Functional and phylogenetic diversity of anaerobic BTEX-degrading microorganisms in contaminated aquifers

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Functional and phylogenetic diversity of anaerobic BTEX-degrading microorganisms in contaminated aquifers

Christian Winderl

Abstract: Microbial communities involved in anaerobic BTEX degradation in contaminated groundwater, and factors which control and limit their metabolic activities are poorly understood. Within this thesis, microbes involved in anaerobic toluene degradation at a number of impacted sites across Germany are investigated using tools of molecular microbial ecology. This is done to circumvent known pitfalls of cultivation-based environmental microbiology, and to provide a better understanding of the true key players in natural attenuation processes.

Due to the phylogenetic dispersal of anaerobic toluene degraders, a specific detection via ribosomal marker genes (e.g. 16S rRNA) is not possible. Therefore, a catabolic marker gene assay was developed in this thesis targeting key enzyme of anaerobic toluene degradation, the benzylsuccinate synthase (Bss). With this newly developed PCR assay, site-specific clusters of *bssA* sequences were shown for three different BTEX contaminated sites (Pasing, Flingern, and Testfeld Süd). At the Pasing site, known geobacterial *bssA* sequence types were exclusively detected, whereas the *bssA* sequences retrieved from the other sites formed distinct and so far unidentified clusters, which were named due to the site Flingern cluster "F1" and "F2" and Testfeld Süd cluster "T1" and "T2", respectively. The Flingern clusters F1 and F2 showed only a remote affiliation to the known proteobacterial lineages and the Testfeld Süd cluster T1/T2 sequence types were deeply branching, indicating a relevance of non-proteobacterial toluene degraders at first glance.

Via DNA-stable isotope probing (DNA-SIP) applied to Testfeld Süd sediments, the key players in anaerobic toluene breakdown at this site were identified as *Desulfosporosinus* related organisms (*Clostridia*), surprisingly not carrying any of the previously detected T1/T2 sequence types. The *bssA* sequences of these degraders were closely related to the previously detected Flingern F2 cluster, which is therefore assumed to represent the "true" clostridial *bssA*. Further *bssA* sequencing efforts applied to cultured *Desulfosporosinus* spp. may prove this hypothesis. The deeply branching T1/T2 sequences thus are putative *bssA*-homologues, which may be involved in other fumarate-adding reactions in anaerobic hydrocarbon degradation.

Furthermore, a *bssA* assay for the specific quantification of F1 cluster *bssA* via quantitative PCR (qPCR) was developed for the Flingern site. With this, it was possible to characterise in detail the spatial distribution of anaerobic toluene degraders over a depth transect of the Flingern contaminant plume. Due to high-resolution sampling made possible by Anneser and colleagues, different plume compartments and redox gradients could be identified to prevail at very small scales. The highest absolute and relative abundance of toluene degraders was detected within the sulfidogenic gradient zone underneath the plume core. This primary field data strongly supports validity of the "plume fringe concept", which predicts that highest degradation activities are occurring at the fringes of contaminant plumes. Bauer and coworkers have previously established this concept via laboratory experiments with two-dimensional (2D) flow-through systems.

Depth-resolved characterisation of total microbial communities at the Flingern site via 16S rRNA gene targeted T-RFLP analyses revealed pronounced shifts in community structure between contaminant and redox compartment. The F1 cluster *bssA* sequence types were

retrieved from the sulfidogenic gradient zone, the “hot spot” of anaerobic toluene degradation at the Flingern site. From the same samples, *Geobacter*-related organisms were identified as dominant and thus to be the likely organisms harbouring the as-yet unaffiliated F1-cluster *bssA* genes. Interestingly, organisms carrying the F1 *bssA* sequences were demonstrated to be capable of coupling toluene degradation to both iron-and sulfate-reduction via preliminary enrichment cultures from the site (Kunapuli et al.).

The *bssA* assay developed within this thesis provides valuable insights in the diversity, the identity, and the spatial distribution of microorganisms responsible for anaerobic toluene degradation in contaminated aquifers. With the help of this approach a broader knowledge of microbes involved in on-site biodegradation processes could be gained and it may be a valuable tool for further investigations of temporal dynamics in communities of anaerobic toluene degraders. To summarise, this data manifest that close correlation exists between the establishment of specialised degrader assemblages and the localisation of degradation and redox processes in contaminated aquifers. A further refinement of these links between intrinsic microbiota and their hydrogeochemical surrounding may help to forward new concepts to assess natural attenuation and for the management of contaminated sites.

Funktionelle und phylogenetische Diversität von anaeroben BTEX abbauenden Mikroorganismen in kontaminierten Grundwasserleitern

Christian Winderl

Kurzfassung: Die für den anaeroben Abbau von BTEX Substanzen verantwortlichen mikrobiellen Gemeinschaften, sowie die Faktoren, welche deren Metabolismus beeinflussen oder limitieren, sind bis jetzt wenig erforscht. In der vorliegenden Arbeit wurden an einigen kontaminierten Standorten in Deutschland mit Hilfe molekularbiologischer Methoden Mikroben untersucht, die am anaeroben Abbau von Toluol beteiligt sind. Diese Methoden wurden gewählt, um die bekannte Problematik bei der Kultivierung von Mikroben aus der Umwelt zu umgehen und um damit die Lebensweise der Mikroorganismen besser verstehen zu können, welche die Hauptrolle in natürlichen Selbstreinigungsprozessen spielen.

Aufgrund ihrer hohen phylogenetischen Diversität ist der spezifische Nachweis anaerober Toluolabbauer über ribosomale Marker Gene (z.B. 16S rRNS Gene) nicht möglich. Daher wurde in dieser Arbeit zunächst eine Nachweismethode über funktionelle (katabolische) Marker Gene entwickelt. Diese Gene kodieren für das Schlüssel-Enzym Benzylsuccinat Synthase (Bss), welches den ersten Schritt im anaeroben Toluolabbauweg katalysiert. An drei verschiedenen BTEX kontaminierten Standorten (Pasing, Flingern und Testfeld Süd) konnten mit der neuen PCR basierten Methode Standort-spezifische Gruppen, so genannte „Cluster", von *bssA* Sequenzen nachgewiesen werden. In Pasing wurden ausschließlich bekannte, zu *Geobacter* verwandte *bssA* Sequenzen detektiert, während die *bssA* Sequenzen der beiden anderen Standorte verschiedene, bis jetzt unbekannte Cluster bildeten, die nach den Standorten Flingern Cluster „F1" und „F2", sowie Testfeld Süd Cluster „T1" und „T2" benannt wurden. Die Flingern Cluster F1 und F2 wiesen nur eine entfernte Abstammung zu den bekannten *Proteobacteria* auf. Die Testfeld Süd Cluster T1/T2 waren besonders tief abzweigend, was auf den ersten Blick auf die Relevanz von nicht-proteobakteriellen Toluolabbauern hindeutete.

Mit Hilfe von „stable isotope probing" (SIP) gelang es eindeutig, *Desulfosporosinus* verwandte Organismen (*Clostridia*) als Hauptakteure des anaeroben Toluolabbaus am Standort Testfeld Süd zu identifizieren. Überraschenderweise trugen diese *Clostridien* nicht die kürzlich zuvor entdeckten T1/T2 Cluster *bssA* Gene, sondern *bssA* Sequenzen, die nah zu dem am Standort Flingern gefundenen F2 Cluster Sequenztyp verwandt sind. Daher wird nun angenommen, dass das F2 Cluster als das „echte" clostridielle *bssA* Cluster darstellt. Diese Hypothese kann durch die zukünftige Sequenzierung der *bssA* Gene kultivierter *Desulfosporosini* bestätigt werden. Die tief abzweigenden T1/T2 Sequenzen stellen hingegen wahrscheinlich *bssA* Homologe dar, welche bei anderen Fumarat addierenden Reaktionen im anaeroben Kohlenwasserstoffabbau beteiligt sein könnten.

Darüber hinaus wurde eine Methode entwickelt, welche die Quantifizierung der F1 Cluster *bssA* Gene am Standort Flingern mit Hilfe von quantitativer PCR (qPCR) erlaubt. Dies ermöglichte erstmals eine detaillierte Charakterisierung der räumlichen Verteilung von anaeroben Toluolabbauern innerhalb eines Tiefenprofils der Schadstofffahne in Flingern. Durch die hochauflösende Beprobung von Anneser und Kollegen, konnten die verschiedenen Bereiche und Redox Gradienten der Schadstofffahne auf sehr kleinen Skalen räumlich aufgelöst werden. Die höchste absolute und relative Abundanz von Toluolabbauern wurde in der sulfidogenen Zone unterhalb des Kernbereichs der Fahne festgestellt. Diese primären Felddaten erhärten die Gültigkeit des „plume fringe Konzeptes", einer Hypothese die besagt,

dass höchste Abbauraten an den Rändern einer Schadstofffahne statt finden. Bauer und Kollegen haben kürzlich dieses Konzept anhand von Laborversuchen mit zweidimensionalen (2D) Durchfluss-Systemen entwickelt.

Die tiefenaufgelöste Beschreibung der mikrobiellen Gemeinschaft am Standort Flingern mittels 16S rRNS T-RFLP Analyse ergab deutliche Veränderungen der Populationsstruktur besonders am Übergang des hoch kontaminierten zum sulfidogenen Bereichs der Schadstofffahne. Die F1 Cluster *bssA* Sequenztypen wurden aus der sulfidogenen Gradienten Zone gewonnen, dem „hot spot" des anaeroben Toluolabbaus am Standort Flingern. In denselben Proben wurden *Geobacter* verwandte Arten als dominante Bakterien identifiziert, daher sind diese mit hoher Wahrscheinlichkeit die Träger der bisher unidentifizierten F1 Cluster *bssA* Gene. Erstaunlicherweise konnte anhand von Anreicherungskulturen (Kunapuli at al.) dieses Standortes gezeigt werden, dass die Organismen, welche F1 Cluster *bssA* Gene tragen dazu in der Lage sind, den Toluolabbau sowohl mit Eisen- als auch mit Sulfat-Reduktion koppeln zu können.

Die in der vorliegenden Arbeit entwickelte *bssA* Nachweismethode ermöglicht wertvolle Einblicke in die Diversität, die Identität und die räumliche Verteilung von Mikroben, welche für den anaeroben Abbau von Toluol in kontaminierten Grundwasserleitern verantwortlich sind. Mit diesem Ansatz konnte das Wissen über Mikroorganismen erweitert werden, welche an biologischen Abbauprozessen vor Ort beteiligt sind und könnte auch ein wertvolles Werkzeug für die zukünftige Erforschung der zeitlichen Dynamiken in mikrobiellen Gemeinschaften anaerober Toluolabbauer darstellen. Zusammenfassend offenbaren diese Ergebnisse, dass in kontaminierten Grundwasserleitern ein enger Zusammenhang zwischen der Etablierung von spezialisierten Abbauer-Gemeinschaften und der Lokalität von Abbau- und Redox-Prozessen besteht. Die weitere Vervollständigung dieser Verbindungen zwischen der inhärenten Biozönose und ihrer hydrogeochemischen Umgebung kann helfen, neue Konzepte voran zu treiben, um im Hinblick auf den Umgang mit Altlasten natürliche Selbstreinigungsprozesse besser bewerten zu können.

