatic hydrocarbons in anaerobic groundwaters. J Contam Hydrol. 9: 325-352.

Aggarwal, P. K. and Hinchee, R. E. (1991): Monitoring *in situ* biodegradation of hydrocarbons by using stable carbon isotopes. Environ Sci Technol. 25: 1178-1180.

Ahad, J. M. E., Lollar, B. S., Edwards, E. A., Slater, G. F. and Sleep, B. E. (2000): Carbon isotope fractionation during anaerobic biodegradation of toluene: Implications for intrinsic bioremediation. Environ Sci Technol. 34: 892-896.

Aksoy, M. (1985): Benzene as a leukemogenic and carcinogenic agent. Am J Ind Med. 8: 9-20.

Alfreider, A., Krössbacher, M. and Psenner, R. (1997): Groundwater samples do not reflect bacterial densities and activity in subsurface systems. Water Res. 31: 832-840.

Alfreider, A. and Vogt, C. (2007): Bacterial diversity and aerobic biodegradation potential in a BTEX contaminated aquifer. Water Air Soil Poll. 183: 415-426.

Alfreider, A., Vogt, C. and Babel, W. (2003): Expression of chlorocatechol 1,2-dioxygenase and chlorocatechol 2,3-dioxygenase genes in chlorobenzene-contaminated subsurface samples. Appl Environ Microbiol. 69: 1372-1376.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990): Basic Local Alignment Search Tool. J Mol Biol. 215: 403-410.

Alvarez, P. J. J. and Vogel, T. M. (1995): Degradation of BTEX and their aerobic metabolites by indigenous microorganisms under nitrate reducing conditions. Water Sci Technol. 31: 15-28.

Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995): Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev. 59: 143-169.

Anderson, R. T. and Lovley, D. R. (2000): Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer. Environ Sci Technol. 34: 2261-2266.

Angerer, J. (1983): Prävention beruflich bedingter Gesundheitsschäden durch Benzol, Toluol, Xylol und Ethylbenzol. Springer-VDI-Verlag. 168.

Arneth, J. D., Milde, G., Kerndorff, H. and Schleyer, R. (1989): Waste deposit influences on groundwater quality as tool for waster type and site selection for final storage quality. In: The Landfill, Lecture Notes in Earth Science. P. Baccini (eds). Springer-Verlag.Berlin. 399-415.

Aronson, D. and Howard, P. H. (1997). Anaerobic biodegradation of organic chemicals in groundwater: A summary of field and laboratory studies, Environmental Science Center.

Atlas, R. M. (1981): Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Mol Biol Rev. 45: 180-209.

Atteia, O. and Franceschi, M. (2002): Kinetics of natural attenuation of BTEX: review of the critical chemical conditions and measurements at bore scale. Sci World J. 2: 1338-1346.

Baedecker, M. J., Cozzarelli, I. M., Eganhouse, R. P., Siegei, D. I., Bennett, P. C. and Applied Geochemistry. Vol., n., pp. (1993): Crude oil in a shallow sand gravel aquifer. III. Biogeochemical reactions and mass balance modeling in anoxic groundwater. Appl Geochem. 8: 569-586.

Baertschi, P. and Kuhn, W. (1957): Dampfdruckunterschiede isotoper Verbindungen. Helv Chim Acta. 40: 1084-1103.

Baertschi, P., Kuhn, W. and Kuhn, H. (1953): Fractionation of isotopes by distillation of some organic substances. Nature. 171: 1018-1020.

Bagneris, C., Cammack, R. and Mason, J. R. (2005): Subtle difference between benzene and toluene dioxygenases of *Pseudomonas putida*. Appl Environ Microbiol. 71: 1570-1580.

Baldwin, B. R., Nakatsu, C. H. and Nies, L. (2003): Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl Environ Microbiol. 69: 3350-3358.

Ball, H. A., Johnson, H. A., Reinhard, M. and Spormann, A. M. (1996): Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J Bacteriol. 178: 5755-5761.

Ball, H. A. and Reinhard, M. (1996): Monoaromatic hydrocarbon transformation under anaerobic conditions at Seal Beach, California: Laboratory studies. Environ Toxicol Chem. 15: 114-122.

Barbaro, J. R., Barker, J. F., Lemon, L. A. and Mayfield, C. I. (1992): Biotransformation of BTEX under anaerobic, denitrifying conditions: field and laboratory observations. J Contam Hydrol. 11: 245-272.

Barker, J. F., Tessmann, J. S., Plotz, P. E. and Reinhard, M. (1986): The organic geochemistry of a sanitary landfill leachate plume. J Contam Hydrol. 1: 171-189.

Bass, D. H., Hastings, N. A. and Brown, R. A. (2000): Performance of air sparging systems: a review of case studies. J Hazard Mater. 72: 101-119.

Bassam, B. J., Caetano-Anolles, G. and Gresshoff, P. M. (1991): Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem. 196: 80-83.

Battersby, N. S. (1997): The ISO headspace CO_2 biodegradation test 1. Chemosphere. 34: 1813-1822.

Beller, H. R. (2000): Metabolic indicators for detecting *in situ* anaerobic alkylbenzene degradation. Biodegradation. 11: 125-139.

Beller, H. R., Ding, W.-H. and Reinhard, M. (1995): Byproducts of anaerobic alkylbenzene metabolism useful as indicators of *in situ* bioremediation. Environ Sci Technol. 29: 2864-2870.

Beller, H. R. and Reinhard, M. (1995): The role of iron in enhancing anaerobic toluene degradation in sulfate-reducing enrichment cultures. Microb Ecol. 30: 105-114.

Beller, H. R. and Spormann, A. M. (1998): Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. J Bacteriol. 180: 5454-5457.

Beller, H. R., Spormann, A. M., Sharma, P. K., Cole, J. R. and Reinhard, M. (1996): Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl Environ Microbiol. 62: 1188-1196.

Beller, H. R., Spormann, A. M. (1997): Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J Bacteriol. 179: 670-676.

Benndorf, D., Balcke, G. U., Harms, H. and von Bergen, M. (2007): Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. Isme J. 1: 224-234.

Bennett, P. C., Hiebert, F. K. and Rogers, J. R. (2000): Microbial control of mineral–groundwater equilibria: Macroscale to microscale. Hydrogeol J. 8: 47-62.

Bhupathiraju, V. K., Hernandez, M., Krauter, P. and Alvarez-Cohen, L. (1999): A new direct microscopy based method for evaluating *in-situ* bioremediation. J Hazard Mater. 67: 299-312.

Blair, N., Leu, A., Muñoz, E., Olsen, J., Kwong, E. and Des Marais, D. (1985): Carbon isotopic fractionation in heterotrophic microbial metabolism. Appl Environ Microbiol. 50: 996-1001.

Bligh, E. G. and Dyer, W. J. (1959): A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 37: 911-917.

Börjesson, G., Sundh, I., Tunlid, A. and Svensson, B. H. (1998): Methane oxidation in landfill cover soils, as revealed by potential oxidation measurements and phospholipid fatty acid analyses. Soil Biol Biochem. 30: 1423-1433.

Boschker, H. T. S. (2004): Linking microbial community structure and functioning: stable isotope (^{13}C) labeling in combination with PLFA analysis. In: Molecular Microbial Ecology Manual II. G. A. Kowalchuk, F. J. de Bruijn, I. M. Head, A. D. Akkermans and J. D. van Elsas (eds). Kluwer Academic Publishers.Dordrecht, The Netherlands. 1673-1688.

Boschker, H. T. S. and Middelburg, J. J. (2002): Stable isotopes and biomarkers in microbial ecology. FEMS Microbiol Ecol. 40: 85-95.

Boschker, H. T. S., Nold, S. C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R., Parkes, R. J. and Cappenberg, T. E. (1998): Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. Nature. 392: 801-805.

Bossio, D. A. and Scow, K. M. (1998): Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microb Ecol. 35: 265-278.

Botton, S. and Parsons, J. R. (2006): Degradation of BTEX compounds under iron-reducing conditions in contaminated aquifer microcosms. Environ Toxicol Chem. 25: 2630-2638.

Botton, S. and Parsons, J. R. (2007): Degradation of BTX by dissimilatory iron-reducing cultures. Biodegradation. 18: 371-381.

Botton, S., van Harmelen, M., Braster, M., Parsons, J. R. and Roling, W. F. M. (2007): Dominance of *Geobacteraceae* in BTX-degrading enrichments from an iron-reducing aquifer. FEMS Microbiol Ecol. 62: 118-130.

Bouchard, D., Höhener, P. and Hunkeler, D. (2008): Carbon isotope fractionation during volatilization of petroleum hydrocarbons and diffusion across a porous medium: a column experiment. Environ Sci Technol. 42: 7801-7806.

Bowman, J. P., Sly, L. I., Nichols, P. D. and Hayward, A. C. (1993): Revised taxonomy of the methanotrophs: Description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. Int J Syst Bacteriol. 43: 735-753.

Burlage, R. S., Hooper, S. W. and Sayler, G. S. (1989): The TOL (pWW0) catabolic plasmid. Appl Environ Microbiol. 55: 1323-1328.

Burland, S. M. and Edwards, E. A. (1999): Anaerobic benzene biodegradation linked to nitrate reduction. Appl Environ Microbiol. 65: 529-533.

Caldwell, M. E. and Suflita, J. M. (2000): Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. Environ Sci Technol. 34: 1216-1220.

Campbell, B. J., Engel, A. S., Porter, M. L. and Takai, K. (2006): The versatile epsilon-proteobacteria: key players in sulphidic habitats. Nature Rev Microbiol. 4: 458-468.

Cavalca, L., Dell'Amico, E. and Andreoni, V. (2004): Intrinsic bioremediability of an aromatic hydrocarbon-polluted groundwater: diversity of bacterial population and toluene monoxygenase genes. Appl Microbiol Biotechnol. 64: 576-587.

Chakraborty, R. and Coates, J. D. (2004): Anaerobic degradation of monoaromatic hydrocarbons. Appl Microbiol Biotechnol. 64: 437-446.

Chakraborty, R. and Coates, J. D. (2005): Hydroxylation and carboxylation-two crucial steps of anaerobic benzene degradation by *Dechloromonas* strain RCB. Appl Environ Microbiol. 71: 5427-5432.

Chakraborty, R., O'Connor, S. M., Chan, E. and Coates, J. D. (2005): Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. Appl Environ Microbiol. 71: 8649-8655.

Chiang, C. Y., Salanitro, J. P., Chai, E. Y., Colthart, J. D. and Klein, C. L. (1989): Aerobic biodegradation of benzene, toluene, and xylene in a sandy aquifer - data analysis and computer modeling. Ground Water. 27: 823-834.

Christensen, T. H., Bjerga, P. L., Banwartb, S. A., Jakobsenc, R., Herona, G. and Albrechtsena, H. J. (2000): Characterization of redox conditions in groundwater contaminant plumes. J Contam Hydrol. 45: 165-241.

Clark, I. D. and Fritz, P. (1997): Environmental isotopes in hydrology. Lewis Publishers. New York. 328 p.

Cline, J. D. (1969): Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol Oceanogr. 14: 454-458.

Coates, J. D., Anderson, R. T., Woodward, J. C., Phillips, E. J. P. and Lovley, D. R. (1996): Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions. Environ Sci Technol. 30: 2784-2789.

Coates, J. D., Bhupathiraju, V. K., Achenbach, L. A., McLnerney, M. J. and Lovley, D. R. (2001a): *Geobacter hydrogenophilus*, *Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. Int J Syst Evol Microbiol. 51: 581-588.

Coates, J. D., Chakraborty, R., Lack, J. G., O'Connor, S. M., Cole, K. A., Bender, K. S. and Achenbach, L. A. (2001b): Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. Nature. 411: 1039-1043.

Coates, J. D., Chakraborty, R. and McInerney, M. J. (2002): Anaerobic benzene biodegradation-a new era. Res Microbiol. 153: 621-628.

Coates, J. D. and Lovley, D. R. (2003): Genus: *Geobacter*. In: Bergey`s manual of systematic bacteriology. D. Brenner, N. Krieg, J. Staley and G. Garrity (eds). Springer-Verlag. New York, Berlin, Heidelberg.

Cole, J. R., Cascarelli, A. L., Mohn, W. W. and Tiedje, J. M. (1994): Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl Environ Microbiol. 60: 3536-3542.

Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Bandela, A. M., Cardenas, E., Garrity, G. M. and Tiedje, J. M. (2007): The Ribosomal Database Project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res. 35: D169-D172.

Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam, S. A., McGarrell, D. M., Garrity, G. M. and Tiedje, J. M. (2005): The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 33: D294-D296.

Collier, J. L. and Campbell, L. (1999): Flow cytometry in molecular aquatic ecology. Hydrobiologia. 401: 34-54.

Connon, S. A., Tovanabootr, A., Dolan, M., Vergin, K., Giovannoni, S. J. and Semprini, L. (2005): Bacterial community composition determined by culture-independent and -dependent methods during propane-stimulated bioremediation in trichloroethene-contaminated groundwater. Environ Microbiol. 7: 165-178.

Conrad, M. E., Daley, P. F., Fischer, M. L., Buchanan, B. B., Leighton, T. and Kashgarian, M. (1997): Combined ^{14}C and $\delta^{13}C$ monitoring of *in situ* biodegradation of petroleum hydrocarbons. Environ Sci Technol. 31: 1463-1469.

Coplen, T. B., Brand, W. A., Gehre, M., Groning, M., Meijer, H. A., Toman, B. and Verkouteren, R. M. (2006): After two decades a second anchor for the VPDB delta^{13}C scale. Rapid Commun Mass Spectrom. 20: 3165-3166.

Cordruwisch, R., Seitz, H. J. and Conrad, R. (1988): The capacity of hydrogenotrophic anaerobic-bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron-acceptor. Arch Microbiol. 149: 350-357.

Cozzarelli, I. M. and Weiss, J. V. (2007): Biogeochemistry of aquifer systems. ASM Press. Washington, DC. 843-859.

Cross, K. M., Biggar, K. W., Semple, K., Foght, J., S.E., G. and Armstrong, J. E. (2006): Intrinsic bioremediation of diesel-contaminated cold groundwater in bedrock. J Environ Eng Sci. 5: 13-27.

Cunningham, J. A., Rahme, H., Hopkins, G. D., Lebron, C. and Reinhard, M. (2001): Enhanced *in situ* bioremediation of BTEX-contaminated groundwater by combined injection of nitrate and sulfate. Environ Sci Technol. 35: 1663-1670.

Da Silva, M. L. and Alvarez, P. J. (2004): Enhanced anaerobic biodegradation of benzene-toluene-ethylbenzene-xylene-ethanol mixtures in bioaugmented aquifer columns. Appl Environ Microbiol. 70: 4720-4726.

Dagley, S. (1981): New perspectives in aromatic catabolism. In: Microbial degradation of xenobiotics and recalcitrant compounds. T. Leisinger, A. M. Cook, R. Hütter and J. Nüesch (eds). Academic Press.New York. 181-186.

Dagley, S. (1986): Biochemistry of aromatic hydrocarbon degradation in *Pseudomonads*. In: The Bacteria. J. R. Sokatch (eds). New York: Academic Press. 527-555.

Davey, J. F. and Gibson, D. T. (1974): Bacterial metabolism of *para*- and *meta*-xylene: oxidation of a methyl substituent. J Bacteriol. 119: 923-929.

de Lipthay, J. R., Johnsen, K., Albrechtsen, H. J., Rosenberg, P. and Aamand, J. (2004): Bacterial diversity and community structure of a sub-surface aquifer exposed to realistic low herbicide concentrations. FEMS Microbiol Ecol. 49: 59-69.

de Lipthay, J. R., Tuxen, N., Johnsen, K., Hansen, L. H., Albrechtsen, H.-J., Bjerg, P. L. and Aamand, J. (2003): *In Situ* exposure to low herbicide concentrations affects microbial population composition and catabolic gene frequency in an aerobic shallow aquifer. Appl Environ Microbiol. 69: 461-467.

Dean, B. J. (1978): Genetic toxicology of benzene, toluene, xylenes and phenols. Mutat Res. 47: 75-97.

Deines, P. (1980): The isotopic composition of reduced organic carbon. Elsevier Sci. Publ. Netherlands. pp. 326-406.

Delong, E. F. (1992): Archaea in coastal marine environments. Proc Natl Acad Sci U S A. 89: 5685-5689.

Dolfing, J., Larter, S. R. and Head, I. M. (2008): Thermodynamic constraints on methanogenic crude oil biodegradation. ISME J. 2: 442-452.

Dolfing, J., Zeyer, J., Binder-Eicher, P. and Schwarzenbach, R. P. (1990): Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch Microbiol. 154: 336-341.

Dou, J., Liu, X., Hu, Z. and Deng, D. (2008): Anaerobic BTEX biodegradation linked to nitrate and sulfate reduction. J Hazard Mater. 151: 720-729.

Dowling, N. J. E., Widdel, F. and White, D. C. (1986): Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulphate-reducers and other sulphide-forming bacteria. J Gen Microbiol. 132: 1815-1825.

Edlung, A., Nichols, P. D., Roffey, R. and White, D. C. (1985): Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. J Lipid Res. 26: 982-988.

Edwards, E. A. and Grbic-Galic, D. (1992): Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl Environ Microbiol. 58: 2663-2666.

Edwards, E. A. and Grbic-Galic, D. (1994): Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl Environ Microbiol. 60: 313-322.

Eganhouse, R. P., Cozzarelli, I. M., Scholl, M. A. and Matthews, L. L. (2001): Natural attenuation of volatile organic compounds (VOCs) in the leachate plume of a municipal landfill: Using alkylbenzenes as process probes. Ground Water. 39: 192-202.

Elshahed, M. S., Bhupathiraju, V. K., Wofford, N. Q., Nanny, M. A. and McInerney, M. J. (2001a): Metabolism of benzoate, cyclohex-1-ene carboxylate, and cyclohexane carboxylate by "*Syntrophus aciditrophicus*" strain SB in syntrophic association with H_2-using microorganisms. Appl Environ Microbiol. 67: 1728-1738.

Elshahed, M. S., Gieg, L. M., McInerney, M. J. and Suflita, J. M. (2001b): Signature metabolites attesting to the *in situ* attenuation of alkylbenzenes in anaerobic environments. Environ Sci Technol. 35: 682-689.

Elsner, M., McKelvie, J., Couloume, G. L. and Sherwood-Lollar, B. (2007): Insight into methyl tert-butyl ether (MTBE) stable isotope fractionation from abiotic reference experiments. Environ Sci Technol. 41: 5693-5700.

Elsner, M., Zwank, L., Hunkeler, D. and Schwarzenbach, R. P. (2005): A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. Environ Sci Technol. 39: 6896-6916.

Evans, P. J., Mang, D. T., Kim, K. S. and Young, L. Y. (1991a): Anaerobic degradation of toluene by a denitrifying bacterium. Appl Environ Microbiol. 57: 1139-1145.

Evans, P. J., Mang, D. T. and Young, L. Y. (1991b): Degradation of toluene and *m*-xylene and transformation of *o*-xylene by denitrifying enrichment cultures. Appl Environ Microbiol. 57: 450-454.

Federle, T. W. (1986): Microbial distribution in soil-new techniques. In: Perspectives in microbial ecology. F. Megusar and M. Gantar (eds). Slovene Society for Microbiology.Ljubljana, Yugoslavia. 493-498.

Field, J. A. (2002): Limits of anaerobic biodegradation. Water Sci Technol. 45: 9-18.

Findlay, R. H. (2004): Determination of microbial community structure using phospholipid fatty acid profiles. In: Molecular microbiology manual. G. A. Kowalchuk, F. J. d. Bruijn, I. M. Head, A. D. L. Akkermans and J. D. v. Elsas (eds). Kluwer Academic Publishers.Dordrecht, The Netherlands. 983-1004.

Findlay, R. H., Trexler, M. B. and White, D. C. (1990): Response of a benthic microbial community to biotic disturbance. Mar Ecol Prog Ser. 62: 135-148.

Fiorenza, S. and Ward, C. H. (1997): Microbial adaptation to hydrogen peroxide and biodegradation of aromatic hydrocarbons. J Ind Microbiol Biotechnol. 18: 140-151.

Fischer, A. (2004): Charakterisierung der Quellen und Senken von Methan in einem BTEX-kontaminierten Grundwasserleiter. Fakultät für Geowissenschaften, Geotechnik und Bergbau. TU-Bergakademie Freiberg. Freiberg, Leipzig. Diplomathesis: 87 p.

Fischer, A., Bauer, J., Meckenstock, R. U., Stichler, W., Griebler, C., Maloszewski, P., Kästner, M. and Richnow, H. H. (2006): A multitracer test proving the reliability of Rayleigh equation-based approach for assessing biodegradation in a BTEX contaminated aquifer. Environ Sci Technol. 40: 4245-4252.

Fischer, A., Herklotz, I., Herrmann, S., Thullner, M., Weelink, S. A. B., Stams, A. J. M., Schlömann, M., Richnow, H. H. and Vogt, C. (2008): Combined carbon and hydrogen isotope fractionation investigations for elucidating benzene biodegradation pathways. Environ Sci Technol. 42: 4356-4363.

Fischer, A., Theuerkorn, K., Stelzer, N., Gehre, M., Thullner, M. and Richnow, H. H. (2007): Applicability of stable isotope fractionation analysis for the characterization of benzene biodegradation in a BTEX-contaminated aquifer. Environ Sci Technol. 41: 3689-3696.

Fischer, A., Vieth, A., Knöller, K., Wachter, T., Dahmke, A. and Richnow, H. H. (2004): Charakterisierung des mikrobiellen Schadstoffabbaus mit Hilfe von isotopenchemischen Methoden. Grundwasser. 3: 159-172.

Fleming, J. T. and Sayler, G. T. (1995): Assessment of gene expression in the environment: Quantitative mRNA analysis in contaminated soils. Florida: CRC Press. Boca Raton. 45-48.

Foght, J. (2008): Anaerobic biodegradation of aromatic hydrocarbons: pathways and prospects. J Mol Microbiol Biotechnol. 15: 93-120.

Friedrich, M. W. (2006): Stable-isotope probing of DNA: insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. Curr Opin Biotechnol. 17: 59-66.

Fries, M. R., Forney, L. J. and Tiedje, J. M. (1997): Phenol- and toluene-degrading microbial populations from an aquifer in which successful trichloroethene cometabolism occurred. Appl Environ Microbiol. 63: 1523-1530.

Fries, M. R., Zhou, J. H., Cheesanford, J. and Tiedje, J. M. (1994): Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl Environ Microbiol. 60: 2802-2810.

Frost, M. R. and Guggenheim, J. A. (1999): Prevention of depurination during elution facilitates the reamplification of DNA from differential display gels. Nucleic Acids Res. 27: e6.

Frostegard, A. and Bååth, E. (1996): The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol Fertil Soils. 22: 59-65.

Fry, V. A., Istok, J. D. and O'Reilly, K. T. (1996): Effect of trapped gas on dissolved oxygen transport - implications for *in situ* bioremediation. Ground Water. 34: 200-210.

Fuchs, G. (2008): Anaerobic metabolism of aromatic compounds. Ann N Y Acad Sci. 1125: 82-99.

Furtak, H. and Langguth, H. R. (1967): Zur hydrochemischen Kennzeichnung von Grundwässern und Grundwassertypen mittels Kennzahlen. Mem IAH-Congress, 1965, Hannover.

Geyer, R., Peacock, A. D., Miltner, A., Richnow, H. H., White, D. C., Sublette, K. L. and Kästner, M. (2005): *In situ* assessment of biodegradation potential using biotraps amended with ^{13}C-labeled benzene or toluene. Environ Sci Technol. 39: 4983-4989.

Gibson, D. T., Cardini, G. E., Maseles, F. C. and Kallio, R. E. (1970): Incorporation of oxygen-18 into benzene by *Pseudomonas putida*. Biochemistry. 9: 1631-1635.

Gibson, D. T., Koch, J. R. and Kallio, R. E. (1968): Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene. Biochemistry. 7: 2653-2662.

Gibson, D. T. and Subramanian, V. (1984): Microbial degradation of aromatic hydrocarbons. In: Microbial degradation of organic compounds. D. T. Gibson (eds). Marcel Dekker, Inc.New York. 181-252.

Gibson, D. T., Zylstra, G. J. and Chauhan, S. (1990): Biotransformation catalyzed by toluene dioxygenase from *Pseudomonas putida* F1. In: *Pseudomonas*: Biotransformations, Pathogenes, and Evolving

Biotechnology. S. Silver, A. M. Chakrabarty, B. Iglewski and S. Kaplan (eds). American Society for Microbiology.Washington DC. 121-132.

Gillham, R. W., Starr, R. C. and Miller, D. J. (1990): A device for *in situ* determination of geochemical transport parameters 2. Biochemical reactions. Ground Water. 28: 858-862.

Glöckner, F. O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebesius, K. and Schleifer, K.-H. (1996): An *in situ* hybridization protocol for detection and identification of planktonic bacteria. System Appl Microbiol. 19: 403-406.

Gödeke, S. (2004): Evaluierung und Modellierung des Natural Attenuation Potentials am Industriestandort Zeitz. Geowissenschaftliche Fakultät. Eberhard Karls Universität. Tübingen, Leipzig. Dissertation: 139 p.

Gödeke, S., Richnow, H. H., Weiss, H., Fischer, A., Vogt, C., Borsdorf, H. and Schirmer, M. (2006): Multi tracer test for the implementation of enhanced *in-situ* bioremediation at a BTEX-contaminated megasite. J Contam Hydrol. 87: 211-236.

Gonfiantini, R., Stichler, W. and Rozanski, K. (1995): Standards and intercomparison materials for stable isotopes of light elements. IAEA-TECDOC-825, Vienna.

Gottschalk, G. (1986): Bacterial Metabolism. Springer. Berlin, Heidelberg, New York. 359.

Gray, N. D. and Head, M. (2001): Linking genetic identity and function in communities of uncultured bacteria. Environ Microbiol. 3: 481-492.

Grbic-Galic, D. and Vogel, T. M. (1987): Transformation of toluene and benzene by mixed methanogenic cultures. Appl Environ Microbiol. 53: 254-260.

Green, C. T. and Scow, K. M. (2000): Analysis of phospholipid fatty acids (PLFA) to characterize microbial communities in aquifers. Hydrogeol J. 8: 126-141.

Griebler, C., Adrian, L., Meckenstock, R. U. and Richnow, H. H. (2004a): Stable carbon isotope fractionation during aerobic and anaerobic transformation of trichlorobenzene. FEMS Microbiol Ecol. 48: 313-321.

Griebler, C., Mindl, B. and Slezak, D. (2001): Combining DAPI and SYBR Green II for the enumeration of total bacterial numbers in aquatic sediments. Int Rev Hydrobiol. 86: 453-465.

Griebler, C., Mindl, B., Slezak, D. and Geiger-Kaiser, M. (2002): Distribution pattern of attached and suspended bacteria in pristine and contaminated shallow aquifers studies with an *in situ* sediment exposure. Aquat Microb Ecol. 28: 117-129.

Griebler, C., Safinowski, M., Vieth, A., Richnow, H. H. and Meckenstock, R. U. (2004b): Combined application of stable carbon isotope analysis and specific metabolites determination for assessing *in situ* degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. Environ Sci Technol. 38: 617-631.

Grootes, P. M., Mook, W. G. and Vogel, J. C. (1969): Isotopic fractionation between gaseous and condensed carbon dioxide. Z Physik. 221: 257-273.

Guckert, J. B., Antworth, C. P., Nichols, P. D. and White, D. C. (1985): Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol Lett. 31: 147-158.

Haas, J. R. and DiChristina, T. J. (2002): Effects of Fe(III) chemical speciation on dissimilatory Fe(III) reduction by *Shewanella putrefaciens*. Environ Sci Technol. 36: 373-380.

Haigler, B. E., Pettigrew, C. A. and Spain, J. C. (1992): Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. Appl Environ Microbiol. 58: 2237-2244.

Hall, B. L., Lachmar, T. E. and Dupont, R. R. (2000): Field monitoring and performance evaluation of an *in situ* air sparging system at a gasoline-contaminated site. J Hazard Mater. 74: 165-186.