Contents

Acknowledgements i
Abstract iii
Kurzfassung v
Contents vii
1. Introduction 1
1.1. Groundwater resources and contamination 1
1.2. Biogeochemistry and microbiota of contaminated aquifers 3
1.3. Biodegradation of BTEX compounds 5
1.4. Novel strategies to detect specific degraders 6
1.5. Aims of this thesis 10
1.6. References 11
2. Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker 19
2.1. Introduction 19
2.2. Experimental procedures 21
2.2.1. Pure cultures of anaerobic toluene degraders 21
2.2.2. Sampling sites and sample acquisition 21
2.2.3. Nucleic acid extraction 21
2.2.4. PCR amplification 22
2.2.5. Cloning and sequencing 22
2.2.6. Sequence data and phylogenetic analysis 22
2.3. Results 24
2.3.1. Primer design and novel *bssA* reference sequences 24
2.3.2. Diversity of *bssA* genes in three contaminated aquifers 27
2.3.3. Comparison of *bssA* and 16S rRNA phylogeny 29
2.4. Discussion 31
2.4.1. Design and potential of novel *bssA* primers 31
2.4.2. Diversity of *bssA* genes in the environment 32
2.5. References 34
3. Stable isotope probing of sulfate-reducing bacteria involved in toluene degradation in a tar-oil contaminated aquifer 39
3.1. Introduction 39
3.2. Experimental procedures 41
3.2.1. Sampling site and sample acquisition 41
3.2.2 Incubation of sediment material 41
3.2.3. Process measurements 41
3.2.4. Nucleic acid extraction and ultracentrifugation 43
3.2.5. Analyses of density-resolved DNA fractions 43
3.2.6. Cloning, sequencing and phylogenetic analysis 43
3.3. Results 44
3.3.1. Exposure of aquifer sediments to ^{13}C-toluene 44
3.3.2. Analysis of DNA-SIP gradient fractions 45
3.3.3. Identification of labelled 16S rRNA genes 47
3.3.4. *bssA* genes detected in labelled DNA fractions 50
3.4. Discussion 51
3.4.1. SIP under close-to-*in situ* conditions 51
3.4.2. Key players in ^{13}C-toluene degradation 51
3.4.3. Catabolic genes of clostridial key players 53
3.5. References 54

4. Depth-resolved microbial community structure and quantitative localisation of anaerobic toluene degraders in distinct redox zones of a tar-oil contaminant plume 57
4.1. Introduction 57
4.2. Experimental procedures 59
4.2.1. Site description, sediment sampling and biogeochemical measurements 59
4.2.2. Nucleic acid extraction 59
4.2.3. Quantitative PCR 59
4.2.4. 16S rRNA and *bssA* gene targeted T-RFLP fingerprinting 61
4.2.5. Cloning, sequencing, and phylogenetic analyses 61
4.3. Results 63
4.3.1. Depth distribution of contaminants and redox species in the plume 63
4.3.2. Quantitative distribution of bacteria and anaerobic toluene degraders 64
4.3.3. Depth-resolved shifts in bacterial communities 66
4.3.4. Bacterial 16S rRNA clone libraries from four depths 69
4.4. Discussion 73
4.4.1. Geochemical and microbial zonation in the Flingern plume 73
4.4.2. Depth-resolved microbial community distribution 74
4.4.3. Identification of zone-specific microbiota 74
4.4.4. Affiliation of the F1-cluster *bssA* genes 76
4.5. References 77
5. General conclusion and outlook 81
5.1. References 85
Appendix I
Curriculum vitae XVII
Publications in peer reviewed journals XVIII
Selected contributions to national and international scientific meetings XVIII

1. Introduction

1.1. Groundwater resources and contamination

Freshwater is one of the most valuable natural resources and an essential feature for life on planet earth. About 97.4% of the total free water is found in oceans as salt water (salinity 3.4%) and only ~2.6% is available as freshwater (salinity lower than 0.2%). From this freshwater (estimated at ~$3.5x10^7$ km^3) ~78% is stored in ice at the poles, ~22% is available as groundwater, and only the least portion of freshwater is present within lakes, rivers, soil moisture, biomass, or as gas in the atmosphere (Seiler, 1994). Mainly driven by the gravitational force of the earth, groundwater flows within an underground layer of water-bearing permeable rock and soil, termed the aquifer. In Europe up to 75% of the drinking water production is accomplished with groundwater (Griebler and Mösslacher, 2003). Furthermore, groundwater and other freshwater resources are used to an increasing degree for agricultural and industrial applications. Due to a fast growing population and increasing industrial development, the need for clean freshwater is permanently rising (EEA, 2005). Thus, groundwater represents one of humankind's most valuable ecological and economic resources. Groundwater exhibits, together with aquifer sediments, an underground habitat for many prokaryotic and eukaryotic organisms (Griebler and Mösslacher, 2003).

Contamination of groundwater is a great issue facing mankind today and will even gain more importance in the near future. Contamination events often occur due to human activities, material fatigue, or accidents (Christensen et al., 2000a; Roling and van Verseveld, 2002) and particular contaminant compounds are found typically correlated to certain industry sectors (Zamfirescu and Grathwohl, 2001b; Brena et al., 2005; Lee et al., 2006). An incredible number of different organic and inorganic substances is known to pollute groundwater world-wide (Coates and Anderson, 2000) e.g. petroleum compounds (Van Hamme et al., 2003b), chlorinated compounds (Marzorati et al., 2006) and pesticides from agricultural usage (Ralebits et al., 2002; Brena et al., 2005), toxic compounds from explosives and ammunition (Preiss et al., 2005), heavy metals (Anderson et al., 2003; Feris et al., 2003; Lee et al., 2006) and nuclear waste (Prohl et al., 2005). Each of these different compounds are characterised by distinct mobility, chemical reactivity and toxicity.

In this thesis, a special focus was put on petroleum contaminations, and all impacted aquifers analysed were at former gasworks sites. These typically tar-oil contaminated sites are characterised by contamination with monoaromatic and polyaromatic hydrocarbons (Anderson and Lovley, 1997b; Widdel and Rabus, 2001a; Zamfirescu and Grathwohl, 2001b; Meckenstock et al., 2004c; Meckenstock et al., 2004a). Hydrocarbons exhibit low chemical reactivity at room temperature and are commonly classified into four groups: alkanes (saturated hydrocarbons), alkenes, alkynes and aromatic hydrocarbons. Among the latter, polyaromatic hydrocarbons (PAH's) and the monoaromatic compounds benzene, toluene, ethylbenzene, and xylene (BTEX) are by far the most prevalent pollutants, and thus have attracted much research focus as model compounds to investigate hydrocarbon degradation in laboratory systems as well as in the field (Spormann and Widdel, 2000; Meckenstock et al., 2004c; Jahn et al., 2005). Especially toluene degradation has been studied in detail since the last decade (Anderson and Lovley, 1997b; Lin et al., 2002b; Chakraborty and Coates, 2004b).
Groundwater contamination with volatile and toxic BTEX compounds comprises a threat to public health. Besides irritating the respiratory tract, eyes and skin by contact, the main target of all BTEX compounds incorporated by humans and animals is the nervous system with

symptoms (dependent on concentration and exposure time) varying from vertigo up to severe neurotoxic effects. Benzene heavily affects the nervous and haematopoietic systems and prolonged exposure can cause the development of leukaemia. Metabolites appear to play a key role in benzene toxicity and carcinogenicity. It is generally believed that benzene metabolites bind covalently to cellular macromolecules (including nucleic acids and proteins) and may induce oxidative DNA damage, thereby leading to dysfunctions in several tissues. For a detailed summary of the toxicology and health effects on humans of both single BTEX compounds and mixtures see the study of the U.S. Agency for Toxic Substances and Disease Registry (ATSDR, 2004). Due to the relatively high solubility and toxicity BTEX compounds comprise a threat particularly to drinking water resources and according to the prevailing legislature the detection of these compounds within the respective catchment may lead to a local shutdown of drinking water supply.

Nevertheless, the microbial communities involved in contaminant degradation in the field and factors, which control and limit their activities, are still poorly understood. A detailed description of the structure and spatial distribution of the microbial communities present at contaminated sites contributes to enhance the current knowledge of biodegradation processes. This is hampered by the phenomenon that respective microorganisms are often hard to cultivate in the laboratory. Microbial ecologists using tools of molecular microbiology can circumvent these pitfalls and are able to characterise involved microorganisms without cultivation, and thus identify putative correlations between the general physico-chemical scenario, degradation processes, and microbial key players involved. E.g. the identification and localisation of specific degraders within different redox compartments of a contaminant plume allows answering the question whether degrader abundances and degradation rates are linked. This provides more insights on the complex mechanisms of biodegradation within contaminated aquifers and may lead to novel concepts for the assessment and prediction of natural attenuation (NA) processes in the field, e.g. based on the detection of microbial potentials and capacities for biodegradation. Comprehensive knowledge of factors, which control or limit contaminant breakdown in the environment is a prerequisite for successful risk assessment and health care (White et al., 1998a).

1.2. Biogeochemistry and microbiota of contaminated aquifers

Processes occurring in aquifer systems play an important role in maintaining water quality and to a large extent the indigenous microorganisms are responsible for natural attenuation. When a petroleum contamination event occurs, the spilled substances are transported along with the groundwater flow (see Fig. 1.1.). A contaminant plume is formed and the fate of these compounds depends on the chemical properties of the contaminants, as well as on the hydrological, geochemical and biological setting of the aquifer at this site. Dispersion and dilution processes influence contaminant transport and contaminant concentrations. However, the mass of contaminants is sustainably reduced only due to *in situ* biological degradation activities (Roling and van Verseveld, 2002). Microorganisms need both electron donors and electron acceptors for successful degradation, therefore degradation activities are expected to be highest in biogeochemical gradient zones, where both compounds meet and are available at the same time (see Fig. 1.1.) (Christensen et al., 2000a).

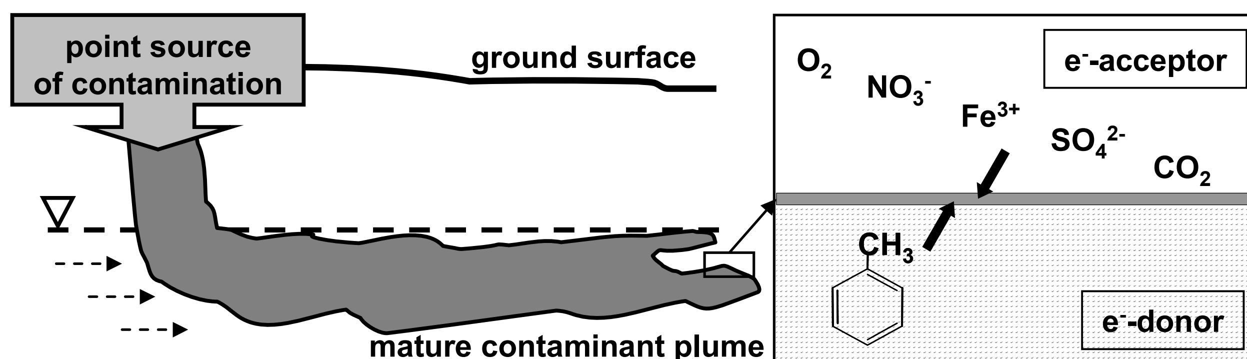

Figure 1.1.: Scheme of a mature contaminant plume transported with the groundwater flow (indicated by three arrows). Highest degradation activities are expected in zones, where electron-donors and electron-acceptors meet (indicated by two bold arrows).

Due to the relatively low amount of oxygen that can be dissolved in water and the slow rates of oxygen replenishment to groundwater systems, oxygen as electron acceptor is rapidly depleted upon contamination by respiratory activities (Anderson and Lovley, 1997b). Particularly relevant in such systems are microbial populations capable of coupling the degradation of contaminants to electron acceptors other than molecular oxygen (Coates and Anderson, 2000; Lovley, 2000), using different anaerobic terminal electron-accepting processes (TEAP's). These are redox half reactions describing only the process of electrons from a reduced electron-donor terminally being accepted by an oxidised species without paying attention to the actual electron-transfer chain and the role of intermediates (Christensen et al., 2000a). Thermodynamics predicts redox reactions to occur according to a "redox ladder" of decreasing possible energy gain for the involved microbes (Bradley and Chapelle, 1998; Christensen et al., 2000a). The most energy gain is coupled to aerobic respiration, followed by denitrification, iron reduction, sulfate reduction and finally methanogenesis, with the lowest yield of energy. Thus from the thermodynamic point of view the most favourable electron acceptors, which yield most energy gain will be depleted first (Christensen et al., 2000a). Notably, different respiratory guilds amongst the total microbial community are specialised to certain TEAP's and therefore, depending on the availability of electron-acceptors, they are (competitively or cooperatively) involved in degradation

processes under different redox conditions in the field (Lin et al., 2002b). Furthermore, fermentation and abiotic transformation processes may also play a role in degradation processes in the field (Maurer and Rittmann, 2004; Knoller et al., 2006). Assuming that within a contaminant plume substrates like e.g. BTEX compounds are available as electron-donors in non-limiting concentrations, the availability of electron-acceptors and their supply by diffusion and dispersion processes becomes a limiting factor for the respiratory activity of degraders and therefore net degradation capacities.