Hallier-Soulier, S., Ducrocq, V., Mazure, N. and Truffaut, N. (1996): Detection and quantification of degradative genes in soils contaminated by toluene. FEMS Microbiol Ecol. 20: 121-133.

Haner, A., Hohener, P. and Zeyer, J. (1995): Degradation of *p*-xylene by a denitrifying enrichment culture. Appl Environ Microbiol. 61: 3185-3188.

Hanson, J. R., Macalady, J. L., Harris, D. and Scow, K. M. (1999): Linking toluene degradation with specific microbial populations in soil. Appl Environ Microbiol. 65: 5403-5408.

Hanzlik, R. P., Hogberg, K. and Judson, C. M. (1984): Microsomal hydroxylation of specifically deuterated monosubstituted benzenes. Evidence for direct aromatic hydroxylation. Biochemistry. 23: 3048-3055.

Harms, G., Rabus, R. and Widdel, F. (1999a): Anaerobic oxidation of the aromatic plant hydrocarbon *p*-cymene by newly isolated denitrifying bacteria. Arch Microbiol. 172: 303-312.

Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rossello-Mora, R. and Widdel, F. (1999b): Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl Environ Microbiol. 65: 999-1004.

Harrington, R. R., Poulson, S. R., Drever, J. I., Colberg, P. J. S. and Kelly, E. F. (1999): Carbon isotope systematics of monoaromatic hydrocarbons: vaporization and adsorption experiments. Org Geochem. 30: 765-775.

Härtig, C. (2008): Rapid identification of fatty acid methyl esters using a multidimensional gas chromatography–mass spectrometry database. Journal Chromatogr A. 1177: 159-169.

Harvey, R. W., Smith, R. L. and George, L. (1984): Effect of organic contamination upon microbial distributions and heterotrophic uptake in a Cape Cod, Mass., aquifer. Appl Environ Microbiol. 48: 1197-1202.

Harwood, D. L. and Russel, N. J. (1984): Lipids in plants and microbes. George Allen and Urwin. London.

Hattori, S. (2008): Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. 23: 118-127.

Hayes, J. M., Freeman, K. H., Popp, B. N. and Hoham, C. H. (1990): Compound-specific isotopic analyses - A novel tool for reconstruction of ancient biogeochemical processes. Org Geochem. 16: 1115-1128.

Hazen, T. C., Jimenez, L., Lopez de Victoria, G. and Fliermans, C. B. (1991): Comparison of bacteria from deep subsurface sediments and adjacent groundwater. Microb Ecol. 22: 293-304.

Heider, J. (2007): Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. Curr Opin Chem Biol. 11: 188-194.

Heider, J., Spormann, A. M., Beller, H. R. and Widdel, F. (1999): Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol Rev. 22: 459-473.

Hendrickx, B., Junca, H., Vosahlova, J., Lindner, A., Ruegg, I., Bucheli-Witschel, M., Faber, F., Egli, T., Mau, M., Schlomann, M., Brennerova, M., Brenner, V., Pieper, D. H., Top, E. M., Dejonghe, W., Bastiaens, L. and Springael, D. (2006): Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. J Microbiol Methods. 64: 250-265.

Hermuth, K., Leuthner, B. and Heider, J. (2002): Operon structure and expression of the genes for benzylsuccinate synthase in *Thauera aromatica* strain K172. Arch Microbiol. 177: 132-138.

Herrmann, S., Kleinsteuber, S., Neu, T. R., Richnow, H. H. and Vogt, C. (2008): Enrichment of anaerobic benzene-degrading microorganisms by *in situ* microcosms. FEMS Microbiol Ecol. 63: 94-106.

Hess, A., Zarda, B., Hahn, D., Haner, A., Stax, D., Hohener, P. and Zeyer, J. (1997): *In situ* analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl Environ Microbiol. 63: 2136-2141.

Hilkert, A. W., Douthitt, C. B., Schluter, H. J. and Brand, W. A. (1999): Isotope ratio monitoring gas chromatography/Mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. Rapid Commun Mass Spectrom. 13: 1226-1230.

Himo, F. (2002): Catalytic mechanism of benzylsuccinate synthase, a theoretical study. J Phys Chem B. 106: 7688-7692.

Hinson, J. A., Freeman, J. P., Potter, D. W., Mitchum, R. K. and Evans, F. E. (1985): Mechanism of microsomal metabolism of benzene to phenol. Mol Pharmacol. 27: 574-577.

Hoefs, J. (1997): Stable isotope geochemistry. Springer Verlag. Berlin. 244.

Hollemann, A. F. and Wieberg, E. (1985): Lehrbuch der anorganischen Chemie. Walter de Gruyter. Berlin-New York. pp. 1451.

Holliger, C., Gaspard, S., Glod, G., Heijman, C., Schumacher, W., Schwarzenbach, R. P. and Vazquez, F. (1997): Contaminated environments in the subsurface and bioremediation: organic contaminants. FEMS Microbiol Rev. 20: 517-523.

Hopper, D. J. (1978): Microbial degradation of aromatic hydrocarbons. Applied Science Publishers. London. 85-112.

Huang, L., Sturchio, N. C., Abrajano, T., Heraty, L. J. and Holt, B. D. (1999): Carbon and chlorine isotope fractionation of chlorinated aliphatic hydrocarbons by evaporation. Org Geochem. 30: 777-785.

Hugenholtz, P., Goebel, B. M. and Pace, N. R. (1998): Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol. 180: 4765-4774.

Hugenholtz, P. and Pace, N. R. (1996): Identifying microbial diversity in the natural environment: A molecular phylogenetic approach. Trends Biotechnol. 14: 190-197.

Hunkeler, D., Andersen, N., Aravena, R., Bernasconi, S. M. and Butler, B. J. (2001): Hydrogen and carbon isotope fractionation during aerobic biodegradation of benzene. Environ Sci Technol. 35: 3462-3467.

Hunkeler, D., Chollet, N., Pittet, X., Aravena, R., Cherry, J. A. and Parker, B. L. (2004): Effect of source variability and transport processes on carbon isotope ratios of TCE and PCE in two sandy aquifers. J Contam Hydrol. 74: 265-282.

Hutchins, S. R. (1991): Biodegradation of monoaromatic hydrocarbons by aquifer microorganisms using oxygen, nitrate, or nitrous oxide as the terminal electron acceptor. Appl Environ Microbiol. 57: 2403-2407.

Hutchins, S. R. (1992): Inhibition of alkylbenzene biodegradation under denitrifying conditions by using the acetylene block technique. Appl Environ Microbiol. 58: 3395-3398.

Hutchins, S. R. (1993): Biotransformation and mineralization of alkylbenzenes under denitrifying conditions. Environ Toxicol Chem. 12: 1413-1423.

Inagaki, F., Takai, K., Nealson, K. H. and Horikoshi, K. (2004): *Sulfurovum lithotrophicum* gen. nov., sp nov., a novel sulfur-oxidizing chemolithoautotroph within the *epsilon-Proteobacteria* isolated from Okinawa Trough hydrothermal sediments. Int J Syst Evol Microbiol. 54: 1477-1482.

Istok, J. D., Humphrey, M. D., Schroth, M. H., Hyman, M. R. and O'Reilly, K. T. (1997): Single-well, "push-pull" test for *in situ* determination of microbial activities. Ground Water. 35: 619-631.

Jackson, B. E. and McInerney, M. J. (2002): Anaerobic microbial metabolism can proceed close to thermodynamic limits. Nature. 415: 454-456.

Jahn, M. K., Haderlein, S. B. and Meckenstock, R. U. (2005): Anaerobic degradation of benzene, toluene, ethylbenzene, and *o*-xylene in sediment-free iron-reducing enrichment cultures. Appl Environ Microbiol. 71: 3355-3358.

Jang, J. Y., Kim, D., Bae, H. W., Choi, K. Y., Chae, J. C., Zylstra, G. J., Kim, Y. M. and Kim, E. (2005): Isolation and characterization of a *Rhodococcus* species strain able to grow on *ortho*- and *para*-xylene. J Microbiol. 43: 325-330.

Jehmlich, N., Schmidt, F., Hartwich, H., von Bergen, M., Richnow, H. H. and Vogt, C. (2008a): Incorporation of carbon and nitrogen atoms into proteins measured by protein-based stable isotope probing (Protein-SIP). Rapid Commun Mass Spectrom. 22: 2889-2897.

Jehmlich, N., Schmidt, F., von Bergen, M., Richnow, H. H. and Vogt, C. (2008b): Protein-based stable isotope probing (Protein-SIP) reveals active species within anoxic mixed cultures. ISME J. 2: 1122-1133.

Jeon, C. O., Park, W., Padmanabhan, P., DeRito, C., Snape, J. R. and Madsen, E. L. (2003): Discovery of a bacterium, with distinctive dioxygenase, that is responsible for *in situ* biodegradation in contaminated sediment. Proc Natl Acad Sci U S A. 100: 13591-13596.

Johnson, R. L., Johnson, P. C., McWhorter, D. B., Hinchee, R. E. and Goodman, I. (1993): An overview of *in situ* air sparging. Ground Water Monit Remediat. 13: 127-135.

Johnson, S. J., Woolhouse, K. J., Prommer, H., Barry, D. A. and Christofi, N. (2003): Contribution of anaerobic microbial activity to natural attenuation of benzene in groundwater. Eng Geol. 70: 343-349.

Johnston, C. D., Rayner, J. L. and Briegel, D. (2002): Effectiveness of *in situ* air sparging for removing NAPL gasoline from a sandy aquifer near Perth, Western Australia. J Contam Hydrol. 59: 87-111.

Johnston, C. D., Rayner, J. L., Patterson, B. M. and Davis, G. B. (1998): Volatilisation and biodegradation during air sparging of dissolved BTEX-contaminated groundwater. J Contamin Hydrol. 33: 377-404.

Jørgensen, C., Nielsen, B., Jensen, B. K. and Mortensen, E. (1995): Transformation of *o*-xylene to *o*-methyl benzoic acid by a denitrifying enrichment culture using toluene as the primary substrate. Biodegradation. 6: 141-146.

Juteau, P., Cote, V., Duckett, M. F., Beaudet, R., Lepine, F., Villemur, R. and Bisaillon, J. G. (2005): *Cryptanaerobacter phenolicus* gen. nov., sp nov., an anaerobe that transforms phenol into benzoate via 4-hydroxybenzoate. Int J Syst Evol Microbiol. 55: 245-250.

Kampara, M., Thullner, M., Richnow, H. H., Harms, H. and Wick, L. Y. (2008): Impact of bioavailability restrictions on microbially induced stable isotope fractionation. 2. experimental evidence. Environ Sci Technol. 42: 6552-6558.

Kao, C. M. and Borden, R. C. (1997): Site-specific variability in BTEX biodegradation under denitrifying conditions. Ground Water. 35: 305-311.

Kasai, Y., Takahata, Y., Hoaki, T. and Watanabe, K. (2005): Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil. Environ Microbiol. 7: 806-818.

Kasai, Y., Takahata, Y., Manefield, M. and Watanabe, K. (2006): RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. Appl Environ Microbiol. 72: 3586-3592.

Kaschl, A., Vogt, C., Uhlig, S., Nijenhuis, I., Weiss, H., Kastner, M. and Richnow, H. H. (2005): Isotopic fractionation indicates anaerobic monochlorobenzene biodegradation. Environ Toxicol Chem. 24: 1315-1324.

Kästner, M., Fischer, A., Nijenhuis, I., Geyer, R., Stelzer, N., Bombach, P., Tebbe, C. C. and Richnow, H. H. (2006): Assessment of microbial *in situ* activity in contaminated aquifers. Eng Life Sci. 6: 234-251.

Kates, M. (1964): Bacterial lipids. Adv Lipid Res. 2: 17-90.

Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R. and Kaushik, R. (2005): Phospholipid fatty acid - a bioindicator of environment monitoring and assessment in soil ecosystem. Curr Sci. 89: 1103-1112.

Kazumi, J., Caldwell, M. E., Suflita, J. M., Lovley, D. R. and Young, L. Y. (1997): Anaerobic degradation of benzene in diverse anoxic environments. Environ Sci Technol. 31: 813-818.

Keller, M. and Zengler, K. (2004): Tapping into microbial diversity. Nat Rev Microbiol. 2: 141-150.

Kelley, C. A., Hammer, B. T. and Coffin, R. B. (1997): Concentrations and stable isotope values of BTEX in gasoline-contaminated groundwater. Environ Sci Technol. 31: 2469-2472.

Kendall, M. M. and Boone, D. R. (2006): The Order *Methanosarcinales*. In: The Prokaryotes - a handbook on the biology of bacteria, third edition. Springer. New York, USA. 244-256.

Kim, H. and Annable, M. D. (2006): Effect of surface tension reduction on VOC removal during surfactant-enhanced air sparging. J Environ Sci Health A Tox Hazard Subst Environ Eng. 41: 2799-2811.

Kim, H., Soh, H. E., Annable, M. D. and Kim, D. J. (2004): Surfactant-enhanced air sparging in saturated sand. Environ Sci Technol. 38: 1170-1175.

Kim, S.-B., Park, C.-H., Kim, D.-J. and Jury, W. A. (2003): Kinetics of benzene biodegradation by *Pseudomonas aeruginosa*: Parameter estimation. Environ Toxicol Chem. 22: 1038-1045.

Kim, S. I., Choi, J.-S. and Kahng, H.-Y. (2007): A proteomics strategy for the analysis of bacterial biodegradation pathways. OMICS: J Integrative Biol. 11: 280-294.

Kleikemper, J., Pombo, S. A., Schroth, M. H., Sigler, W. V., Pesaro, M. and Zeyer, J. (2005): Activity and diversity of methanogens in a petroleum hydrocarbon-contaminated aquifer. Appl Environ Microbiol. 71: 149-158.

Kleikemper, J., Schroth, M. H., Sigler, W. V., Schmucki, M., Bernasconi, S. M. and Zeyer, J. (2002): Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. Appl Environ Microbiol. 68: 1516-1523.

Kleinsteuber, S., Riis, V., Fetzer, I., Harms, H. and Muller, S. (2006): Population dynamics within a microbial consortium during growth on diesel fuel in saline environments. Appl Environ Microbiol. 72: 3531-3542.