1.3. Biodegradation of BTEX compounds

BTEX degradation is known to occur under both aerobic and anaerobic conditions. Aerobic degraders transfer electrons gained from the substrates to oxygen. Of the BTEX compounds, toluene is degraded by many strains and different aerobic toluene degradation pathways have been described in several microorganisms, most of them *Pseudomonas* species or close relatives (Fang et al., 2004; Andreoni and Gianfreda, 2007). Certain steps of these pathways were redesigned with genetic engineering in order to create mutants with enhanced biodegradation capacities (Lee et al., 1995; Fishman et al., 2004; Tao et al., 2004). This may offer new possibilities for remediation by enhanced natural attenuation (ENA) (Godeke et al., 2006).

P. putida F1 oxidises toluene to toluene dihydrodiol, which is transformed to 3-methylcatechol and further metabolised through *meta* ring fission via enzymes encoded by the *tod* operon (Zylstra et al., 1988). *P. putida* mt-2 was reported to oxidise the methyl group of toluene to form benzoic acid prior to *meta* cleavage using genes encoded on the TOL plasmids (Assinder and Williams, 1990; Lee et al., 1995). *Burkholderia vietnamensis* G-4 (Newman and Wackett, 1995) and *B. pickettii* PKO1 (Olsen et al., 1994) transform toluene to o-cresol and m-cresol, respectively, which are metabolised to 3-methylcatechol further undergoing *meta* ring cleavage, whereas in *P. mendocina* KR1 toluene is transformed over p-cresol to 4-hydroxybenzoate, which is further degraded via *ortho* ring fission (Whited and Gibson, 1991a, b). To date, BTEX degradation was described for strains either only under aerobic or only under anaerobic conditions. Interestingly, *Thauera* sp. strain DNT-1 could be demonstrated recently to degrade toluene under aerobic as well as under denitrifying conditions when oxygen was depleted (Shinoda et al., 2004b).

Anaerobic BTEX degradation was first reported in the late 1980's under methanogenic and denitrifying conditions (Vogel and Grbic-Galic, 1986; Zeyer et al., 1986; Grbic-Galic and Vogel, 1987). Furthermore, respective transformation reactions could be demonstrated recently under manganese-reducing, iron-reducing, and sulfate-reducing conditions, in syntrophic associations, or by anoxygenic photosynthetic bacteria (Heider et al., 1998; Meckenstock, 1999b; Zengler et al., 1999; Christensen et al., 2000a; Van Hamme et al., 2003b). Our current knowledge of anaerobic BTEX degraders is almost exclusively based on isolation studies (Grbic-Galic and Vogel, 1987; Evans et al., 1991; Beller and Edwards, 2000; Meckenstock et al., 2004a; Jahn et al., 2005), and pure cultures are mostly available within different lineages of the *Proteobacteria* (for a review see (Widdel and Rabus, 2001a)). Recently, anaerobic toluene degraders have also been reported within the *Clostridia* (Liu et al., 2004b; Morasch et al., 2004a). Thus it is still unclear, whether the strains available in pure culture are of relevance for on-site toluene breakdown, but it has to be hypothesised that the real environmental key players in anaerobic BTEX degradation are still poorly described. Answering this question contributes to a comprehensive process understanding at contaminated sites. Based upon available information on the cultured proteobacterial lineages, field applicable assays to monitor the potential and capacity for intrinsic bioremediation processes can be developed. In this thesis the author is presenting the development and application of methods for detecting and characterisation of microbial degrader communities at contaminated sites via tools of molecular microbiology, in order to obtain monitoring environmental degradation processes.

1.4. Novel strategies to detect specific degraders

During the last two decades microbial ecology was revolutionised by the development of modern culture-independent methods like PCR-based detection assays combined with gene identification via phylogenetic analysis. Utilising the ribosomal rRNA gene as universal phylogenetic marker was one major strategy for microbiologists to overcome the “great plate count anomaly” (Amann et al., 1995) and to describe the whole diversity of microbial communities within their natural habitats. However, an rRNA approach is not feasible for investigating environmental microorganisms, which have a common physiological function in the environment, but are phylogenetically diverse (see Fig. 1.2.). In these cases, functional marker genes coding for characteristic key enzymes, which convey a certain catabolic or respiratory trait can be used to detect specific functional guilds involved in certain processes within their natural habitat in a cultivation-independent manner (Rotthauwe et al., 1997; Braker et al., 2000; Friedrich, 2005; Selesi et al., 2005; Wagner et al., 2005; Sipila et al., 2006).

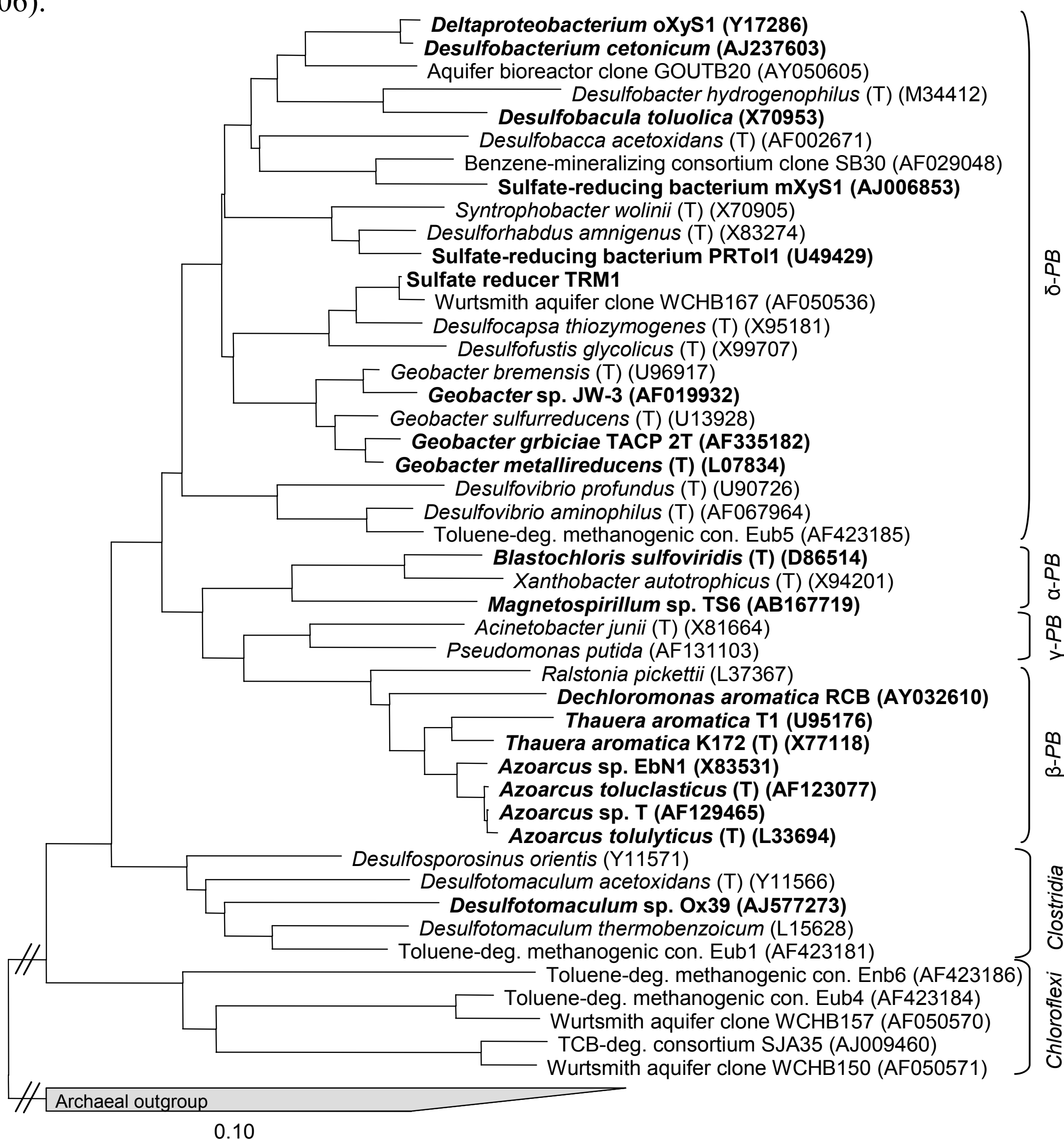

Figure 1.2.: Phylogeny of anaerobic toluene degraders within the Bacteria as reconstructed via their 16S rRNA genes. Anaerobic toluene degrading organisms are marked bold and phylogenetic affiliation is indicated. The scale bar represents 10% sequence divergence, branch lengths to the outgroup were scaled down by a factor of 8.

The key enzyme for anaerobic toluene and xylene degradation is the benzylsuccinate synthase (Bss) (Biegert et al., 1996b). It has a $\alpha_2\beta_2\gamma_2$ heterohexameric structure whose subunits are encoded by the *bssCAB* genes (Beller and Spormann, 1998; Leuthner et al., 1998b; Achong et al., 2001a; Kane et al., 2002b). Homologues in *Thauera aromatica* T1 were termed *tutFDG* genes (Coschigano, 2000). This enzyme is a highly oxygen sensitive glycyl radical enzyme (GRE) and belongs to the family of pyruvate formiate lyases (PFL, for an overview see (Lehtio and Goldman, 2004)). It catalyses the addition of the methyl group of the aromatic ring to the double bond of a fumarate cosubstrate leading to (R)-benzylsuccinate. With this initial reaction the Bss is rendering toluene accessible for ring cleavage (Fig. 1.3.). The subsequent beta oxidation catalysed by proteins encoded by another operon consisting of bbs (beta-oxidation of benzylsuccinate) genes leads to the central intermediate benzoyl coenzyme A (benzoyl-CoA) (Heider et al., 1998; Leuthner and Heider, 2000; Boll et al., 2002a).

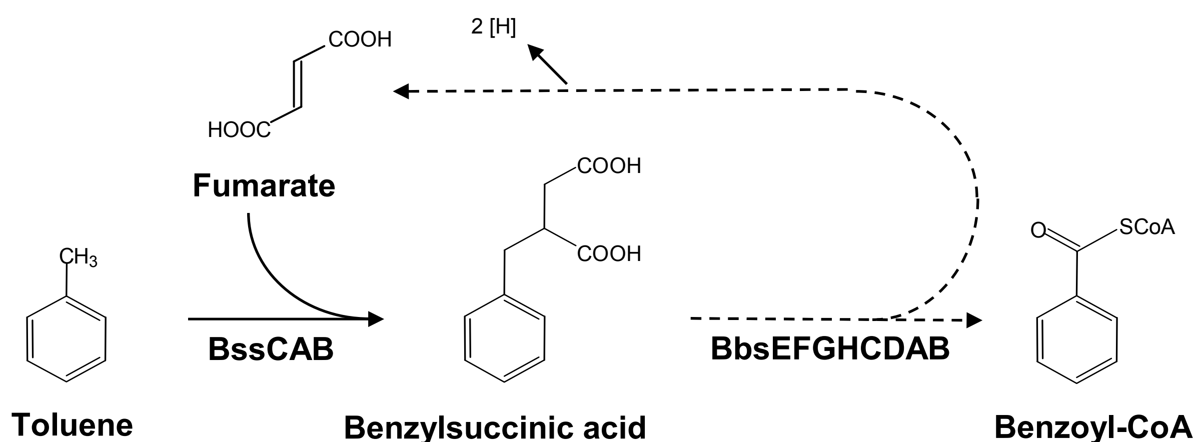

Figure 1.3.: Proposed initial reaction of toluene degradation via fumarate addition (simplified from (Leuthner and Heider, 2000; Boll et al., 2002a)). Stepwise β-oxidation and recycling of fumarate are indicated by dashed arrows.

However, only a limited number of *bssA* sequences from pure cultures are available: *Thauera aromatica* K172 (Leuthner et al., 1998b), *Thauera aromatica* T1 (Coschigano et al., 1998b), *Azoarcus* sp. T (Achong et al., 2001a), *Geobacter metallireducens* (Kane et al., 2002b), *Azoarcus* sp. EbN1 (Kube et al., 2004b), *Thauera* sp. DNT-1 (Shinoda et al., 2004b) and *Magnetospirillum* sp. TS-6 (Shinoda et al., 2005a). All of these reference cultures belong to the *Proteobacteria* and in most of these strains the *bss* genes are organised similarly within a *bssDCABEFGH* operon and upstream of the *bbsA-H* operon (Kube et al., 2004b). Upon start of this thesis work, only one primer (and qPCR probe) set was reported in the literature, useful only to detect *bssA* sequence types closely related to denitrifying *Azoarcus* and *Thauera* species (Beller et al., 2002a; Da Silva and Alvarez, 2004a; Shinoda et al., 2004b). More recently, *bssA* primer sets targeting a methanogenic culture were also published (Washer and Edwards, 2007a), concomitant to the publication of the primer sets developed in the present work (Winderl et al., 2007b). The sequence information of reference strains can be combined and used to establish a *bssA* gene based sequence alignment. Such a database allows for the development of PCR assays hopefully capable of covering a wide phylogenetic radiation to detect also unknown members of the functional guilds in the environment which carry *bssA* genes. Thus they can be identified as putatively capable of performing anaerobic toluene degradation via fumarate addition at contaminated aquifer sites.

Targeting the respective genes as described above leads to the information whether degraders are present or not within a sample. Additionally, the retrieved sequences can be identified via phylogenetic analysis. Moreover, with the information about abundances of bacteria and specific degraders, their importance within the environment can be inferred. For quantification of genes the real-time PCR technology can be used (Zhang and Fang, 2006). In quantitative PCR (qPCR), amplification is monitored after each PCR cycle via signal increase of a fluorescent dye. Quantification is accomplished via comparison of sample signals versus signals of serially diluted standard DNA measured simultaneously and generating a certain signal intensity after a certain cycle within PCR ("threshold cycle") according to initial template concentration (Heid et al., 1996). As with all methods, it is of special importance to be aware of the efficiency and biases the chosen methods exhibit for appropriate data interpretation. For qPCR strategies, DNA extraction and detection efficiency is a major source of bias (Mumy and Findlay, 2004).

Further, the diversity of a mixed gene pool present within a sample is assessable with genetic fingerprinting. One frequently used fingerprinting technique is terminal restriction length polymorphism (T-RFLP) (Marsh, 1999). Mixed amplicons, which were generated using a total community's metagenome as template for PCR, are discriminated via their phylogenetically conserved restriction sites. After restriction, fragments of different length are obtained and these fragments are labelled at one end due to the use of one fluorescent primer in the PCR. With sequencing technology, the different fragments can be linked to single species. Thus, the identification of shifts within the total community of different samples is possible in high throughput, which may yield important information on microbial distribution patterns of different samples (Ludemann et al., 2000; Buckley and Schmidt, 2001; Lueders and Friedrich, 2003a). Additionally, the identification of microbes is possible via signature T-RF's, which they may produce (Derakshani et al., 2001). However, T-RFLP fingerprinting can also be subject to different biases, e.g. in semi-quantitative approaches (Frey et al., 2006), or due to the formation of pseudo-T-RF's (Egert and Friedrich, 2003a).

As mentioned above, the detection of functional marker genes (e.g. *bssA*) implies the presence of microorganisms potentially capable of degrading toluene or xylene via fumarate addition. Yet, the question whether the organisms carrying the detected genes are active degrading the contaminants *in situ* remains unanswered. Stable isotope probing (SIP) allows for the detection and identification of microorganisms, which were assimilating carbon from a given substrate, and thus actively utilising the compound. Isotopically labelled contaminants serve as tracer for microbes metabolising them, and incorporate the isotopic label into different biomarker molecules (e.g. DNA, RNA, and PLFA). Finally, it is possible to discriminate between "utilisers" and "non-utilisers" via their biomarker molecules (Griffiths et al., 2004; Lueders et al., 2004a; Lueders et al., 2004d; Miller et al., 2004; Ginige et al., 2005; Tillmann et al., 2005; Leake et al., 2006; Buckley et al., 2007a, b; Lu et al., 2007; Manefield et al., 2007). Especially for the investigation of biodegradation, which is a complex environmental process conducted often by as-yet unidentified microbes, different SIP approaches can be helpful tools. For example, the aerobic degradation of naphthalene could be assigned to specific microbes in the field using SIP (Jeon et al., 2003b). For reviews of the application of SIP strategies in bioremediation see (Manefield et al., 2004; Wackett, 2004; Madsen, 2006). However, there are very few examples to date of the application of SIP under anaerobic conditions, and especially the application of SIP to questions of anaerobic contaminant breakdown remains promising, but also very challenging (Chang et al., 2005; Geyer et al., 2005; Kasai et al., 2006; Kunapuli et al., 2007)

With the described research strategies it is feasible to offer potential solutions for hitherto unanswered questions about microbial key players and the flow of carbon and energy in anaerobic hydrocarbon degradation processes in the field. This may, ultimately lead to a better understanding of natural attenuation processes occurring within the environment and thus contribute to successful risk assessment of contaminated sites.

1.5. Aims of this thesis

The aim of the present thesis is to characterise natural microbial communities involved in anaerobic toluene degradation in aquifer environments. Particularly, the use of the *bssA* gene as a specific functional marker for anaerobic BTEX degraders stands in a central focus, to provide cultivation-independent insights to intrinsic degrader communities in aquifers. Together with further tools of molecular microbial ecology, several research questions about on-site anaerobic BTEX degradation are tackled:

- Which bacteria carrying the genetic potential for degrading toluene are detectable at contaminated sites and how diverse are degrader assemblages?
- Are known (=cultured) toluene degraders of relevance in environmental degradation processes?
- Can novel toluene-degrading microorganisms be detected at contaminated sites (and possibly enriched and isolated)?
- Can the key players responsible for active breakdown of toluene be identified within polluted aquifers?
- What fraction of the total microbial community do specific degraders constitute in a given sample.
- How are contaminant-degrading microbial communities structured within the different redox zones of a contaminant plume and how does their spatial distribution reflect degradation processes?

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2. Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker

2.1. Introduction

Microbial degradation of contaminants is a major mechanism that protects groundwater resources against anthropogenic perturbations. Due to high carbon loads, contaminated aquifers are often anoxic. Particularly relevant in such systems are microbial populations capable of coupling the degradation of contaminants to electron acceptors other than molecular oxygen (Christensen et al., 2000). In many cases of anthropogenic pollution, petroleum hydrocarbons like polycyclic aromatic hydrocarbons (PAH) and benzene, toluene, ethylbenzene, xylene (BTEX) mixtures are of serious concern, and toluene is a frequent major constituent (Anderson and Lovley, 1997; Lin et al., 2002; Chakraborty and Coates, 2004).

A detailed characterisation of specific microbial community members responsible for degradation capacities is a prerequisite for a better understanding of biodegradation processes occurring at contaminated sites. However, the current knowledge of anaerobic toluene degraders is almost exclusively based on isolation studies, and pure cultures are mostly available within different lineages of the *Proteobacteria* (for a review see (Widdel and Rabus, 2001). Recently, anaerobic toluene degraders have also been reported within the *Clostridia* (Liu et al., 2004; Morasch et al., 2004). Due to this large phylogenetic diversity, a selective characterisation of anaerobic toluene degraders in the environment based on ribosomal marker genes is not possible. Here, as in other microbially mediated carbon transformation reactions (Galvao et al., 2005), it is helpful to target a conserved key enzyme in the unifying catabolic pathway of a functional guild, a so-called functional marker gene.

The benzylsuccinate synthase (Bss) is the key enzyme of anaerobic toluene degradation (Biegert et al., 1996). This highly oxygen sensitive glycyl radical enzyme (GRE) belongs to the family of pyruvate formiate lyases (PFL, for an overview see (Lehtiö and Goldman, 2004) and catalyses the addition of fumarate to the methyl group of the aromatic ring, thus rendering toluene accessible for ring cleavage and subsequent beta oxidation (Heider et al., 1999; Boll et al., 2002). However, only a limited number of *bssA* sequences from pure cultures are available to date (Coschigano et al., 1998; Leuthner et al., 1998; Achong et al., 2001; Kane et al., 2002; Kube et al., 2004; Shinoda et al., 2004; Shinoda et al., 2005). In order to develop a degenerate primer pair capable of detecting a potentially wide phylogenetic radiation of *bssA* genes in environmental samples, it is crucial to expand the number of reference *bssA* genes from pure cultures to search for conserved sequence motifs. In an iterative process, designed primers can be optimised and tested for their diversity coverage using both pure cultures and environmental samples. Reports in the current literature state only one primer (and qPCR probe) set that has been used to detect and quantify partial *bssA* genes in contaminated aquifer mesocosms (Beller et al., 2002; Da Silva and Alvarez, 2004). Due to primer and probe specificities, this assay is mainly useful for the detection of *bssA* genes closely related to that of denitrifying *Thauera* and *Azoarcus* species (H.R. Beller, personal communication and own findings).

In this study different degenerate *bssA*-targeted primer sets applicable to pure culture and environmental DNA extracts have been developed and tested. New *bssA* reference sequences from 6 anaerobic toluene degraders are presented. One developed primer pair, showing the best amplification efficiency and diversity coverage, was applied to DNA

extracted from three hydrocarbon contaminated aquifer sites characterised by different geochemical conditions. It can be demonstrated that this is reflected in distinct local communities of anaerobic toluene and hydrocarbon degraders, and that *bssA* genes closely related to those of known degraders as well as new and deeply-branching putative *bssA*-homologues can be detected. The presented results shed new light on the considerable environmental diversity of anaerobic hydrocarbon degraders, which is of great relevance for the understanding of biodegradation processes in polluted aquifers.

2.2. Experimental procedures

2.2.1. Pure cultures of anaerobic toluene degraders

Azoarcus sp. T DSM 9506, *Geobacter grbiciae* DSM 13689, *Desulfobacula toluolica* DSM 7467, *Desulfobacterium cetonicum* DSM 7267, and *Geobacter metallireducens* DSM 7210 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The strains were grown in 120 ml serum bottles sealed with butyl rubber stoppers in anaerobic media as specified by the DSMZ, except for *Azoarcus* sp. T, which was grown aerobically as specified by the DSMZ. *Geobacter* sp. TMJ1 (Kunapuli et al., unpublished), the *Desulfocapsa*-related sulfate reducer TRM1 (Meckenstock, 1999) and *Desulfotomaculum* sp. OX39 (Morasch et al., 2004) were from the institute culture collection. These strains were grown in anaerobic freshwater medium (Widdel and Bak, 1992) with addition of toluene as carbon source, and with 50 mM ferric citrate or 10 mM Na_2SO_4 as electron acceptors, respectively.

2.2.2. Sampling sites and sample acquisition

Aquifer sediment samples where taken from three distinct sites, one at the "Weyl area", a former gasworks site in Pasing near Munich, Germany (Totsche et al., 2006). Fresh sediment samples from different horizons of this tar-oil polluted aquifer were obtained in May 2005. Five monitoring wells (WYBR1-WYBR5) were constructed, distributed across increasingly contaminated zones (Zosseder, 2007). From each borehole, fresh sediment samples were taken at ~6 m, ~12 m, and ~18 m depth below ground (water table was at ~10 m depth). Samples for nucleic acid extraction were immediately frozen on dry ice. A description of the contaminated site and local biogeochemical conditions is available elsewhere (Totsche et al., 2006; Zosseder, 2007).

The second site was a tar-oil contaminated aquifer in Flingern near Duesseldorf, Germany, where samples from fresh sediment cores taken during the installation of multilevel monitoring well (ML19222) were obtained in June 2005. Sediment cores were taken between ~6 and ~13 m (groundwater table at ~6.40 m), immediately frozen on dry ice and stored at –20°C until DNA extraction. Details on the site can be obtained from (Wisotzky and Eckert, 1997; Eckert and al., 2000).

The third location was also a tar-oil contaminated former gasworks site (Testfeld Süd) near Stuttgart, Germany. Samples were taken in August 2004 in the form of sediment from the bottom of a monitoring well (B49, ~10 m depth, groundwater table at ~3.50 m depth), in an area in which anaerobic toluene degradation has been shown to occur (Griebler et al., 2004). Well sediment and groundwater samples were taken by pumping into sterile 1 l glass bottles, closed anoxically, and transported to the lab, where sub samples were immediately frozen for DNA extraction. For detailed site characteristics, see (Herfort et al., 1998; Bockelmann et al., 2001; Zamfirescu and Grathwohl, 2001).