Kleinsteuber, S., Schleinitz, K. M., Breitfeld, J., Harms, H., Richnow, H. H. and Vogt, C. (2008): Molecular characterization of bacterial communities mineralizing benzene under sulfate-reducing conditions. FEMS Microbiol Ecol. 66: 143-157.

Kniemeyer, O., Fischer, T., Wilkes, H., Glockner, F. O. and Widdel, F. (2003): Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl Environ Microbiol. 69: 760-768.

Koizumi, Y., Kojima, H. and Fukui, M. (2005): Potential sulfur metabolisms and associated bacteria within anoxic surface sediment from saline meromictic Lake Kaiike (Japan). FEMS Microbiol Ecol. 52: 297-305.

Kölbel-Boelke, J., Anders, E. M. and Nehrkorn, A. (1988): Microbial communities in the saturated groundwater environment II: diversity of bacterial communities in a pleistocene sand aquifer and their *in vitro* activities. Microb Ecol. 16: 31-48.

Kopinke, F. D., Georgi, A., Voskamp, M. and Richnow, H. H. (2005): Carbon isotope fractionation of organic contaminants due to retardation on humic substances: implications for natural attenuation studies in aquifers. Environ Sci Technol. 39: 6052-6062.

Korte, J. E., Hertz-Picciotto, I., Schulz, M. R., Ball, L. M. and Duell, E. J. (2000): The Contribution of Benzene to Smoking-Induced Leukemia. Environ Health Perspect. 108: 333-339.

Korzekwa, K. R., Swinney, D. C. and Trager, W. F. (1989): Isotopically labeled chlorobenzenes as probes for the mechanism of cytochrome P-450 catalyzed aromatic hydroxylation. Biochemistry. 28: 9019-9027.

Krieger, C. J., Beller, H. R., Reinhard, M. and Spormann, A. M. (1999): Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. J Bacteriol. 181: 6403-6410.

Krieger, C. J., Roseboom, W., Albracht, S. P. J. and Spormann, A. M. (2001): A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp. strain T. J Biol Chem. 276: 12924-12927.

Krumholz, L. R., Caldwell, M. E. and Suflita, J. M. (1996): Biodegradation of BTEX compounds under anaerobic conditions. In: Bioremediation: Principles and Applications. R. L. Crawford, Crawford, D.L. (eds). Cambridge University Press, Cambridge. 61-99.

Kube, M., Heider, J., Amann, J., Hufnagel, P., Kuhner, S., Beck, A., Reinhardt, R. and Rabus, R. (2004): Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1. Arch Microbiol. 181: 182-194.

Kuder, T., Philp, P. and Allen, J. (2009): Effects of volatilization on carbon and hydrogen isotope ratios of MTBE. Environ Sci Technol. 43: 1763-1768.

Kuder, T., Wilson, J. T., Kaiser, P., Kolhatkar, R., Philp, P. and Allen, J. (2005): Enrichment of stable carbon and hydrogen isotopes during anaerobic biodegradation of MTBE: microcosm and field evidence. Environ Sci Technol. 39: 213-220.

Kuever, J., Rainey, F. A. and Widdel, F. (2006): Validation of publication of new names and new combinations previously effectively published outside the IJSEM - List no. 107. Int J Syst Evol Microbiol. 56: 1-6.

Kuhn, E. P., Colberg, P. J., Schnoor, J. L., Wanner, O., Zehnder, A. J. B. and Schwarzenbach, R. P. (1985): Microbial transformations of substituted benzenes during infiltration of river water to groundwater: laboratory column studies. Environ Sci Technol. 19: 961-968.

Kuhn, E. P., Zeyer, J., Eicher, P. and Schwarzenbach, R. P. (1988): Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. Appl Environ Microbiol. 54: 490-496.

Kunapuli, U., Griebler, C., Beller, H. R. and Meckenstock, R. U. (2008): Identification of intermediates formed during anaerobic benzene degradation by an iron-reducing enrichment culture. Environ Microbiol. 10: 1703-1712.

Kunapuli, U., Lueders, T. and Meckenstock, R. U. (2007): The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. ISME J. 1: 643-653.

Kuntze, K., Shinoda, Y., Moutakki, H., McInerney, M. J., Vogt, C., Richnow, H. H. and Boll, M. (2008): 6-Oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolases from obligately anaerobic bacteria: characterization and identification of its gene as a functional marker for aromatic compounds degrading anaerobes. Environ Microbiol. 10: 1547-1556.

Laban, N. A., Selesi, D., Jobelius, C. and Meckenstock, R. U. (2009): Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria. FEMS Microbiol Ecol. 68: 300-311.

Landmeyer, J. E., Vroblesky, D. A. and Chapelle, F. H. (1996): Stable carbon isotope evidence of biodegradation zonation in a shallow jet-fuel contaminated aquifer. Environ Sci Technol. 30: 1120-1128.

Lane, D. J. (1991): 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. E. Stackebrandt and M. Goodfellow (eds). Wiley.Chichester, New York. 115-175.

Langenhoff, A. A. M., Zehnder, A. J. B. and Schraa, G. (1996): Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. Biodegradation. 7: 267-274.

LAWA, L. W. (1998-2008): AQS-Merkblätter für die Wasser-, Abwasser- und Schlammuntersuchung. Erich Schmidt Verlag GmbH. Berlin.

Leeson, A., Johnson, P. C., Johnson, R. L., Vogel, C. M., Hinchee, R. E., Marley, M., Peargin, T., Bruce, C. L., Amerson, I. L., Coonfare, C. T., Gillespie, R. D. and McWhorter, D. B. (2002): Air sparging design paradigm. Ohio. 150 p.

Lehmann, R. M., Colwell, F. S. and Bala, G. A. (2000): Attached and unattached microbial communities in a simulated basalt aquifer under fracture- and porous-flow conditions. Appl Environ Microbiol. 67: 2799-2809.

Leuthner, B., Leutwein, C., Schulz, H., Horth, P., Haehnel, W., Schiltz, E., Schagger, H. and Heider, J. (1998): Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol. 28: 615-628.

Li, L. and Marsh, E. N. (2006a): Mechanism of benzylsuccinate synthase probed by substrate and isotope exchange. J Am Chem Soc. 128: 16056-16057.

Li, L. and Marsh, E. N. G. (2006b): Deuterium isotope effects in the unusual addition of toluene to fumarate catalyzed by benzylsuccinate synthase. Biochemistry. 45: 13932-13938.

Lin, B., Van Verseveld, H. W. and Roling, W. F. (2002): Microbial aspects of anaerobic BTEX degradation. Biomed Environ Sci. 15: 130-144.

Liou, J. S. C., DeRito, C. M. and Madsen, E. L. (2008): Field-based and laboratory stable isotope probing surveys of the identities of both aerobic and anaerobic benzene-metabolizing microorganisms in freshwater sediment. Environ Microbiol. 10: 1964-1977.

Liu, A., Garcia-Dominguez, E., Rhine, E. D. and Young, L. Y. (2004): A novel arsenate respiring isolate that can utilize aromatic substrates. FEMS Microbiol Ecol. 48: 323-332.

Liu, W. T., Marsh, T. L., Cheng, H. and Forney, L. J. (1997): Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol. 63: 4516-4522.

Lo, I., Denef, V. J., Verberkmoes, N. C., Shah, M. B., Goltsman, D., DiBartolo, G., Tyson, G. W., Allen, E. E., Ram, R. J., Detter, J. C., Richardson, P., Thelen, M. P., Hettich, R. L. and Banfield, J. F. (2007): Strain-resolved community proteomics reveals recombining genomes of acidophilic bacteria. Nature. 446: 537-541.

Lovley, D. R. (2000): Anaerobic benzene degradation. Biodegradation. 11: 107-116.

Lovley, D. R., Baedecker, M. J., Lonergan, D. J., Cozzarelli, I. M., Phillips, E. J. P. and Siegel, D. I. (1989): Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature. 339: 297-299.

Lovley, D. R. and Coates, J. D. (1997): Bioremediation of metal contamination. Curr Opin Biotechnol. 8: 285-289.

Lovley, D. R., Coates, J. D., Woodward, J. C. and Phillips, E. (1995): Benzene oxidation coupled to sulfate reduction. Appl Environ Microbiol. 61: 953-958.

Lovley, D. R. and Lloyd, J. R. (2000): Microbes with a mettle for bioremediation. Nat Biotechnol. 18: 600-601.

Lovley, D. R. and Lonergan, D. J. (1990): Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl Environ Microbiol. 56: 1858-1864.

Lovley, D. R., Woodward, J. C. and Chapelle, F. H. (1994): Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. Nature. 370: 128-131.

Lovley, D. R., Woodward, J. C. and Chapelle, F. H. (1996): Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. Appl Environ Microbiol. 62: 288-291.

Lueders, T., Manefield, M. and Friedrich, M. W. (2004): Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environ Microbiol. 6: 73-78.

Mace, R. E., Fisher, R. S., Welch, D. M. and Parra, S. P. (1997). Extent, mass and duration of hydrocarbon plumes from leaking petroleum storage tank sites in Texas. Austin, Texas, Bureau of Economic Geology, University of Texas at Austin.

Madigan, M. T., Martinenko, J. M. and Parker, J. (1997): Brock - Biology of microorganisms. Prentice Hall International.

Madsen, E. L. (2000): Nucleic-acid characterization of the identity and activity of subsurface microorganisms. Hydrogeol J. 8: 112-125.

Madsen, E. L. (2005): Identifying microorganisms responsible for ecologically significant biogeochemical processes. Nat Rev Microbiol. 3: 439-446.

Maher, N., Dillon, H. K., Vermund, S. H. and Unnasch, T. R. (2001): Magnetic bead capture eliminates PCR inhibitors in samples collected from the airborne environment, permitting detection of *Pneumacystis carinii* DNA. Appl Environ Microbiol. 67: 449-452.

Mahmood, S., Paton, G. I. and Prosser, J. I. (2005): Cultivation-independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. Environ Microbiol. 7: 1349-1360.

Major, D. W., Mayfield, C. I. and Baker, J. F. (1998): Biotransformation of benzenes by denitrification in aquifer sand. Ground Water. 28: 8-14.

Makula, R. A. (1978): Phospholipid composition of methane-utilizing bacteria. J Bacteriol. 134: 771-777.

Mancini, S. A., Devine, C. E., Elsner, M., Nandi, M. E., Ulrich, A. C., Edwards, E. A. and Lollar, B. S. (2008): Isotopic evidence suggests different initial reaction mechanisms for anaerobic benzene biodegradation. Environ Sci Technol. 42: 8290-8296.

Mancini, S. A., Hirschorn, S. K., Elsner, M., Lacrampe-Couloume, G., Sleep, B. E., Edwards, E. A. and Lollar, B. S. (2006): Effects of trace element concentration on enzyme controlled stable isotope fractionation during aerobic biodegradation of toluene. Environ Sci Technol. 40: 7675-7681.

Mancini, S. A., Lacrampe-Couloume, G., Jonker, H., van Breukelen, B. M., Groen, J., Volkering, F. and Lollar, B. S. (2002): Hydrogen isotopic enrichment: an indicator of biodegradation at a petroleum hydrocarbon contaminated field site. Environ Sci Technol. 36: 2464-2470.

Mancini, S. A., Ulrich, A. C., Lacrampe-Couloume, G., Sleep, B., Edwards, E. A. and Lollar, B. S. (2003): Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. Appl Environ Microbiol. 69: 191-198.

Mandelbaum, R. T., Shati, M. R. and Ronen, D. (1997): *In situ* microcosms in aquifer bioremediation studies. FEMS Microbiol Rev. 20: 489-502.

Manefield, M., Griffiths, R. I., Bailey, M. J. and Whiteley, A. S. (2006): Stable isotope probing: a critique of its role in linking phylogeny and function. In: Nucleic Acids and Proteins in Soil. P. Nannipieri and K. Smalla (eds). Springer-Verlag.Berlin, Heidelberg. 205-216.

Manefield, M., Whiteley, A. S., Griffiths, R. I. and Bailey, M. J. (2002a): RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl Environ Microbiol. 68: 5367-5373.

Manefield, M., Whiteley, A. S., Ostle, N., Ineson, P. and Bailey, M. J. (2002b): Technical considerations for RNA-based stable isotope probing: an approach to associating microbial diversity with microbial community function. Rapid Commun Mass Spectrom. 16: 2179-2183.

Mariotti, A., Germon, J. C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A. and Tardieux, P. (1981): Experimental determination of nitrogen kinetic isotope fractionation: some principles, illustration for the denitrification and nitrification processes. Plant Soil. 62: 413-430.

Massol-Deya, A. A., Odelson, D. A., Hickey, R. F. and Tiedje, J. M. (1995): Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In: Molecular microbial ecology manual. A. D. L. Akkermans, van Elsas, J.D., deBruijn, F.J. (eds). Kluwer, Dordrecht. 1-8.

Mauclaire, L., Pelz, O., Thullner, M., Abraham, W. and Zeyer, J. (2003): Assimilation of toluene carbon along a bacteria-protist food chain determined by ^{13}C-enrichment of biomarker fatty acids. J Microbiol Methods. 55: 635-649.

Maurer, M. and Rittmann, B. E. (2004): Modeling intrinsic bioremediation for interpret observable biogeochemical footprints of BTEX biodegradation: the need for fermentation and abiotic chemical processes. Biodegradation. 15: 405-417.

McKelvie, J. R., Lindstrom, J. E., Beller, H. R., Richmond, S. A. and Sherwood Lollar, B. (2005): Analysis of anaerobic BTX biodegradation in a subarctic aquifer using isotopes and benzylsuccinates. J Contam Hydrol. 81: 167-186.

Mechichi, T., Stackebrandt, E., Gad'on, N. and Fuchs, G. (2002): Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. Arch Microbiol. 178: 26-35.

Meckenstock, R. U. (1999): Fermentative toluene degradation in anaerobic defined syntrophic cocultures. FEMS Microbiol Lett. 177: 67-73.

Meckenstock, R. U., Annweiler, E., Warthmann, R., Schink, B., Michaelis, W. and Richnow, H. H. (1999a): $^{13}C/^{12}C$ stable isotope fractionation of toluene by anaerobic degradation: A new method to monitor biological degradation *in situ*? In: Novel approaches for bioremediation of organic pollution. R. Fass, Y. Flashner and S. Reuveny (eds). Kluwer Academic Publishers.New York. 219-227.