2.2.3. Nucleic acid extraction

Grown strains (~75 ml culture in 120 ml serum bottles) were centrifuged five minutes at 5000 rpm in sterile plastic tubes. Medium supernatant was discarded and ~ 500 µl cell pellets were used for nucleic acid extraction. Similarly, ~ 500 µl aliquots of thawed wet

sediment material were taken for DNA extraction, following a bead beating protocol as previously described (Lueders et al., 2004). To compensate for the low amount of microbial biomass in aquifer sediments, up to 4 replicate extractions were combined into pooled 50 µl nucleic acid extracts and stored frozen (-20°C) in elution buffer (EB, Qiagen). The integrity and yield of extracted nucleic acids was checked by standard agarose gel electrophoresis and ethidium bromide staining, and by UV quantification (ND-1000 Spectrophotometer, NanoDrop Technologies).

2.2.4. PCR amplification

BssA fragments were PCR amplified using different primer sets (see Tab. 2.1.) and a mastercycler ep gradient (Eppendorf) with standard thermal cycling (3 min of initial denaturation (94°C), 30 - 35 cycles of amplification (30 s at 94°C, 30 s at 58°C, 60 s at 72°C), and 5 min of terminal extension (72°C) unless otherwise indicated. To optimise PCR for newly designed primer sets, annealing temperature gradients were run between 50°C and 60°C. One 50 µl PCR reaction contained nuclease-free H_2O, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 1 U Taq polymerase (all Promega), 10 µg BSA (Roche), 0.5 µM of each primer (MWG Biotech), and 1 µl of template DNA. These PCR conditions were adequate to amplify partial *bssA* amplicons from all tested pure culture and environmental DNA extracts with two exceptions: (I) a *bssA* amplicon of *Desulfotomaculum* sp. OX39 could only be obtained by 'quasi-nested' PCR (2 x 25 cycles, 5µl product of reaction 1 served as template for reaction 2). (II) MasterAmp PCR premix C (Epicentre Technologies) was used to amplify Testfeld Süd *bssA* amplicons. In both cases, only a mixture of unspecific and specific PCR products was obtained. The appropriately sized amplicons (~773 bp) were obtained by excision from a low-melt agarose gel (PeqLab) and purified (QIAquick Gel Extraction Kit, Qiagen) prior to cloning. All other pure culture amplicons were purified using the QIAquick PCR purification kit (Qiagen) for subsequent sequencing reactions. Environmental *bssA* amplicons from Pasing and Flingern were generated for cloning with the above standard reaction chemistry.

2.2.5. Cloning and sequencing

BssA fragments amplified from pure culture DNA were directly sequenced using the respective amplification primers, and internal primers (Fig. 2.1.), where possible. PCR products from environmental samples and from *Desulfotomaculum* sp. OX39 were cloned into *E. coli* JM109 prior to sequencing using the pGEM-T cloning kit (Promega). Clones were randomly selected and screened for correct insert size via vector targeted PCR and agarose gel electrophoresis. Sequencing was performed on a 3730 DNA Analyzer (Applied Biosystems) using BigDye terminator v3.1 chemistry according to the manufacturer's instructions. If a number of clones from a library showed no or only minimal diversity, amplicons from further clones were first screened by *Msp* I digestion for restriction fragment pattern similarities, to reduce redundancies in sequence generation.

2.2.6. Sequence data and phylogenetic analysis

All publicly available benzylsuccinate synthase operons and *bssA* gene sequences were integrated into an alignment using the ARB software (Ludwig et al., 2004). *De novo*

sequencing reads from pure cultures and clones were manually assembled and checked for quality using the SeqMan II software module (Lasergene 6 suite, DNAstar). Contigs were subsequently screened for similarities to published sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and integrated into the *bssA*-derived amino acid sequence alignment. An amino acid filter was generated from the data set to include only the BssA amino acid positions available for environmental clones (250 positions) into phylogenetic analyses (see also Appendix Fig. A.1.). Distance matrices were calculated using PROTDIST with the Dayhoff PAM 001 matrix as amino acid replacement model. Phylogenetic dendrograms of partial BssA sequences were reconstructed from distance matrices using Fitch analysis as previously described (Lueders et al., 2001), and verified by neighbour-joining, maximum likelihood, and treepuzzle methods. For phylogenetic analyses of 16S rRNA genes, the sequence alignment of the ARB 16S rRNA database was used. A phylogenetic tree of bacterial 16S rDNA sequences was reconstructed from a distance matrix (Kimura 2 parameter model as evolutionary distance correction) of almost complete 16S rRNA genes (~ 1300 bp; n = 45) using Fitch (with global rearrangement of branches and randomised species input order: random number seed 7, jumble 7 times) and validated by neighbour-joining, maximum likelihood, and treepuzzle methods as implemented in ARB. To avoid treeing artefacts caused by highly variable nucleotide positions or those that do not align unambiguously, only positions that were present in at least 50% of all sequences analysed were included for treeing. All pure culture and clone sequences from this study were deposited with GenBank under the Accession Numbers EF123662 to EF123703.

2.3. Results

2.3.1. Primer design and novel *bssA* reference sequences

A sequence alignment of all *bss* operon sequences from pure cultures available in the public databases has been established: *Thauera aromatica* K172 (Leuthner et al., 1998), *Thauera aromatica* T1 (Coschigano et al., 1998), *Azoarcus* sp. T (Achong et al., 2001), *Geobacter metallireducens* (Kane et al., 2002), *Azoarcus* sp. EbN1 (Kube et al., 2004), *Thauera* sp. DNT-1 (Shinoda et al., 2004), and *Magnetospirillum* sp. TS-6 (Shinoda et al., 2005). This alignment, which almost exclusively contains betaproteobacterial *bssA* references, was subsequently screened for conserved sequence motifs to design a number of degenerate primer sets. A summary of the designed PCR primers, their location within the *bss* operon of *Thauera aromatica* K172, and employed primer combinations are given in Tab. 2.1. and Fig. 2.1., respectively.

Table 2.1.: Primer sequences that target different sites and motifs of the *bssA* gene.

Primer	5' – 3' sequence	Reference
*bssA*f	ACG ACG GYG GCA TTT CTC	(Beller et al., 2002)
*bssA*r	GCA TGA TSG GYA CCG ACA	(Beller et al., 2002)
6759f	CCG GCA TCG AGC GCA TG	this study
6884f	GAC GAA TTC ATC GTC GGC TA	this study
6888f	AAT TCA TCG TCG GCT ACC ACG	this study
7772f	GAC ATG ACC GAC GCS ATY CT	this study
8546r	TCG TCG TCR TTG CCC CAY TT	this study
8816r	TTC TGG TTY TTC TGC ACY TT	this study
8828r	AGC AGR TTG SCC TTC TGG TT	this study

Numbering of *bssA* primer binding sites according to *Thauera aromatica* K172 *bss* operon nucleotide numbering (Leuthner et al., 1998).

With these, pure cultures of anaerobic toluene degraders were further tested for *bssA* amplicons, to expand the phylogenetic framework of *bssA* reference sequences especially within the *Deltaproteobacteria*, and also towards non-*Proteobacteria*.
Amplicons for *Desulfobacterium cetonicum* (Galushko and Rozanova, 1991), 794 bp), *Desulfobacula toluolica* (Rabus et al., 1993) 1805 bp), the sulfate reducer TRM1 (Meckenstock, 1999) 1836 bp), *Geobacter grbiciae* (Coates et al., 2001) 1911 bp), and *Geobacter sp.* TMJ1 (U. Kunapuli et al., unpublished, 1953 bp) were generated and directly sequenced (Tab. 2.2., Fig. 2.2.).

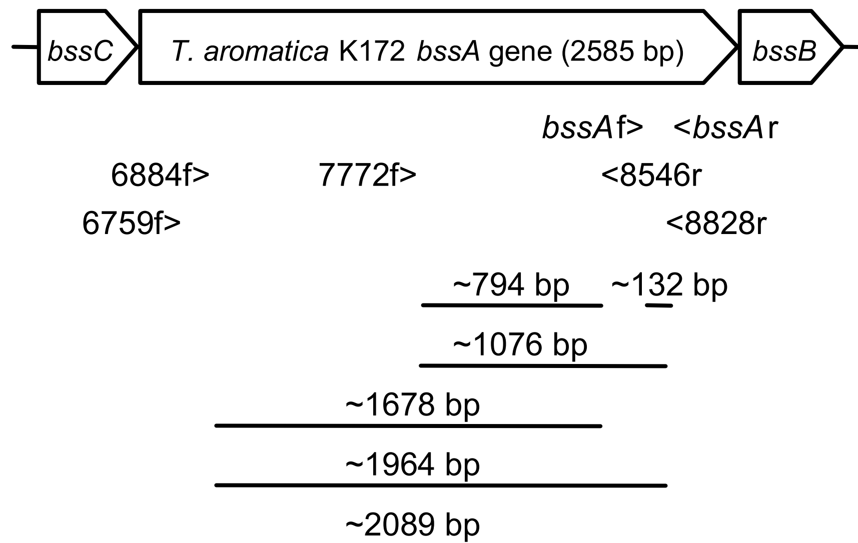

Figure 2.1.: Scheme of the localisation and possible combinations of primers tested in this study on the *bssA* gene of *Thauera aromatica* K172. Numbering of primer binding sites as in Tab. 2.1.

Table 2.2.: Various *bssA* primer combinations tested for the amplification of *bssA* amplicons (size, bp) from pure culture DNA extracts of anaerobic toluene degraders.

f-primer	*bssAf*	6759f	6884f	6884f	6888f	7772f	7772f
r-primer	*bssAr*	8828r	8816r	8828r	8546r	8546r	8828r
Amplicon [~ bp]	132	2089	1952	1964	1678	794	1076
Thauera aromatica K172	+[a]	–	–	–	+	++	+
Azoarcus sp. T	+[a]	+	++	+	++	++	++
Geobacter grbiciae	–	–	+[b]	n.a.	++	++	–
Geobacter sp. TMJ1	–	–	+[b]	n.a.	–	++	+
Desulfobacula toluolica	–	–	+[a]	+[b]	–	++	++
Desulfobacter cetonicum	–	–	–	–	–	+	–
Sulfate Reducer TRM1	n.a.	+[b]	+[a]	n.a.	+[a]	+[a]	++[a]
Desulfotomaculum sp. Ox39	n.a.	n.a.	n.a.	–	–	+/x	n.a.

Numbering of *bssA* primer binding sites as in Tab. 2.1.
++ = strong specific *bssA* amplicon; + = weak specific *bssA* amplicon;
x = unspecific products; – = no product; n.a. = not analysed
[a] annealing temperature = 52°C
[b] annealing temperature = 55°C

As shown in Tab. 2.2., the primer pair 7772f/8546r, generating ~794 bp amplicons, was most successful in generating specific PCR products with the full diversity of tested anaerobic toluene degraders. The only exception was the non-proteobacterial *Desulfotomaculum* sp. OX39 (Morasch et al., 2004), where a mixture of *bssA*-like and unspecific amplicons had to be cloned prior to sequencing. The clone library generated from these PCR products showed that most clones contained a 722 bp non-*bssA* amplicon with distant BLAST similarity to a *Desulfitobacterium hafniense* Y51 hypothetical protein (GenBank locus DSY2295, similarity to an activator of 2-hydroxyglutaryl-CoA dehydratase), which were apparently generated by primer mismatching to other gene targets on the *Desulfotomaculum* sp. OX39 genome. Nevertheless, clones with a slightly longer insert (773 bp) contained a sequence with clear *bssA* homology (Fig. 2.2.).

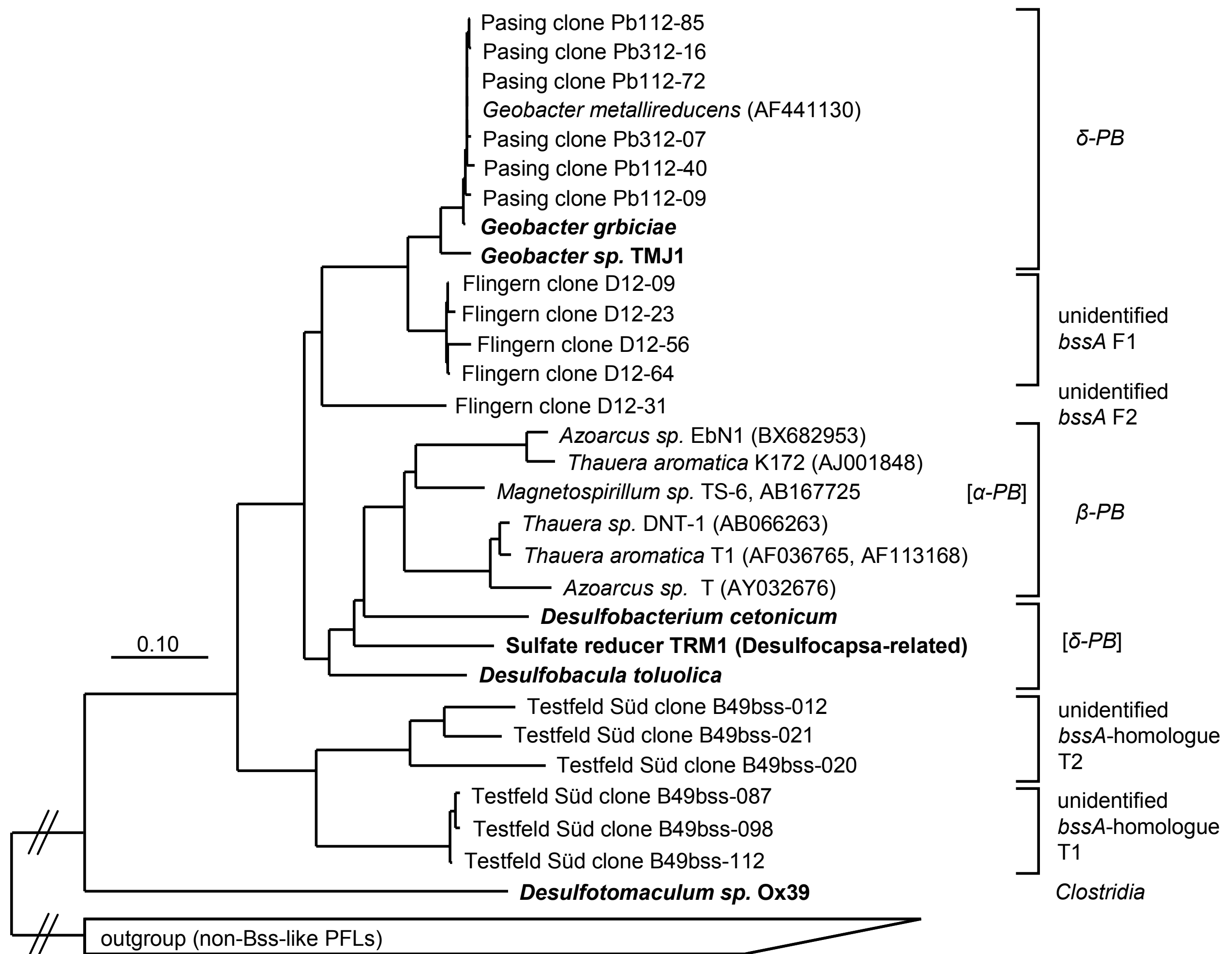

Figure 2.2.: Phylogenetic tree showing the affiliation of pure culture and environmental deduced BssA and putative homologous amino acid sequences. Samples were from three tar-oil contaminated aquifers (Pasing, Flingern, and Gaisburg). The tree was reconstructed with inferred amino acid sequences from aligned partial (~794 bp) *bssA* genes using Fitch distance matrix calculations. New pure culture reference sequences generated in this study are marked bold. GenBank accession numbers of available sequences are indicated. Major lineages of affiliation are given; *PB* = *Proteobacteria*. Brackets [ ] point out incongruencies to SSU rRNA phylogeny and indicate putative cases of lateral gene transfer. The tree was rooted with the following non-Bss PFL paralogues as outgroup: *Desulfitobacterium hafniense* (NCBI protein identifier numbers 23114478, 23114695) and *Desulfovibrio desulfuricans* (23473769, 23475526). The scale bar represents 10% sequence divergence, branch lengths to the outgroup were scaled down by a factor of 4.

This has only a remote affiliation with the known proteobacterial homologues (< 55% identity for inferred amino acids), but is still clearly connected to the Bss branch of the PFL gene family and distinct from other, more distantly related GREs (Fig. 2.2., see also (Lehtiö and Goldman, 2004)). However, based merely on primary sequence data, an unambiguous functional identification of this novel sequence type as 'true' Bss is clearly not possible. Nevertheless, the generation of this sequence may be an important prerequisite for the detection and affiliation of further deeply-branching *bssA*-homologues in environmental DNA extracts.

2.3.2. Diversity of *bssA* genes in three contaminated aquifers

In order to test the utility of the new 7772f/8546r primer set for the characterisation of natural communities of anaerobic toluene degraders, *bssA* amplicons from DNA extracts of three distinct hydrocarbon contaminated aquifers were generated. A brief characterisation of the three sites is summarised in Tab. 2.3.

Table 2.3.: Hydrogeochemical and contamination parameters of the three tar-oil contaminated aquifer sites investigated in this study.

Site	Munich-Pasing [a]	Duesseldorf-Flingern [b]	Stuttgart-Gaisburg [c]
Thickness of unsaturated zone [m]	10	6.4	3.5
Thickness of saturated zone [m]	10	7.5	3.5
Geology of saturated zone	gravel	coarse and fine sand, gravel	mostly gravel
Seepage velocity [m/d]	11 – 20	0.8 – 1.1	0.5 – 3.9
Typical contaminants	PAH, BTEX	BTEX, PAH	BTEX, PAH, phenol, cyanide
Dominant contamination (max.; min. conc.)	PAH	benzene (100 mg/l; 10 µg/l)	naphthalene (86 mg/l; 14 µg/l)
Dominant redox process	unknown	sulfate reduction	sulfate reduction

Data taken from [a] (Totsche et al., 2006; Zosseder, 2007), [b] (Wisotzky and Eckert, 1997; Eckert and al., 2000; Liebich et al., 2000), and [c] (Herfort et al., 1998; Bockelmann et al., 2001; Zamfirescu and Grathwohl, 2001).

Most importantly, the degradation of BTEX compounds has been shown to depend on sulfate reduction at two of the sites (Wisotzky and Eckert, 1997; Griebler et al., 2004), while the dominating respiratory process is unknown at the third site (Tab. 2.3.). Environmental *bssA* amplicons were easily amplified from several DNA extracts of the Pasing and Flingern sites. In contrast, the sediment DNA from Testfeld Süd produced only a faint band of amplicons with the expected size, again in combination with a smear of unspecific PCR products similar to the *Desulfotomaculum* sp. OX39 amplicons. The observed amplification problems were not caused by PCR inhibition in this extract, since all obtained DNA extracts were routinely checked with bacterial 16S rRNA gene-targeted PCR using standard protocols (data not shown), and no inhibition was observed. Rather, it appeared to be an indication that the local *bssA* or *bssA*-homologous gene pool at Testfeld Süd was more distinct from the proteobacterial *bssA* genes that has been used for primer design.

The phylogenetic affiliation of representative *bssA* sequences from the three sites within the framework of pure culture references is depicted in Fig. 2.2. From the Pasing, Flingern, and Testfeld Süd *bssA* libraries, 186, 92 and 26 clones were obtained, respectively. To reduce the sequencing of redundant clones, amplicons from Pasing and Flingern were screened for *Msp* I restriction fragment patterns, and only a small number of representative clones (16, Pasing; 10, Flingern; and 26, Testfeld Süd) for each restriction pattern were fully sequenced. All clones from Pasing were identified as being very closely related to *Geobacter* spp. *bssA* genes (over 99% deduced amino acid identity, respectively). The two wells (WYBR5 and WYBR2) investigated at this site were ~75 m apart, the first characterised by lower and the second by higher contamination (Zosseder, 2007). Still, they both showed identical *bssA* community composition (Pb112 and Pb312 clones, respectively, see Fig. 2.2.). The clone library from a highly contaminated saturated zone sample of the Flingern aquifer (D12 clones, 6.8 m depth) showed a distinct *bssA* gene pool (Fig. 2.2.). Here, the detected *bssA*-like genes originated almost exclusively from a novel lineage, branching between the known delta- and betaproteobacterial references. This cluster showed ~90 % amino acid identity to geobacterial *bssA* genes and was entitled the F1 cluster of unidentified *bssA* genes. Furthermore, one distinct clone was retrieved that showed only ~ 79% deduced amino acid sequence identity to the other Flingern *bssA* clones (F2 lineage, Fig. 2.2.). It was also attempted to generate *bssA* amplicons for different horizons of the Flingern depth transect through the contaminant plume. Despite the saturated zone here being completely reduced and dominated by sulfate reduction (Wisotzky and Eckert, 1997), appreciable amounts of *bssA* amplicons were only detected in zones of high contamination between 6.5 and 7.4 m depth, while below the plume (below 7.4 m), or in sediments from the vadose zone (above 6.3 m), they were either hardly detectable or completely absent.

Finally, 10 *bssA*-homologous clones retrieved from the Testfeld Süd formed two deeply branching clusters outside the known proteobacterial *bssA* genes (T1 and T2 clusters, Fig. 2.2.). They were < 70% related to the proteobacterial reference genes, and also only distantly related to the novel *bssA* homologue that was found within the Gram positive *Desulfotomaculum* sp. OX39 (< 53% identity). Furthermore, despite correct insert size, the Testfeld Süd clone library contained substantial ratios of non-*bssA*-like amplicons (16 clones). By BLAST, these were found to show low sequence similarities to ABC transporter ATP-binding proteins of diverse origin. Therefore, no systematic overlap was observed to the unspecific 722 bp clones retrieved from OX39 genomic DNA described above, and there is no evidence for a targeted mispriming of the new *bssA* primer pair to non-Bss-like PFL paralogues that may be present in microbial contaminant degraders.

2.3.3. Comparison of *bssA* and 16S rRNA phylogeny

Due to the considerable phylogenetic radiation of *bssA* genes detected within the pure cultures and in environmental samples, the phylogeny reconstructed for the *bssA* marker gene has been compared with the 16S rRNA based phylogeny of anaerobic toluene degraders. All currently available 16S rRNA gene sequences of described anaerobic toluene degraders and selected clones retrieved from anaerobic toluene-degrading enrichment cultures (Ficker et al., 1999) were entered into a database to reconstruct their phylogeny (Fig. 2.3.).