Meckenstock, R. U., Morasch, B., Griebler, C. and Richnow, H. H. (2004a): Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated acquifers. J Contam Hydrol. 75: 215-255.

Meckenstock, R. U., Morasch, B., Kästner, M., Vieth, A. and Richnow, H. H. (2002): Assessment of bacterial degradation of aromatic hydrocarbons in the environment by analysis of stable carbon isotope fractionation. Water, Air, Soil Pollut Focus. 2: 141-152.

Meckenstock, R. U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W. and Richnow, H. H. (1999b): $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ Microbiol. 1: 409-414.

Meckenstock, R. U., Safinowski, M. and Griebler, C. (2004b): Anaerobic degradation of polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol. 49: 27-36.

Meier-Augenstein, W. (1999): Applied gas chromatography coupled to isotope ratio mass spectrometry. J Chromatogr A. 842: 351-371.

Melander, L. and Saunders, W. H. (1980): Reaction rates of isotopic molecules. John Wiley & Sons. New York.

Mesarch, M. B., Nakatsu, C. H. and Nies, L. (2000): Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. Appl Environ Microbiol. 66: 678-683.

Meselson, M. and Stahl, F. W. (1958): The replication of DNA in *Escherichia coli*. Proc Natl Acad Sci U S A. 44: 671-682.

Mikesell, M. D., Kukor, J. J. and Olsen, R. H. (1993): Metabolic diversity of aromatic hydrocarbon-degrading bacteria from a petroleum-contaminated aquifer. Biodegradation. 4: 249-259.

Mitchell, K. H., Rogge, C. E., Gierahn, T. and Fox, B. G. (2003): Insight into the mechanism of aromatic hydroxylation by toluene 4-monooxygenase by use of specifically deuterated toluene and *p*-xylene. Proc Natl Acad Sci USA. 100: 3784-3789.

Morasch, B., Annweiler, E., Warthmann, R. J. and Meckenstock, R. U. (2001a): The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. J Microbiol Methods. 44: 183-191.

Morasch, B., Hohener, P. and Hunkeler, D. (2007): Evidence for *in situ* degradation of mono-and polyaromatic hydrocarbons in alluvial sediments based on microcosm experiments with C-13-labeled contaminants. Environ Pollut. 148: 739-748.

Morasch, B. and Meckenstock, R. U. (2005): Anaerobic degradation of *p*-xylene by a sulfate-reducing enrichment culture. Curr Microbiol. 51: 127-130.

Morasch, B., Richnow, H. H., Schink, B. and Meckenstock, R. U. (2001b): Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects. Appl Environ Microbiol. 67: 4842-4849.

Morasch, B., Richnow, H. H., Schink, B., Vieth, A. and Meckenstock, R. U. (2002): Carbon and hydrogen stable isotope fractionation during aerobic bacterial degradation of aromatic hydrocarbons. Appl Environ Microbiol. 68: 5191-5194.

Morasch, B., Richnow, H. H., Vieth, A., Schink, B. and Meckenstock, R. U. (2004a): Stable isotope fractionation caused by glycyl radical enzymes during bacterial degradation of aromatic compounds. Appl Environ Microbiol. 70: 2935-2940.

Morasch, B., Schink, B., Tebbe, C. C. and Meckenstock, R. U. (2004b): Degradation of *o*-xylene and *m*-xylene by a novel sulfate-reducer belonging to the genus *Desulfotomaculum*. Arch Microbiol. 181: 407-417.

Morgan, P., Lewis, S. T. and Watkinson, R. J. (1993): Biodegradation of benzene, toluene, ethylbenzene and xylenes in gas-condensate-contaminated ground-water. Environ Pollut. 82: 181-190.

Morgan, P. and Watkinson, R. J. (1992): Factors limiting the supply and efficiency of nutrient and oxygen supplements for the *in situ* biotreatment of contaminated soil and groundwater. Water Res. 26: 73-78.

Morrison, R. T. and Boyd, R. N. (1986): Lehrbuch der Organischen Chemie. Verlag Chemie. Weinheim. 1535 p.

Mottram, H. R. and Evershed, R. P. (2003): Practical considerations in the gas chromatography/combustion/isotope ratio monitoring mass spectrometry of ^{13}C-enriched compounds: detection limits and carryover effects. Rapid Commun Mass Spectrom. 17: 2669-2674.

Muccio, Z. and Jackson, G. P. (2009): Isotope Ratio Mass Spectrometry. Analyst. 134: 213-222.

Murray, W. A., Lunardini, R. C., Jr., Ullo, F. J., Jr. and Davidson, M. E. (2000): Site 5 air sparging pilot test, Naval Air Station Cecil Field, Jacksonville, Florida. J Hazard Mater. 72: 121-145.

Musat, F. and Widdel, F. (2008): Anaerobic degradation of benzene by a marine sulfate-reducing enrichment culture, and cell hybridization of the dominant phylotype. Environ Microbiol. 10: 10-19.

Muyzer, G., de Waal, E. C. and Uitterlinden, A. G. (1993): Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol. 59: 695-700.

Muyzer, G. and Smalla, K. (1998): Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek. 73: 127-141.

Na, K. S., Kuroda, A., Takiguchi, N., Ikeda, T., Ohtake, H. and Kato, J. (2005): Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains. J Biosci Bioeng. 99: 378-382.

Nakagawa, S. and Takai, K. (2008): Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance. FEMS Microbiol Ecol. 65: 1-14.

Nakagawa, S., Takaki, Y., Shimamura, S., Reysenbach, A. L., Takai, K. and Horikoshi, K. (2007): Deep-sea vent epsilon-proteobacterial genomes provide insights into emergence of pathogens. Proc Natl Acad Sci U S A. 104: 12146-12150.

Nakagawa, T., Sato, S. and Fukui, M. (2008): Anaerobic degradation of *p*-xylene in sediment-free sulfate-reducing enrichment culture. Biodegradation. 19: 909-913.

Nales, M., Butler, B. J. and Edwards, E. A. (1998): Anaerobic benzene biodegradation: a microcosm survey. Bioremediation J. 2: 125-144.

Neufeld, J. D., Dumont, M. G., Vohra, J. and Murrell, J. C. (2007a): Methodological considerations for the use of stable isotope probing in microbial ecology. Microb Ecol. 53: 435-442.

Neufeld, J. D., Vohra, J., Dumont, M. G., Lueders, T., Manefield, M., Friedrich, M. W. and Murrell, J. C. (2007b): DNA stable-isotope probing. Nat Protoc. 2: 860-866.

Nichols, P. D., Mancuso, C. A. and White, D. C. (1987): Measurement of methanotroph and methanogen signature phosopholipids for use in assessment of biomass and community structure in model systems. Org Geochem. 11: 451-461.

Nijenhuis, I., Andert, J., Beck, K., Kastner, M., Diekert, G. and Richnow, H.-H. (2005): Stable isotope fractionation of tetrachloroethene during reductive dechlorination by *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S and abiotic reactions with cyanocobalamin. Appl Environ Microbiol. 71: 3413-3419.

Nijenhuis, I., Stelzer, N., Kastner, M. and Richnow, H. H. (2007): Sensitive detection of anaerobic monochlorobenzene degradation using stable isotope tracers. Environ Sci Technol. 41: 3836-3842.

Nikolausz, M., Nijenhuis, I., Ziller, K., Richnow, H. H. and Kastner, M. (2006): Stable carbon isotope fractionation during degradation of dichloromethane by methylotrophic bacteria. Environ Microbiol. 8: 156-164.

Northrop, D. B. (1981): The expression of isotope effects on enzyme-catalyzed reactions. Annu Rev Biochem. 50: 103-131.

Nusslein, B., Chin, K. J., Eckert, W. and Conrad, R. (2001): Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical Lake Kinneret (Israel). Environ Microbiol. 3: 460-470.

O'leary, W. M. and Wilkinson, S. G. (1988): Gram-positive *Bacteria*. In: Microbial lipids. C. Ratledge and S. G. Wilkinson (eds). Academic Press.London. 117-201.

Ogram, A., Sun, W., Brockman, F. J. and Fredrickson, J. K. (1995): Isolation and characterization of RNA from low-biomass deep-subsurface sediments. Appl Environ Microbiol. 61: 763-768.

Oka, A. R., Phelps, C. D., McGuinness, L. M., Mumford, A., Young, L. Y. and Kerkhof, L. J. (2008): Identification of critical members in a sulfidogenic benzene-degrading consortium by DNA stable isotope probing. Appl Environ Microbiol. 74: 6476-6480.

Olsen, R. H., Kukor, J. J. and Kaphammer, B. (1994): A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J Bacteriol. 176: 3749-3756.

Olsen, R. H., Mikesell, M. D., Kukor, J. J. and Byrne, A. M. (1995): Physiological attributes of microbial BTEX degradation in oxygen-limited environments. Environ Health Perspect. 103 Suppl 5: 49-51.

Ommedal, H. and Torsvik, T. (2007): *Desulfotignum toluenicum* sp. nov., a novel toluene-degrading, sulphate-reducing bacterium isolated from an oil-reservoir model column. Int J Syst Evol Microbiol. 57: 2865-2869.

Ong, S.-E. and Mann, M. (2005): Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol. 1: 252-262.

Pace, N. R. (1997): A molecular view of microbial diversity and the biosphere. Science. 276: 734-740.

Pace, N. R., Stahl, D. A., Lane, D. J. and Olsen, G. J. (1986): The analysis of natural microbial populations by ribosomal RNA sequences. Adv Microb Ecol. 9: 1-55.

Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J. R., Tsai, C. S., Park, W., Jeon, C. and Madsen, E. L. (2003): Respiration of ^{13}C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of ^{13}C-labeled soil DNA. Appl Environ Microbiol. 69: 1614-1622.

Parker, J. H., Smith, G. A., Fredrickson, H. L., Vestal, J. R. and White, D. C. (1982): Sensitive assay, based on hydroxy fatty acids from lipopolysaccharide lipid A, for Gram-negative bacteria in sediments. Appl Environ Microbiol. 44: 1170-1177.

Parkes, R. J. and Calder, A. G. (1985): The cellular fatty acids of three strains of *Desulfobulbus*, a propionate-utilising sulphate-reducing bacterium. FEMS Microbiol Lett. 31: 361-363.

Parkes, R. J. and Taylor, J. (1983): The relationship between fatty acid distributions and bacterial respiratory types in contemporary marine sediments. Estuar Coast Shelf Sci. 16: 173-189.

Peacock, A. D., Chang, Y. J., Istok, J. D., Krumholz, L., Geyer, R., Kinsall, B., Watson, D., Sublette, K. L. and White, D. C. (2004): Utilization of microbial biofilms as monitors of bioremediation. Microb Ecol. 47: 284-292.

Pelz, O., Chatzinotas, A., Andersen, N., Bernasconi, S. M., Hesse, C., Abraham, W. R. and Zeyer, J. (2001a): Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations. Arch Microbiol. 175: 270-281.

Pelz, O., Chatzinotas, A., Zarda-Hess, A., Abraham, W. R. and Zeyer, J. (2001b): Tracing toluene-assimilating sulfate-reducing bacteria using ^{13}C-incorporation in fatty acids and whole-cell hybridization. FEMS Microbiol Ecol. 38: 123-131.

Pernthaler, A., Pernthaler, J. and Amann, R. (2002a): Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl Environ Microbiol. 68: 3094-3101.

Pernthaler, A., Preston, C. M., Pernthaler, J., DeLong, E. F. and Amann, R. (2002b): Comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. Appl Environ Microbiol. 68: 661-667.

Peterson, J. W., DeBoer, M. J. and Lake, K. L. (2000): A laboratory simulation of toluene cleanup by air sparging of water-saturated sands. J Hazard Mater. 72: 167-178.

Pfennig, N., Eimhjellen, K. E. and Liaaen Jensen, S. (1965): A new isolate of the *Rhodospirillum fulvum* group and its photosynthetic pigments. Arch Microbiol. 51: 258-266.

Phelps, C. D., Kazumi, J. and Young, L. Y. (1996): Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction. FEMS Microbiol Lett. 145: 433-437.

Phelps, C. D., Kerkhof, L. J. and Young, L. Y. (1998): Molecular characterization of a sulfate-reducing consortium which mineralizes benzene. FEMS Microbiol Lett. 27: 269-279.

Phelps, C. D. and Young, L. Y. (1999): Anaerobic biodegradation of BTEX and gasoline in various aquatic sediments. Biodegradation. 10: 15-25.

Phelps, C. D. and Young, L. Y. (2001): Biodegradation of BTEX under anaerobic conditions: A review. In: Advances in Agronomy(eds). 329-357.

Phelps, C. D. and Young, L. Y. (2002): Metabolic biomarkers for detecting anaerobic PAH biodegradation in groundwater and sediments. Soil Sed Contam. 11: 1023.

Phelps, C. D., Zhang, Z. M. and Young, L. Y. (2001): Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulphidogenic consortium. Environ Microbiol. 3: 600-603.

Pickup, R. W., Rhodes, G., Alamillo, M. L., Mallinson, H. E. H., Thornton, S. F. and Lerner, D. N. (2001): Microbiological analysis of multi-level borehole samples from a contaminated groundwater system. J Contam Hydrol. 53: 269-284.

Plumb, R. H. (1987): A comparison of ground water monitoring data from CERCLA and RCRA sites. Ground Water Monit Remediat. 7: 94-100.

Porter, M. L. and Engel, A. S. (2008): Diversity of uncultured *Epsilonproteobacteria* from terrestrial sulfidic caves and springs. Appl Environ Microbiol. 74: 4973-4977.

Poulson, S. R. and Drever, J. I. (1999): Stable isotope (C, Cl, and H) fractionation during vaporization of trichloroethylene. Environ Sci Technol. 33: 3689-3694.

Powell, S. M., Ferguson, S. H., Snape, I. and Siciliano, S. D. (2006): Fertilization stimulates anaerobic fuel degradation of antarctic soils by denitrifying microorganisms. Environ Sci Technol. 40: 2011-2017.

Qiu, Y. L., Sekiguchi, Y., Hanada, S., Imachi, H., Tseng, I. C., Cheng, S. S., Ohashi, A., Harada, H. and Kamagata, Y. (2006): *Pelotomaculum terephthalicum* sp nov and *Pelotomaculum isophthalicum* sp nov.: two anaerobic bacteria that degrade phthalate isomers in syntrophic association with hydrogenotrophic methanogens. Arch Microbiol. 185: 172-182.

Qiu, Y. L., Sekiguchi, Y., Imachi, H., Kamagata, Y., Tseng, I. C., Cheng, S. S., Ohashi, A. and Harada, H. (2003): *Sporotomaculum syntrophicum* sp. nov., a novel anaerobic, syntrophic benzoate-degrading

bacterium isolated from methanogenic sludge treating wastewater from terephthalate manufacturing. Arch Microbiol. 179: 242-249.

Rabenhorst, M. C., Wilding, L. P. and West, L. T. (1984): Identification of pedogenic carbonates using stable carbon isotope and microfabric analyses. Soil Sci Soc Am J. 48: 125-132.

Rabus, R. and Heider, J. (1998): Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Arch Microbiol. 170: 377-384.

Rabus, R., Nordhaus, R., Ludwig, W. and Widdel, F. (1993): Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol. 59: 1444-1451.

Rabus, R. and Widdel, F. (1995): Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol. 163: 96-103.

Radajewski, S., Ineson, P., Parekh, N. R. and Murrell, J. C. (2000): Stable-isotope probing as a tool in microbial ecology. Nature. 403: 646-649.

Radajewski, S., McDonald, I. R. and Murrell, J. C. (2003): Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. Curr Opin Biotechnol. 14: 296-302.

Ram, R. J., Verberkmoes, N. C., Thelen, M. P., Tyson, G. W., Baker, B. J., Blake, R. C., 2nd, Shah, M., Hettich, R. L. and Banfield, J. F. (2005): Community proteomics of a natural microbial biofilm. Science. 308: 1915-1920.

Ramos, J. L., Wasserfallen, A., Rose, K. and Timmis, K. N. (1987): Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science. 235: 593-596.

Rappe, M. S. and Giovannoni, S. J. (2003): The uncultured microbial majority. Annu Rev Microbiol. 57: 369-394.

Rayleigh, J. W. S. (1896): Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos Mag. 42: 493-498.

Reardon, C. L., Cummings, D. E., Petzke, L. M., Kinsall, B. L., Watson, D. B., Peyton, B. M. and Geesey, G. G. (2004): Composition and diversity of microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer. Appl Environ Microbiol. 70: 6037-6046.

Reasoner, D. J. and Geldreich, E. E. (1985): A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol. 49: 1-7.

Reinhard, M. (1993): *In-situ* bioremediation technologies for petroleum-derived hydrocarbons based on alternate electron acceptors (other than molecular oxygen). In: Handbook of Bioremediation. R. D. Norris, Hinchee, R. E., Brown, R., McCarty, P. L., Semprini, L., Wilson, K. J. T., D. H., Reinhard, M., Bouwer, E. J., Borden, R. and V. C., T. M., Thomas, J. M., Ward, C. H., (eds). Lewis Publishers.Ann Arbor, MI. 131-147.

Reinhard, M., Hopkins, G. D., Steinle-Darling, E. and LeBron, C. A. (2005): *In situ* biotransformation of BTEX compounds under methanogenic conditions. Ground Water Monitor Rem. 25: 50-59.

Reusser, D. E. and Field, J. A. (2002): Determination of benzylsuccinic acid in gasoline-contaminated groundwater by solid-phase extraction coupled with gas chromatography-mass spectrometry. J Chromatogr A. 953: 215-225.

Reusser, D. E., Istok, J. D., Beller, H. R. and Field, J. A. (2002): *In situ* transformation of deuterated toluene and xylene to benzylsuccinic acid analogues in BTEX-contaminated aquifers. Environ Sci Technol. 36: 4127-4134.

Rice, D. W., Grose, R. D., Michaelsen, J. C., Dooher, B. P., MacQueen, D. H. and Cullen, S. J. (1995). California leaking underground fuel tank (LUFT) historical case analyses. Sacramento, California, California State Water Resource Control Board: 22.

Richnow, H. H., Annweiler, E., Michaelis, W. and Meckenstock, R. U. (2003a): Microbial *in situ* degradation of aromatic hydrocarbons in a contaminated aquifer monitored by carbon isotope fractionation. J Contam Hydrol. 65: 101-120.

Richnow, H. H., Eschenbach, A., Mahro, B., Kastner, M., Annweiler, E., Seifert, R. and Michaelis, W. (1999): Formation of nonextractable soil residues: A stable isotope approach. Environ Sci Technol. 33: 3761-3767.

Richnow, H. H., Meckenstock, R. U., Reitzel, L. A., Baun, A., Ledin, A. and Christensen, T. H. (2003b): *In situ* biodegradation determined by carbon isotope fractionation of aromatic hydrocarbons in an anaerobic landfill leachate plume (Vejen, Denmark). J Contam Hydrol. 64: 59-72.

Rikken, G. B., Kroon, A. G. M. and Van-Ginkel, C. G. (1996): Transformation of (per)chlorate into chloride by a newly isolated bacterium: Reduction and dismutation. Appl Environ Microbiol. 45: 420-426.

Ringelberg, D. B., Talley, J. W., Perkins, E. J., Tucker, S. G., Luthy, R. G., Bouwer, E. J. and Fredrickson, H. L. (2001): Succession of phenotypic, genotypic, and metabolic community characteristics during *in vitro* bioslurry treatment of polycyclic aromatic hydrocarbon-contaminated sediments. Appl Environ Microbiol. 67: 1542-1550.

Rohwerder, T., Gehrke, T., Kinzler, K. and Sand, W. (2003): Bioleaching review part A: Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl Microbiol Biotechnol. 63: 239-248.

Rooney-Varga, J. N., Anderson, R. T., Fraga, J. L., Ringelberg, D. and Lovley, D. R. (1999): Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. Appl Environ Microbiol. 65: 3056-3063.

Rosell, M., Barcelo, D., Rohwerder, T., Breuer, U., Gehre, M. and Richnow, H. H. (2007): Variations in $^{13}C/^{12}C$ and D/H enrichment factors of aerobic bacterial fuel oxygenate degradation. Environ Sci Technol. 41: 2036-2043.

Ruske, R., Böhme, O. and Falke, P. (2001): Dokumentationsbericht Geo-Standortmanagement SAFIRA Zeitz. unpublished work.

Salanitro, J. P., Wisniewski, H. L., Byers, D. L., Neaville, C. C. and Schroder, R. A. (1997): Use of aerobic and anaerobic microcosms to assess BTEX biodegradation in aquifers. Ground Water Monit Remediat. 17: 210-221.

Sambrook, J. and Russell, D. W. (2001): Molecular cloning: a laboratory handbook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandrin, S. K., Brusseau, M. L., Piatt, J. J., Bodour, A. A., Blanford, W. J. and Nelson, N. T. (2004): Spatial variability of *in situ* microbial activity: biotracer tests. Ground Water. 42: 374-383.

Scheuerbrandt, G. and Bloch, K. (1962): Unsaturated fatty acids in microorganisms. J Biol Chem. 237: 2064-2068.

Schildkraut, C. L. (1967): The preparation and properties of ^{15}N-deuterated DNA from bacteria. In: Methods in Enzymology. L. Grossman and K. Moldave (eds). Academic Press.New York. 695-699.

Schink, B. (1997): Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev. 61: 262-280.

Schink, B., Brune, A. and Schnell, S. (1992): Anaerobic degradation of aromatic compounds. In: Microbial degradation of natural products. G. Winkelmann (eds). Weinheim, New York, Basel, Cambridge: VCH. 219-242.

Schirmer, M., Dahmke, A., Dietrich, P., Dietze, M., Gödeke, S., Richnow, H. H., Schirmer, K., Weiß, H. and Teutsch, G. (2006): Natural attenuation research at the contaminated megasite Zeitz. J Hydrol. 328: 393-407.

Schmidt, T. C., Zwank, L., Elsner, M., Berg, M., Meckenstock, R. U. and Haderlein, S. B. (2004): Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. Anal Bioanal Chem. 378: 283-300.

Schocher, R. J., Seyfried, B., Vazquez, F. and Zeyer, J. (1991): Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch Microbiol. 157: 7-12.

Schreiber, M. E. and Bahr, J. M. (2002): Nitrate-enhanced bioremediation of BTEX-contaminated groundwater: parameter estimation from natural-gradient tracer experiments. J Contam Hydrol. 55: 29-56.

Schroth, M. H., Istok, J. D., Conner, G. T., Hyman, M. R., Haggerty, R. and O'Reilly, K. T. (1998): Spatial variability in *in situ* aerobic respiration and denitrification rates in a petroleum-contaminated aquifer. Ground Water. 36: 924-937.

Schüth, C., Taubald, H., Bolano, N. and Maciejczyk, K. (2003): Carbon and hydrogen isotope effects during sorption of organic contaminants on carbonaceous materials. J Contam Hydrol. 64: 269-281.

Schulze, S. and Tiehm, A. (2004): Assessment of microbial natural attenuation in groundwater polluted with gasworks residues. Water Sci Technol. 50: 347-353.

Schwieger, F. and Tebbe, C. C. (1998): A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl Environ Microbiol. 64: 4870-4876.

Sessions, A. L. (2006): Isotope-ratio detection for gas chromatography. J Sep Sci. 29: 1946-1961.

Shaw, N. (1974): Lipid composition as a guide to the classification of bacteria. Adv Appl Microbiol. 17: 63-108.

Sherwood Lollar, B., Slater, G. F., Ahad, J., Sleep, B., Spivack, J., Brennan, M. and MacKenzie, P. (1999): Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: Implications for intrinsic bioremediation. Org Geochem. 30: 813-820.

Shields, M. S., Montgomery, S. O., Cuskey, S. M., Chapman, P. J. and Pritchard, P. H. (1991): Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl Environ Microbiol. 57: 1935-1941.

Shim, H., Hwang, B., Lee, S.-S. and Kong, S.-H. (2005): Kinetics of BTEX biodegradation by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* under hypoxic conditions. Biodegradation. 16: 319-327.

Shinoda, Y., Akagi, J., Uchihashi, Y., Hiraishi, A., Yukawa, H., Yurimoto, H., Sakai, Y. and Kato, N. (2005): Anaerobic degradation of aromatic compounds by *Magnetospirillum* strains: isolation and degradation genes. Biosci Biotechnol Biochem. 69: 1483-1491.

Shinoda, Y., Sakai, Y., Uenishi, H., Uchihashi, Y., Hiraishi, A., Yukawa, H., Yurimoto, H. and Kato, N. (2004): Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. Appl Environ Microbiol. 70: 1385-1392.

Shirai, K. (1987): Catechol production from benzene through reaction with resting and immobilized cells of a mutant strain of *Pseudomonas*. Agric Biol Chem. 51: 121-128.

Siering, P. L. (1998): The double helix meets the crystal lattice; the power and pitfalls of nucleic acid approaches for biomineralogical investigations. Am Mineral. 83: 1593-1607.

Sikkema, J., de Bont, J. A. and Poolman, B. (1995): Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev. 59: 201-222.

Slater, G. F., Ahad, J. M. E., Sherwood Lollar, B., Allen-King, R. and Sleep, B. (2000): Carbon isotope effects resulting from equilibrium sorption of dissolved VOCs. Anal Chem. 72: 5669-5672.

Smith, M. R. (1990): The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation. 1: 191-206.

Smith, M. T. (1999): Benzene, NQO1, and genetic susceptibility to cancer. Proc Natl Acad Sci USA. 96: 7624-7626.

Snijders, A. P. L., Walther, J., Peter, S., Kinnman, I., M.G.J., d. V., van de Werken, H. J. G., Brouns, S. J. J., van der Oost, J. and Wright, P. C. (2006): Reconstruction of central carbon metabolism in central carbon metabolism in *Sulfolobus solfataricus* using a two-dimensional gel electrophoresis map, stable isotope labelling and DNA microarray analysis. Proteomics. 6: 1518-1529.

Söhngen, N. L. (1913): Benzin, Petroleum, Paraffinol und Paraffin als Kohlenstoff- und Energiequelle für Mikroben. Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten. 37: 595-609.

Spormann, A. M. and Widdel, F. (2000): Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation. 11: 85-105.

Squillace, P. J., Moran, M. J. and Price, C. V. (2004): VOCs in shallow groundwater in new residential/commercial areas of the United States. Environ Sci Technol. 38: 5327-5338.

Stahl, W. J. (1980): Compositional changes and $^{13}C/^{12}C$ fractionations during the degradation of hydrocarbons by bacteria. Geochim Cosmochim Acta. 44: 1903-1907.

Stapleton, R. D. and Sayler, G. S. (1998): Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system. Microb Ecol. 36: 349-361.

Steinbach, A. (2003): Isotopenfraktionierung als Indikator für den biotischen und abiotischen Abbau von Xenobiotika in Grundwasserleitern. Fachbereich Geowissenschaften. Universität Hamburg. Hamburg. Dissertation: 183 p.

Steinbach, A., Seifert, R., Annweiler, E. and Michaelis, W. (2004): Hydrogen and carbon isotope fractionation during anaerobic biodegradation of aromatic hydrocarbons - a field study. Environ Sci Technol. 38: 609-616.

Stelzer, N. (2008): Entwicklung eines Testsystems zur Erfassung der *in situ* Biodegradation im Grundwasser. Faculty of Mathematics and Natural Sciences. Christian-Albrechts-Universität. Kiel. Dissertation: 179.

Stelzer, N., Buning, C., Pfeifer, F., Dohrmann, A. B., Tebbe, C. C., Nijenhuis, I., Kastner, M. and Richnow, H. H. (2006): *In situ* microcosms to evaluate natural attenuation potentials in contaminated aquifers. Org Geochem. 37: 1394-1410.

Streitwieser, A. and Heathcock, C. H. (1980): Organische Chemie. Verlag Chemie. Weinheim, Deerfield Beach, Basel. 1490 p.

Struchtemeyer, C. G., Elshahed, M. S., Duncan, K. E. and McInerney, M. J. (2005): Evidence for aceticlastic methanogenesis in the presence of sulfate in a gas condensate-contaminated aquifer. Appl Environ Microbiol. 71: 5348-5353.

Suchomel, K. H., Kreamer, D. K. and Long, A. (1990): Production and transport of carbon dioxide in a contaminated vadose zone: a stable and radioactive carbon isotope study. Environ Sci Technol. 24: 1824-1831.