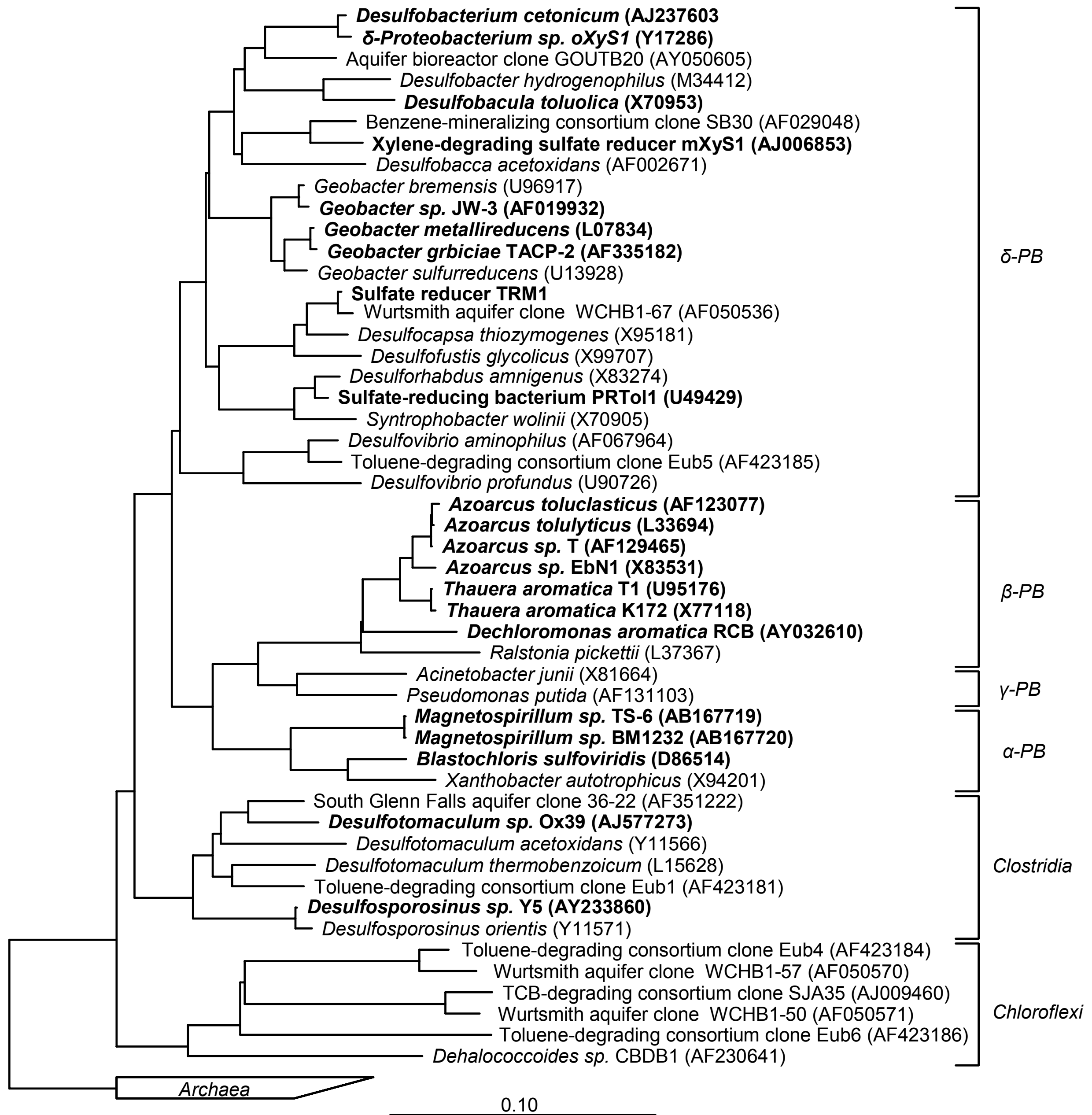

Figure 2.3.: Phylogeny of anaerobic toluene degraders within the *Bacteria* as reconstructed via their SSU rRNA genes. Known strains of anaerobic toluene degraders are marked in bold. Major lineages of affiliation are given; *PB* = *Proteobacteria*. The scale bar represents 10% sequence divergence. GenBank accession numbers are indicated.

As mentioned above, most anaerobic toluene degraders are affiliated to the *Proteobacteria*, particularly to the delta- und beta-subdivisions. Surprisingly, some of the new reference *bssA* sequences determined for members of the *Deltaproteobacteria* (i.e. *D. cetonicum*, *D. toluolica*, and the strain TRM1) were shown to carry *bssA* genes more closely related to that of the *Betaproteobacteria*. The Flingern *bssA*-like clones are related to deltaproteobacterial *bssA* genes (Fig. 2.2.), and there are further anaerobic toluene degraders described for the *Deltaproteobacteria* (Fig. 2.3.) not yet represented on *bssA* level. However, based on this phylogenetic comparison, no affiliation of the unidentified Flingern *bssA* or Testfeld Süd *bssA*-homologous sequences was possible.

2.4. Discussion

2.4.1. Design and potential of novel *bssA* primers

In this study a new and targeted detection system for anaerobic toluene and hydrocarbon degraders is presented. This offers new assessment possibilities for this important guild of BTEX degraders directly within their natural habitats, and means to investigate how such communities are composed and distributed at contaminated sites. Degradation gene-based assays provide an important tool for monitoring intrinsic or enhanced bioremediation processes and for risk assessment at perturbed groundwater environments (White et al., 1998; Röling and van Verseveld, 2002; Meckenstock et al., 2004). In contrast to the copious assays available for detecting and quantifying specific genes involved in aerobic BTEX degradation (Baldwin et al., 2003; Junca and Pieper, 2004; Taylor and Janssen, 2005; Hendrickx et al., 2006), reports on anaerobic key players are very limited. The benzoyl coenzyme A reductase (BCR), a central enzyme in the anaerobic degradation of aromatic compounds, is one suitable functional marker (Hosoda et al., 2005; Song and Ward, 2005). Only one assay has been described to date for the amplification and quantification of *bssA* sequences (Beller et al., 2002; Da Silva and Alvarez, 2004). However, the design of an 'optimum' primer pair particularly depends upon the available reference genes. Thus it is not surprising that the first published *bssA* assay (Beller et al., 2002) and also some of the new primer sets developed in this study (Tab. 2.2.) displayed only a limited capacity to detect *bssA* genes outside the denitrifying *Betaproteobacteria*.

Here, new *bssA* sequence information from anaerobic toluene degraders was generated, especially among the sulfate reducers. The phylogenetic comparison of ribosomal and *bssA* marker genes for anaerobic toluene degraders presented in this study (Figs. 2.2. & 2.3.) seem to indicate that lateral gene transfer for this catabolic marker has occurred in several events. The deltaproteobacterial *D. cetonicum*, *D. toluolica*, and TRM1 were shown to carry *bssA* genes more closely related to that of the *Betaproteobacteria*. Similarly, this has been reported for the *bssA* gene of the alphaproteobacterial *Magnetospirillum* sp. TS-6, which clusters amidst other betaproteobacterial *bssA* genes (Fig. 2.2.; (Shinoda et al., 2005). Cases of lateral gene transfer, even between domains, are well known for other GREs (Lehtiö et al., 2006), and have been reported also for aromatics degradation genes in aquifer microbes (Zhou and Tiedje, 1995; Herrick et al., 1997; Hendrickx et al., 2006). Such lateral gene transfers may, during the months and years, allow microbial populations to adapt to drastically changing environmental conditions such as the impact of organic contaminants.

The 7772f/8546r primer combination displayed the best diversity coverage for the tested pure cultures (Tab. 2.2.) and was therefore selected for detecting *bssA* in the environment. It must be noted here that for the putative non-proteobacterial deeply-branching *bssA*-homologue of *Desulfotomaculum* sp. OX39, specific amplicons remain hard to generate, and their identity remains uncertain. A functional identification of such novel Bss-homologous sequence types by phylogenetic extrapolation is clearly not possible. Fumarate addition is becoming more and more recognised as a common strategy in anaerobic hydrocarbon degradation and succinate adducts have been detected in sulfate reducers while degrading ethylbenzene (Kniemeyer et al., 2003) and alkanes (Cravo-Laureau et al., 2005). Although still unknown, it can be expected that the involved key enzymes will be close homologues of Bss. Benzylsuccinate has been detected in the supernatant of toluene-grown OX39 cells (Morasch et al., 2004), which clearly points towards the presence of Bss in this

strain. But still, although OX39 does not utilise ethylbenzene (Morasch et al., 2004), it remains unclear whether its 'true' *bssA* gene has been detected or just the alpha-subunit of a closely related fumarate-adding homologue.

In this short sequence, a lack of seven amino acids could be observed (positions 466-474 in the *T. aromatica* K172 BssA sequence (Leuthner et al., 1998); see also Appendix Fig. A.1.) just a few amino acids upstream of the highly conserved cysteine (K172 position 489). This cysteine, together with the highly conserved glycine located towards the C-terminus of GREs, forms part of the enzymes catalytic site (Selmer et al., 2005). In a homology analysis of glycyl radical enzymes, Leuthner et al. (1998) describe a similar gap of amino acids for non-Bss PFLs. On the other hand, the OX39 sequence shares many characteristic sequence motifs with the known BssA proteins, but not with other more distantly related non-Bss PFLs (Appendix Fig. A.1.). Thus, although a role in anaerobic hydrocarbon degradation seems highly likely, more detailed investigations are necessary to identify the true functionality and substrate specificity of the detected *bssA*-homologue in strain OX39.

2.4.2. Diversity of *bssA* genes in the environment

The clone libraries showed that different tar-oil contaminated aquifer sediments can harbour distinct populations of intrinsic anaerobic toluene and hydrocarbon degraders. This provides evidence that the diversity of this functional guild within contaminated environments is far greater than previously recognised. In both Flingern and Testfeld Süd aquifer sediments, BTEX degradation has been shown to proceed via sulfate reduction (Wisotzky and Eckert, 1997; Griebler et al., 2004). The detection of the corresponding hydrocarbon degradation genes provides evidence that yet unidentified key players are responsible for BTEX degradation at both sites. The *bssA*-like genes detected at the Flingern site are very likely to be functional as 'true' Bss enzymes, as they are very closely related to geobacterial *bssA* genes and share almost all conserved amino acids with other well-known BssA sequences (see Appendix Fig. A.1.).

Still, as already discussed above, conclusions on substrate-specificity for these genes have to be cautioned as long as further evidence is not available. Enrichment cultures grown on toluene from Flingern sediments, where the expression of the detected *bssA*-like genes could be investigated are currently under way.

Even more, such additional functional information is necessary for the deeply-branching Testfeld Süd *bssA*-homologous sequences, which share less of the conserved sequence motifs with 'typical' BssA (Appendix Fig. A.1.). However, it can be concluded from the results that well-known proteobacterial *bssA* genes are absent at this site, since a certain preferential amplification of such sequence types due to PCR selection by the degenerate primers (Wagner et al., 1994; von Wintzingerode et al., 1997) can be postulated.

At the Pasing site, the detected gene pool is very closely related to well-known *Geobacter* spp. *bssA* and thus provides evidence for an importance of iron reduction for toluene degradation in these sediments. Here however, the dominating redox process is not known (Tab. 2.3.). Due to the high groundwater flow velocity of the Munich gravel plain it remains questionable, whether anaerobic degradation processes can be expected to dominate here. Oxygen for aerobic degradation may be readily replenished with groundwater flow. Consequently, anaerobic toluene degradation by *Geobacter* spp. may only be of relevance in anaerobic micro-environments or at the fringe of the underlying aquitard (~20 m depth).

The observed high phylogenetic redundancy and the complete lack of overlap discovered for local *bssA* or -homologous gene pools points towards highly specialised communities of anaerobic toluene and hydrocarbon degraders, which have established themselves at the three sites in close coupling to the local biogeochemical regime. Such 'specialised' local communities of BTEX degraders within aquifers had not been apparent from other screenings of aerobic (Taylor and Janssen, 2005; Hendrickx et al., 2006) or anaerobic (Hosoda et al., 2005) BTEX degradation genes, yet may have important ecological implications. It is not currently known what time scales are required for the establishment of such degrader communities (after contamination), and how dynamic these populations are to changes in electron acceptor availability or local hydrological regime (i.e. changes in groundwater flow or hydraulic shifts).

Particularly relevant is the question whether the detection of a given contaminant degrader can be taken as an indication of ongoing natural attenuation processes. Most of the available literature regarding aquifers concerns oxygen-dependent degrader communities, and these reports are partially incongruous. It has been shown that specific degradation gene pools can be distinct within different zones of a contaminated aquifer (Bakermans and Madsen, 2002; Hosoda et al., 2005; Taylor and Janssen, 2005). Specific genes can be present only in contaminated zones (Bakermans and Madsen, 2002; Hosoda et al., 2005; Hendrickx et al., 2006) or also in pristine samples (Hendrickx et al., 2006), and can be positively (Dionisi et al., 2004; Hosoda et al., 2005) or negatively (Taylor and Janssen, 2005) correlated in abundance to specific contaminants. With the Flingern samples, where it was not possible to detect *bssA* amplicons above or below the contaminated sediment layers, the hypothesis of a specific allocation of defined contaminant degraders to particular zones within the aquifer can be supported.

2.5. References

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3. Stable isotope probing of sulfate-reducing bacteria involved in toluene degradation in a tar-oil contaminated aquifer

3.1. Introduction

The identification of microbial key players and the linking of their ecophysiological capabilities to respective functions in the environment is a major task in modern day microbial ecology. The potential of specific microorganisms in the environment to transform a given substrate can be demonstrated by detecting genes encoding specific catabolic key enzymes (Wilson et al., 1999; Beller et al., 2002b; Watanabe et al., 2002; Baldwin et al., 2003; Junca and Pieper, 2004; Galvao et al., 2005; Hendrickx et al., 2006). Accordingly, microbial potentials for anaerobic degradation of the BTEX compounds toluene and xylene can be detected in the environment via genes of the benzylsuccinate synthase (Bss), the key enzyme of anaerobic toluene degradation (Biegert et al., 1996a). Anaerobic BTEX degradation is known to occur under different terminal electron accepting processes (Anderson and Lovley, 1997a; Christensen et al., 2000b; Van Hamme et al., 2003a), yet to the current knowledge, Bss is always involved.