Sundh, I., Borgå, P., Nilsson, M. and Svensson, B. H. (1995): Estimation of cell numbers of methanotrophic bacteria in boreal peatlands based on analysis of specific phospholipid fatty acids. FEMS Microbiol Ecol. 18: 103-112.

Suthersan, S. S. (1997): Remediation engineering: design concepts. Lewis Publishers (CRC Press). Boca Raton, FL.

Sweed, H. G., Bedient, P. B. and Hutchins, S. R. (1996): Surface application system for *in situ* ground-water bioremediation: site characterization and modeling. Ground Water. 34: 211-222.

Tan, H. M. and Mason, J. R. (1990): Cloning and expression of the plasmid-encoded benzene dioxygenase genes from *Pseudomonas putida* ML2. FEMS Microbiol Lett. 60: 259-264.

Tao, Y., Fishman, A., Bentley, W. E. and Wood, T. K. (2004): Oxidation of benzene to phenol, catechol, and 1,2,3-trihydroxybenzene by toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 and toluene 3-monooxygenase of *Ralstonia pickettii* PKO1. Appl Environ Microbiol. 70: 3814-3820.

Taylor, J. and Parkes, R. J. (1983): The cellular fatty acids of the sulphate-reducing bacteria *Desulfobacter* sp., *Desulfobulbus* sp., and *Desulvovibrio desulfuricans*. J Gen Microbiol. 129: 3303-3309.

Thierrin, J., Davis, G. B. and Barber, C. (1995): A ground-water tracer test with deuterated compounds for monitoring *in situ* biodegradation and retardation of aromatic hydrocarbons. Ground Water. 33: 469-475.

Tobler, N. B., Hofstetter, T. B. and Schwarzenbach, R. P. (2008): Carbon and hydrogen isotope fractionation during anaerobic toluene oxidation by *Geobacter metallireducens* with different Fe(III) phases as terminal electron acceptors. Environ Sci Technol. 42: 7786-7792.

Tomaszewski, J. E., Jerina, D. M. and Daly, J. W. (1975): Deuterium isotope effects during formation of phenols by hepatic monoxygenases. Evidence for an alternative to arene oxide pathway. Biochemistry. 14: 2024-2031.

Trüper, H. G. and Pfennig, N. (1992): The family *Chlorobiaceae*. In: The Prokaryotes, a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. A. Ballows, H. G. Trüper, M. Dworkin, W. Harder and K. H. Schleifer (eds). Springer-Verlag.Berlin. 3583-3592.

Tunlid, A. and White, D. C. (1992): Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In: Soil Biochemistry. G. Stotzky and J. M. Bollag (eds). Marcel Dekker.New York. 229-262.

Ulrich, A. C., Beller, H. R. and Edwards, E. A. (2005): Metabolites detected during biodegradation of $^{13}C_6$-benzene in nitrate-reducing and methanogenic enrichment cultures. Environ Sci Technol. 39: 6681-6691.

Ulrich, A. C. and Edwards, E. A. (2003): Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. Environ Microbiol. 5: 92-102.

Urey, H. C., Brickwedde, F. G. and Murphy, G. M. (1932): A H isotope of mass 2 and its concentration. Physical Review. 40: 1-15.

US-EPA (1998): Carcinogenic effects of benzene: an update. Washington, DC. 1-45.

van Agteren, M. H., Keuning, S. and Janssen, D. B. (1998): Handbook on biodegradation and biological treatment of hazardous organic compounds. Kluwer Academic Publishers. Dordrecht, The Netherlands.

Van Hamme, J. D., Singh, A. and Ward, O. P. (2003): Recent advances in petroleum microbiology. Microbiol Mol Biol Rev. 67: 503-549.

Vandamme, P. and Coenye, T. (2004): Taxonomy of the genus *Cupriavidus*: a tale of lost and found. Int J Syst Evol Microbiol. 54: 2285-2289.

Vestal, J. R. and White, D. C. (1989): Lipid analysis in microbial ecology: Quantitative approaches to the study of microbial communities. Bioscience. 39: 535-541.

Vieth, A., Kästner, M., Morasch, B., Meckenstock, R. U. and Richnow, H. H. (2001): Charakterisierung des biologischen Abbaus von BTEX im Grundwasser über Isotopenfraktionierung - Feldstudien. TerraTech. 5: 37-41.

Vieth, A., Kästner, M., Schirmer, M., Weiss, H., Godeke, S., Meckenstock, R. U. and Richnow, H. H. (2005): Monitoring *in situ* biodegradation of benzene and toluene by stable carbon isotope fractionation. Environ Toxicol Chem. 24: 51-60.

Villatoro-Monzon, W. R., Mesta-Howard, A. M. and Razo-Flores, E. (2003): Anaerobic biodegradation of BTEX using Mn(IV) and Fe(III) as alternative electron acceptors. Water Sci Technol. 48: 125-131.

Vogel, T. M. and Grbic-Galic, D. (1986a): Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl Environ Microbiol. 52: 200-202.

Vogel, T. M. and Grbic-Galic, D. (1986b): Transformation of toluene and benzene by mixed methanogenic cultures. Appl Environ Microbiol. 53: 254-260.

Vogt, C., Alfreider, A., Lorbeer, H., Ahlheim, J., Feist, B., Böhme, O., Weiss, H., Babel, W. and Wünsche, L. (2002): Two pilot plant reactors designed for the *in situ* bioremediation of chlorobenzene-contaminated ground water: hydrogeological and chemical characteristics and bacterial consortia. Water, Air, Soil Pollut Focus. 2: 161-170.

Vogt, C., Alfreider, A., Lorbeer, H., Hoffmann, D., Wuensche, L. and Babel, W. (2004): Bioremediation of chlorobenzene-contaminated ground water in an *in situ* reactor mediated by hydrogen peroxide. J Contam Hydrol. 68: 121-141.

Vogt, C., Cyrus, E., Herklotz, I., Schlosser, D., Bahr, A., Herrmann, S., Richnow, H.-H. and Fischer, A. (2008): Evaluation of toluene degradation pathways by two-dimensional stable isotope fractionation. Environ Sci Technol. 42: 7793-7800.

Vogt, C., Gödeke, S., Treutler, H. C., Weiss, H., Schirmer, M. and Richnow, H. H. (2007): Benzene oxidation under sulfate-reducing conditions in columns simulating *in situ* conditions. Biodegradation. 18: 625-636.

Wachter, T., Dethlefsen, F. and Dahmke, A. (2001): Neubewertung der Eisen (III)-Oxidationskapazität bei NA-Prozessen anhand Literatur- und Feldstandort-Daten. Tagungshandbuch - 3 Symposum Natural Attenuation, Dechema eV.

Waduge, W. A., Soga, K. and Kawabata, J. (2004): Effect of NAPL entrapment conditions on air sparging remediation efficiency. J Hazard Mater. 110: 173-183.

Wang, Y. and Huang, Y. (2001): Hydrogen isotope fractionation of low molecular weight *n*-alkanes during progressive vaporization. Org Geochem. 32: 991-998.

Wang, Y. and Huang, Y. (2003): Hydrogen isotopic fractionation of petroleum hydrocarbons during vaporization: implications for assessing artificial and natural remediation of petroleum contamination. Appl Geochem. 18: 1641-1651.

Ward, J. A. M., Ahad, J. M. E., Lacrampe-Couloume, G., Slater, G. F., Edwards, E. A., Sherwood Lollar, B. and Technology, E. S. (2000): Hydrogen isotope fractionation during methanogenic degradation of toluene: potential for direct verification of bioremediation. Environ Sci Technol. 34: 4577-4581.

Washer, C. E. and Edwards, E. A. (2007): Identification and expression of benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. Appl Environ Microbiol. 73: 1367-1369.

Weber, F. J., Isken, S. and de Bont, J. A. M. (1994): Cis/trans isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiology. 140: 2013-2017.

Weelink, S. A. B. (2008): Degradation of benzene and other hydrocarbons by anaerobic bacteria. Wageningen University. Wageningen, The Netherlands. Dissertation: 125 p.

Weelink, S. A. B., Tan, N. C. G., ten Broeke, H., van den Kieboom, C., van Doesburg, W., Langenhoff, A. A. M., Gerritse, J., Junca, H. and Stams, A. J. M. (2008): Isolation and characterization of *Alicycliphilus denitrificans* strain BC, which grows on benzene with chlorate as the electron acceptor. Appl Environ Microbiol. 74: 6672-6681.

Weelink, S. A. B., Tan, N. C. G., ten Broeke, H., van Doesburg, W., Langenhoff, A. A. M., Gerritse, J. and Stams, A. J. M. (2007): Physiological and phylogenetic characterization of a stable benzene-degrading, chlorate-reducing microbial community. FEMS Microbiol Ecol. 60: 312-321.

Weiner, J. M. and Lovley, D. R. (1998a): Anaerobic benzene degradation in petroleum-contaminated aquifer sediments after inoculation with a benzene-oxidizing enrichment. Appl Environ Microbiol. 64: 775-778.

Weiner, J. M. and Lovley, D. R. (1998b): Rapid benzene degradation in methanogenic sediments from a petroleum-contaminated aquifer. Appl Environ Microbiol. 64: 1937-1939.

Weismann, D. and Lohse, M. (2007): Sulfid-Praxishandbuch der Abwassertechnik- biogene Korrosion, Geruch, Gefahr verhindern und Kosten beherrschen. Vulkan-Verlag. 733.

Weiss, J. V. and Cozzarelli, I. M. (2008): Biodegradation in contaminated aquifers: incorporating microbial/molecular methods. Ground Water. 46: 305-322.

White, D. C. (1988): Validation of quantitative analysis for microbial biomass, community structure, and metabolic activity. Adv Limnol. 31: 1-18.

White, D. C. (1994): Is there anything else you need to understand about the microbiota that cannot be derived from analysis of nucleic acids? Microb Ecol. 28: 163-166.

White, D. C., Gouffon, J. S., Peacock, A. D., Geyer, R., Biernacki, A., Davis, G. A., Pryor, M., Tabacco, M. B. and Sublette, K. L. (2003): Forensic analysis by comprehensive rapid detection of pathogens and contamination concentrated in biofilms in drinking water systems for water resource protection and management. Environ Forensics. 4: 63-74.

White, D. C., Smith, G. A., Gehron, M. J., Parker, J. H., Findlay, R. H., Martz, R. F. and Fredrickson, H. L. (1983): The groundwater aquifer microbiota: Biomass, community structure, and nutritional status. Dev Ind Microbiol. 24: 201-212.

Whited, G. M. and Gibson, D. T. (1991): Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. J Bacteriol. 173: 3017-3020.

Widdel, F. and Rabus, R. (2001): Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol. 12: 259-276.

Wiedemeier, T. H., Miller, R. N., Wilson, J. T. and Kampbell, D. H. (1995): Significance of anaerobic processes for the intrinsic bioremediation of fuel hydrocarbons. In: Proceedings of the petroleum hydrocarbons and organic chemicals in ground water conference: prevention, detection, and remediation(eds). National Ground Water Association.Dublin, Ohio: NGWA. 49-61.

Wiedemeier, T. H., Rifai, H. S., Newell, C. J. and Wilson, J. T. (1999): Natural attenuation of fuels and chlorinated solvents in the subsurface. John Wiley & Sons, I. New York, Chichester, Weinheim, Brisbane, Singapore, Toronto. 617.

Wiesner, M. R., Grant, M. C. and Hutchins, S. R. (1996): Reduced permeability in groundwater remediation systems: role of mobilized colloids and injected chemicals. Environ Sci Technol. 30: 3184-3191.

Wilkes, H., Boreham, C., Harms, G., Zengler, K. and Rabus, R. (2000): Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. Org Geochem. 31: 101-115.

Wilkins, P. C., Dalton, H., Samuel, C. J. and Green, J. (1994): Further evidence for multiple pathways in soluble methane-monooxygenase-catalysed oxidations from the measurement of deuterium kinetic isotope effects. Eur J Biochem. 226: 555-560.

Wilkinson, S. G. (1988): Gram-negative bacteria. In: Microbial lipids. C. Ratledge and S. G. Wilkinson (eds). Academic Press.London. 299-488.

Wilmes, P. and Bond, P. L. (2004): The application of two-dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. Environ Microbiol. 6: 911-920.

Wilmes, P. and Bond, P. L. (2006a): Metaproteomics: studying functional gene expression in microbial ecosystems. Trends Microbiol. 14: 92-97.

Wilmes, P. and Bond, P. L. (2006b): Towards exposure of elusive metabolic mixed-culture processes: the application of metaproteomic analyses to activated sludge. Water Sci Technol. 54: 217-226.

Wilson, L. P. and Bouwer, E. J. (1997): Biodegradation of aromatic compounds under mixed oxygen/denitrifying conditions: a review. J Ind Microbiol Biotechnol. 18: 116-130.

Wilson, M. S., Bakermans, C. and Madsen, E. L. (1999): *In situ*, real-time catabolic gene expression: Extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. Appl Environ Microbiol. 65: 80-87.

Winderl, C., Schaefer, S. and Lueders, T. (2007): Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. Environ Microbiol. 9: 1035-1046.

Woese, C. R. and Fox, G. E. (1977): Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci U S A. 74: 5088-5090.

Worsey, M. J. and Williams, P. A. (1975): Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. J Bacteriol. 124: 7-13.

Yang, X., Beckmann, D., Fiorenza, S. and Niedermeier, C. (2005): Field study of pulsed air sparging for remediation of petroleum hydrocarbon contaminated soil and groundwater. Environ Sci Technol. 39: 7279-7286.

Yarmoff, J.-J., Kawakami, Y., Yago, T. and Nishimura, H. (1988): Benzene toxicity and yield improvement in a *cis*-benzeneglycol production process. J Ferment Technol. 66: 313-321.

Yerushalmi, L., Lascourreges, J. F. and Guiot, S. R. (2002): Kinetics of benzene biotransformation under microaerophilic and oxygen-limited conditions. Biotechnol Bioeng. 79: 347-355.

Yerushalmi, L., Lascourreges, J. F., Rhofir, C. and Guiot, S. R. (2001): Detection of intermediate metabolites of benzene biodegradation under microaerophilic conditions. Biodegradation. 12: 379-391.

Yu, C. P. and Chu, K. H. (2005): A quantitative assay for linking microbial community function and structure of a naphthalene-degrading microbial consortium. Environ Sci Technol. 39: 9611-9619.

Zelles, L. (1999): Fatty acid patterns of phospholipids and liposaccharides of microbial communities in soil: A review. Biol Fertil Soils. 29: 111-129.

Zengler, K., Richnow, H. H., Rossello-Mora, R., Michaelis, W. and Widdel, F. (1999): Methane formation from long-chain alkanes by anaerobic microorganisms. Nature. 401: 266-269.

Zhang, T., Ke, S. Z., Liu, Y. and Fang, H. P. (2005): Microbial characteristics of a methanogenic phenol-degrading sludge. Water Sci Technol. 52: 73-78.

Zhilina, T. N., Zavarzina, D. G., Kolganova, T. V., Tourova, T. P. and Zavarzin, G. A. (2005): "*Candidatus contubernalis alkalaceticum,*" an obligately syntrophic alkaliphilic bacterium capable of anaerobic acetate oxidation in a coculture with *Desulfonatronum cooperativum*. Microbiology. 74: 695-703.

Zucchi, M., Angiolini, L., Borin, S., Brusetti, L., Dietrich, N., Gigliotti, C., Barbieri, P., Sorlini, C. and Daffonchio, D. (2003): Response of bacterial community during bioremediation of an oil-polluted soil. J Appl Microbiol. 94: 248-257.

Zwank, L., Berg, M., Elsner, M., Schmidt, T. C., Schwarzenbach, R. P. and Haderlein, S. B. (2005): New evaluation scheme for two-dimensional isotope analysis to decipher biodegradation processes: Application to groundwater contamination by MTBE. Environ Sci Technol. 39: 1018-1029.

Zylstra, G. J. and Kim, E. (1997): Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. J Ind Microbiol Biotechnol. 19: 408-414.

Acknowledgments

I am indebted to Hans Richnow for the opportunity to prepare this thesis at the Department of Isotope Biogeochemistry working on the BEOQUE project and all the other projects he enabled me to be part of. Thank you for your continuing support and especially your constructive remarks during my thesis preparation. Many thanks go to Prof. Michael Schlömann for giving me the opportunity to submit and defend my thesis at the TU Bergakademie Freiberg. His lectures provided the basis for this thesis.

My very special thanks go to Carsten Vogt for best possible supervision of this thesis. He guided me through lab work, troubleshooting, writing, presentations, as if I had been his only student. Carsten, I owe so much to you. A thousand thanks to you. I'll keep in my mind: "Everything will be fine."

Thanks to Sabine Kleinsteuber and Antonis Chatzinotas for all the help and guidance during my molecular genetic studies. I have saved your numbers for future questions.

I also thank S. Hinke, K. Ethner, A. Heidtmann, V. Jaschik, C. Süring, A. Wichmann and U. Lohse for expert technical assistance. Special thanks go to Ursula Günther for backing me up whenever I needed one of the mass spectrometers and for being reachable at any time. I also thank our heart and soul of the Department, Liane Paul, who helped whenever bureaucracy was in sight.

Thanks to all people of the Departments ISOBIO, UMB and UBT and the people, I had the luck of getting to know during my time in Leipzig, for the wonderful time we spent together, especially Julia, Steffen, Petra, Danny, Axel, Benjamin, Martin, Florian and Jana.

My loving thanks go to Anke, Marie, Chucki, Stefan, Anja, Dori and Ben for the extraordinary straightforward and loving friendship.

I especially give my loving thanks to my family for all their love, faith, and continuing support. Without you I would never have come so far. I owe my warmest thanks to a very special person for everything. This is for you and (actually) because of you. Deine.

Publications

Herrmann S., Kleinsteuber S., Neu T.R., Richnow H.H. and Vogt C. (2008):
Enrichment of anaerobic benzene-degrading microorganisms by *in situ* microcosms.
FEMS Microbiol. Ecol. 63: 94–106

Fischer A., Herklotz I., Herrmann S., Thullner M., Weelink S.A.B., Stams A.J.M., Schlömann M., Richnow H.H. and Vogt C. (2008):
Combined carbon and hydrogen isotope fractionation investigations for elucidating benzene biodegradation pathways.
Environ. Sci. Technol., 42 (12): 4356–4363

Vogt C., Cyrus E., Herklotz I., Schlosser D., Bahr A., Herrmann S., Richnow H.H. and Fischer A. (2008):
Evaluation of toluene degradation pathways by two-dimensional stable isotope fractionation.
Environ. Sci. Technol., 42(21): 7793-7800

Herrmann S., Kleinsteuber S., Chatzinotas A., Kuppardt S., Lüders T., Richnow H.H. and Vogt C.:
Identification of anaerobic benzene-assimilating organisms by DNA stable isotope probing.
submitted to Environ. Microbiol.

Herrmann S., Vogt C., Fischer A., Kuppardt A., and Richnow H.H.:
Characterization of anaerobic xylene biodegradation by two dimensional isotope fractionation analysis. submitted to Environ. Microbiol.

Curriculum vitae

Steffi Herrmann
Ludwig-Wucherer-Straße 25
06108 Halle

Date of birth: 16 June 1980
German nationality

EDUCATION

11/2004 – 06/2005	**Diploma thesis**: „Investigations into benzene degradation under sulphate-reducing conditions at the contaminated test field Zeitz“ at the Department of Environmental Microbiology, Helmholtz- Centre for Environmental Research (Leipzig-Halle)
10/1999 – 06/2005	**Studies of Geoecology** at the Technische Universität Bergakademie Freiberg Majors: Environmental microbiology/biotechnology Environmental analytical- and geochemistry Pedology Minors: Environmental management and law Geoinformatics
1987 - 1999	**Abitur** at the `Manfred von Ardenne'-Gymnasium, Riesa

PROFESSIONAL EXPERIENCE

since 10/2005	**PhD student** at the Department of Isotope Biogeochemistry, Helmholtz- Centre for Environmental Research (Leipzig-Halle); Subject: "Molecular biological, physiological and isotope chemical analyses of BTEX (bio)degradation processes within a contaminated aquifer"
04/2004 – 05/2004 and 11/2003 – 01/2004	**Research assistant** at the National Research Centre for Environmental Toxicology in Brisbane, Australia; subject: Evaluation of pollutants in the water of Redcliffe using passive samplers
09/2003 – 10/2003	**Practical training** at the Department Groundwater Remediation, Helmholtz- Centre for environmental research (Leipzig-Halle); Subject: „Investigations into microbial degradation and ecotoxicological effects of monochloro-benzene within a constructed wetland (Bitterfeld)“
09/2002 – 12/2002	**Research assistant** at the Technische Universität Bergakademie Freiberg: "Preparation of soil samples for heavy metal analyses"

PUBLICATIONS

Peer-reviewed publications

Mueller J., Kapernick A., Wackwitz A., Herrmann S., Stephens S. (2005): Passive sampling of polar and nonpolar halogenated pollutants in aquatic environments of Redcliffe, Australia. Organohalogen Compounds (Electronic) 67: 363-366

Herrmann S., Kleinsteuber S., Neu T.R., Richnow H.H. and Vogt C. (2008): Enrichment of anaerobic benzene-degrading microorganisms by *in situ* microcosms. FEMS Microbiol. Ecol. 63: 94–106

Fischer A., Herklotz I., Herrmann S., Thullner M., Weelink S.A.B., Stams A.J.M., Schlömann M., Richnow H.H. and Vogt C. (2008): Combined carbon and hydrogen isotope fractionation investigations for elucidating benzene biodegradation pathways. Environ. Sci. Technol. 42 (12): 4356–4363

Vogt C., Cyrus E., Herklotz I., Schlosser D., Bahr A., Herrmann S., Richnow H.H. and Fischer A. (2008): Evaluation of toluene degradation pathways by two-dimensional stable isotope fractionation. Environ. Sci. Technol. 42(21): 7793-7800

Herrmann S., Kleinsteuber S., Chatzinotas A., Kuppardt S., Lüders T., Richnow H.H. and Vogt C. (2010): Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. Environ. Microbiol. 12(2): 401-411

Herrmann S., Vogt C., Fischer A., Kuppardt A., Richnow H.H. (2009): Characterization of anaerobic xylene biodegradation by two-dimensional isotope fractionation analysis. Environ. Microbiol. Rep. 1(6): 535-544

CONFERENCE CONTRIBUTIONS

VAAM
19/03/2006-22/03/2006
Jena, Germany

Poster: Enrichment of benzene degrading microorganisms under sulphate reducing conditions by *in situ* microcosms
*Herrmann S., Kleinsteuber S., and Vogt C.

VAAM
19/03/2006-22/03/2006
Germany, Jena

Poster: Monitoring *in situ*-benzene biodegradation within an anoxic BTEX contaminated aquifer using stable isotope fractionation analysis (SIFA)
*Fischer A., Vogt C., Herrmann S., Stelzer N., Theuerkorn K., Eisenmann H., and Richnow H. H

VAAM
01/04/2007-04/04/2007
Osnabrück, Germany

Poster: Detection of phenol as metabolite of benzene degradation in a mixed culture under sulphate reducing conditions
*Herrmann S., Vogt C., Weber S., and Richnow H.H.

VAAM 01/04/2007-04/04/2007 Osnabrück, Germany	Poster: Stable hydrogen and carbon isotope fractionation during microbial benzene degradation under aerobic and anaerobic conditions – a comparative study *Vogt C., Herklotz I., Herrmann S., Richnow H.H, and Fischer A.
EGU 15/04/2007-20/04/2007 Vienna, Austria	Presentation: Monitoring *in situ* benzene biodegradation in contaminated aquifers using compound-specific stable isotope analysis (CSIA) *Fischer A., Vogt C., Herrmann S., Theuerkorn K., Herklotz I., Thullner M., and Richnow H.-H.
ESIR 23/06/2007-28/06/2007 Napoca, Romania	Presentation: Compound-specific stable isotope analysis (CSIA) for monitoring *in situ* benzene biodegradation in Cluj-contaminated aquifers *Fischer A., Vogt C., Herrmann S., Theuerkorn K., Herklotz I., Thullner M., and Richnow H.-H.
SIMSUG 20/06/2007-22/06/2007 Newcastle, England	Presentation: Compound-specific stable isotope analysis (CSIA) for monitoring *in situ* benzene biodegradation in contaminated aquifers *Fischer A., Vogt C., Herrmann S., Theuerkorn K., Herklotz I., Thullner M., and Richnow H.-H.
BAGECO 23/06/2007-27/06/2007 Wernigerode, Germany	Poster: Detection of phenol as metabolite of benzene degradation in a mixed culture under sulphate reducing conditions *Herrmann S., Vogt C., Weber S., and Richnow H.H.
ISEB – ESEB – JSEB 09/07/2006-13/07/2006 Leipzig, Germany	Poster: Enrichment of benzene degrading microorganisms under sulphate reducing conditions by *in situ* microcosms *Herrmann S., Kleinsteuber S., and Vogt C.
HydroEco 11/09/2006-14/09/2006 Prague, Czech Republic	Poster: Characterization and assessment of BTEX bio-degradation within aquifers using compound-specific stable isotope analysis (CSIA) *Fischer A., Vogt C., Herrmann S., Stelzer N., Theuerkorn K., Herklotz I., Thullner M., and Richnow H. H.
Conference on Natural Attenuation 19/11/2007-21/11/2007 Frankfurt, Germany	Poster: Monitoring *in situ* benzene biodegradation in contaminated aquifers using compound-specific stable isotope analysis (CSIA) *Fischer A., Vogt C., Herrmann S., Theuerkorn K., Herklotz I., Thullner M., and Richnow H.-H.
Symposium Natural Attenuation 21/11/2007-22/11/2007 Frankfurt, Germany	Poster: Charakterisierung des Benzolabbaus mittels zweidimensionaler Isotopenfraktionierungsanalyse *Fischer A., Stelzer N., Theuerkorn K., Herklotz I., Herrmann S., Vogt C., and Richnow H.-H.
VAAM 09/03/2008-11/03/2008 Frankfurt, Germany	Poster: Two dimensial isotope fractionation as a tool to elucidate biodegradation pathways of toluene and xylene isomers under sulphate reducing conditions *Herrmann S., Kleinsteuber S., Wackwitz A., Fischer A., Richnow H.H., and Vogt C.

* presenting author

Scientific workshops and courses

Spring School 02/04/2006-06/04/2006 Leipzig, Germany	Microbial Activity at Biogeochemical Gradients Poster: Enrichment of benzene degrading microorganisms under sulphate reducing conditions by *in situ* microcosms *Herrmann S., Kleinsteuber S., and Vogt C.
International Course 13/11/2006-17/11/2006 Leipzig, Germany	Moderne Methoden der Analytik stabiler Isotope leichter Elemente
International Course 09/12/2006-15/12/2006 Munich, Germany	*In situ* detection of microbial structure and function in contaminated and pristine aquatic surface and subsurface systems
Spring School 04/03/2008-07/03/2008 Leipzig, Germany isome	Electron transfer processes at biogeochemical gradients Poster: Two-dimensional isotope fractionation as a tool to elucidate biodegradation pathways of toluene and xylene isomers under sulfate-reducing conditions *Herrmann S., Kleinsteuber S., Wackwitz A., Fischer A., Richnow H.H., and Vogt C.

* presenting author

Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:

Carsten Vogt (Chapter 1, 2, 3, 4, 5, 6)
Hans-Hermann Richnow (Chapter 1, 3, 5, 6)
Sabine Kleinsteuber (Chapter 2, 3, 5)
Antonis Chatzinotas (Chapter 3, 5)
Kai Müller (Chapter 6)
Anke Kuppardt (Chapter 5)
Steffen Kuppardt (Chapter 3)
Thomas Neu (Chapter 2)
Tillmann Lüders (Chapter 3)
Claus Härtig (Chapter 6)
Benjamin Remy (Chapter 5)

Weitere Personen waren an der Abfassung der vorliegenden Arbeit nicht beteiligt. Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen. Weitere Personen haben von mir keine geldwerten Leistungen für Arbeiten erhalten, die nicht als solche kenntlich gemacht worden sind.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

_____
Datum, Unterschrift