At a former gasworks site in Germany with a tar-oil contaminated aquifer, anaerobic BTEX degradation has been reported to occur mostly under sulfate-reducing conditions (Griebler et al., 2004). The Gram-positive toluene-degrading sulfate reducer *Desulfotomaculum sp.* Ox39 was previously isolated from this site (Morasch et al., 2004b). Also, previously detected deeply-branching environmental *bssA* homologues, which were not affiliated to any of the known proteobacterial *bssA* sequences and also not to a putative *bssA* homologue detected in strain Ox39 *bssA*, dominate at this site (Winderl et al., 2007a). Yet, based purely on PCR gene detection, the identity of the respective organisms carrying these genes was unknown. Furthermore, the question whether they are actively involved in on-site degradation of toluene, or possibly also other hydrocarbons, remained unanswered.

In recent years, especially the use of stable isotopes in combination with molecular methods has enabled researchers to answer such questions, not only for hydrocarbon degradation in groundwater environments, but for a wide variety of processes and environments (for recent reviews see (Madsen, 2005; Whiteley et al., 2006; Kreuzer-Martin, 2007; Neufeld et al., 2007a). Stable isotope probing (SIP) of nucleic acids allows for the specific identification of microorganisms utilising and assimilating carbon from a particular substrate, and if done for DNA, allows to affiliate also the involved catabolic genes marker (Jeon et al., 2003a). The aim of the present study was to identify, by means of DNA-based SIP, microbial key players responsible for anaerobic toluene degradation under sulfate-reducing conditions in Testfeld Süd aquifer samples. A further aim was to elucidate whether a functional role can be ascertained for the deeply-branching environmental *bssA* homologues, which were previously found at this site (Winderl et al., 2007a).

Therefore, freshly obtained aquifer sediments were exposed to fully labelled ^{13}C-toluene ($^{13}C_7H_8$) under close-to-*in situ* .conditions. $^{13}CO_2$ accumulation, the production of sulfide, and the depletion of toluene were monitored continuously. After an incubation period of 64 days a significant increase of both $^{13}CO_2$ and sulfide values was observed, indicating stimulated toluene turnover via sulfate-reduction. The most active microbes in sulfate-dependent toluene degradation in these samples were affiliated to the *Desulfosporosinus* and *Desulfotomaculum* genera (*Clostridia, Peptococcaceae*). Moreover, they harboured a novel

bssA sequence type related to an as-yet unaffiliated *bssA* gene previously detected at a different site (Winderl et al., 2007a), but clearly distinct from the more deeply branching *bssA* homologues found previously at the site of investigation. These findings substantiate that the environmental importance of non-proteobacterial key players in anaerobic BTEX degradation may be largely underestimated.

3.2. Experimental procedures

3.2.1. Sampling site and sample acquisition

Sampling was performed within a tar-oil contaminated site in Germany anonymised Testfeld Süd. This former gasworks site has been intensively studied and sulfate has been identified as a major electron acceptor for BTEX degradation as described elsewhere (Herfort et al., 1998; Bockelmann et al., 2001; Zamfirescu and Grathwohl, 2001a; Griebler et al., 2004). Aquifer sediment from the bottom of monitoring well B49 (at ~7.50 m depth, groundwater table at ~3.25 m bgs) was retrieved in December 2006 with an aqua-sampler (Bürkle, Germany). Sampled sediments and groundwater were immediately filled into autoclaved glass bottles without gaseous headspace to minimise oxygen exposure and transported to the lab under cooling.

3.2.2 Incubation of sediment material

Replicates of ~8 g (wet weight) of freshly sampled sediment material were anoxically incubated in sterile 120 ml serum bottles containing 50 ml of artificial groundwater medium. The low salt mineral medium contained all components of the freshwater mineral medium described by (Widdel and Bak, 1992) in a 1:10 dilution, except bicarbonate buffer, which was added to 30 mM final concentration. Additionally, 1mM Na_2S was used as reducing agent, 10 mM Na_2SO_4 was added as electron acceptor, and 5µM cAMP was added to stimulate degradation (Bruns et al., 2002). To ensure constantly low *in situ* concentrations of toluene during SIP incubation, 0.3 g of Amberlite XAD7 adsorber resin was added to each bottle (Morasch et al., 2001) as an inert carrier phase. 5 µl of either non-labelled (^{12}C) or fully ^{13}C-labelled toluene (Sigma) was injected through butyl rubber stoppers with a gastight syringe (Hamilton Co.) and allowed to adsorb to the carrier for 2 days. This resulted in a reservoir of ~0.96 mM toluene in each bottle, however actual concentrations can be expected to be only ~0.05 – 0.1 times as high due to adsorption to the carrier. Finally, after sediment addition, the bottles were gassed with N_2/CO_2 (80:20 v/v), sealed again anoxically with butyl stoppers, and incubated statically for over 133 days at 16°C in the dark. A total of 15 replicate bottles were prepared for each series (^{12}C & ^{13}C) for replicate biogeochemical measurements and successive time-dependent termination. Sediment un-amended controls were also run.

3.2.3. Process measurements

Liquid and gaseous samples were taken from replicate ^{12}C- and ^{13}C-toluene and negative control bottles for monitoring toluene degradation on a weekly basis. Aqueous toluene concentrations were determined via headspace analysis of 1 ml subsamples with a GC-MS (Finnigan Trace GC Ultra, connected to a Finnigan Trace DSQ, Thermo Electron Coorperation) over a DB-5ms fused silica capillary column (0.5 µm film thickness, 0.25 i.d., 30 m length, J & W Scientific) and standardised with 0.67 µg/ml internal standard (EPA 524 internal standard mix, Supelco). Sample injection was on split mode (1:10 ml min^{-1}) and helium carrier gas flow rate was 1.5 ml min^{-1}. Oven temperature was 40°C for 1 min, then ramped at a rate of 16°C min^{-1} to 145°C, then at a rate of 45°C min^{-1} to 300°C, and held for 1 min. The MS was operated at 350°C in the SIM scan mode for the masses 91, 92 (^{12}C-

toluene), 98, 99 (^{13}C-toluene), and 96, 150 (fluorobenzene and 1, 4-dichlorobenzene-D4 of the EPA standard, respectively).

Sulfide concentrations in 200 µl liquid samples were monitored spectrophotometrically as described by (Cline, 1969) using a Cary 50 Bio UV-Vis photometer (Varian) at a wavelength of 670 nm. A calibration curve was generated with sulfide (Na_2S x 9 H_2O, Sigma) of known concentrations (0, 0.5, 1, 1.5, 2, 2.5, and 5 mM).

Additionally, the formation of ^{13}C labelled CO_2 was followed via gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). 15 µl samples were taken from the head space of each bottle and stable carbon isotope ratios of CO_2 were determined with the following setup: a Trace GC Ultra coupled to a Finnigan GC combustion III interface and a Finnigan MAT 253 IRMS (all Thermo Electron Corporation) via open split. The GC was equipped with a DB-5 column (0.25 µm film thickness, 0.25 mm in diameter, 30 m length, Agilent Technologies). Helium grade 5.0 was used as carrier gas with a constant flow rate of 1.4 ml/min. Injector split flow was set to 14 ml/min, i.e. a split-ratio of 1:10 and the temperature was held isothermally at 180°C. The GC oven temperature was programmed as follows: 100°C for 2.5 min, then ramped at 45°C min^{-1} to 150°C, and held for 1.4 min). Carbon isotope signatures of CO_2 are reported in AT% (atom percent, AT% = $^{13}C/(^{12}C + ^{13}C)$ * 100). Calibration and calculation of mass balances was achieved using the δ-notation relative to Vienna PeeDee Belemnite (V-PDB): $\delta^{13}C = [(^{13}C/^{12}C_{Sample} - ^{13}C/^{12}C_{V\text{-}PDB\ Standard}) / ^{13}C/^{12}C_{V\text{-}PDB\ Standard}] \times 1000$ by measurement of the analytes against a laboratory CO_2 reference gas that was calibrated to V-PDB by referenced CO_2 isotope standards (RM 8562, RM 8563, RM 8564) provided by the International Atomic Energy Agency (IAEA, Vienna). The calculations were done with the following assumptions:

1.) $^{12}CO_2$ pool is constant
2.) C-isotope fractionation between TIC-species within gaseous phase and liquid phase within the microcosms is negligible due to the strong ^{13}C-label.

$^{13}CO_2$ values were measured within each microcosm at day 1 and were taken as t_0 for further measurements according to equation 1:

$$n\left(^{13}CO_{2\,toluene}\right) = n\left(^{13}CO_2\right)_t - n\left(^{13}CO_2\right)_{t0}, \text{ def: t = measurement day; } t_0 = \text{day 1} \quad (1)$$

The total amount of $^{13}CO_2$ was calculated via the measured ^{13}C values of total inorganic carbon (TIC) referenced to Vienna PeeDee Belemnite according to equation 2:

$$n\left(^{13}CO_2\right) = \frac{R \cdot n(TIC)_{initial}}{(1+R)}, \text{ def: } R = \frac{^{13}C}{^{12}C} = \left(1 + \frac{\delta^{13}C}{1000}\right) \cdot R_{PDB} \quad (2)$$

Finally the values of $n\left(^{13}CO_{2\,toluene}\right)$ were devided by 7, because 7 moles of CO_2 are obtained from 1 mole of toluene, if toluene is completely oxidised.

3.2.4. Nucleic acid extraction and ultracentrifugation

At selected time points, a pair of bottles (^{12}C & ^{13}C) was sacrificed for DNA-SIP analyses. Sediment and biomass was collected by centrifugation at 4000 rpm at 4°C for 10 min with a Megafuge 1.0 R (Heraeus Intsruments). Pellets were frozen immediately at -20°C until subsequent nucleic acid extraction. Total nucleic acids were extracted from ~1-2 ml of incubated sediment samples following a previously described protocol (Winderl et al., 2007c). 5µg of PicoGreen (Invitrogen) quantified DNA extracts were loaded into a gradient medium of CsCl (Calbiochem) dissolved in gradient buffer (GB) (Lueders et al., 2004b) of an average density 1.71 g ml^{-1} prepared in accordance to (Neufeld et al., 2007b). Centrifugation was done in 5 ml polyallomer quick seal tubes in a VTI 65.2 vertical rotor (both Beckman) using a Centrikon T-2190 ultra centrifuge (Kontron Instruments). Centrifugation runs were at 44,500 rpm (180 000 x g) at 20°C over 65 h. 13 fractions of each gradient were collected from 'heavy' to 'light' using a Perfusor V syringe pump (B.Braun Melsungen AG) as described (Kunapuli et al., 2007). The refractometric measurement fraction densities and the recovery of DNA from gradient fractions was also done as described (Lueders et al., 2004b).

3.2.5. Analyses of density-resolved DNA fractions

Re-extracted DNA from CsCl gradient fractions was analysed via bacterial 16S rRNA gene targeted qPCR in the presence of 0.1 x SYBR Green as described (Kunapuli et al., 2007). From each gradient, 10 DNA fractions were selected for 16S rRNA gene targeted T-RFLP fingerprinting. Fluorescently labelled amplicons were generated as detailed elsewhere (Winderl et al., 2007c), and electrophoresis on an ABI 3730 DNA Analyzer and data evaluation with the Gene Mapper 5.1 software (both Applied Biosystems) was also as previously described (Lueders et al., 2006).

3.2.6. Cloning, sequencing and phylogenetic analysis

From selected gradient fractions, amplicons generated with the primer sets Ba27f (Weisburg et al., 1991) / 907r (Muyzer et al., 1995) (targeting bacterial 16S rRNA genes, same as for T-RFLP) and 7772f / 8546r (Winderl et al., 2007a) (targeting *bssA* genes) were generated, cloned and sequenced as published (Winderl et al., 2007c; Winderl et al., 2007a). Selected T-RFs predicted *in silico* for representative clone sequences were checked *in vitro* (see Tab. 3.1.). Chimeric sequences were checked for using CHIMERA_CHECK 2.7 of RDP-II version 8.1 (http://rdp8.cme.msu.edu/html/) and by manual inspection of the alignment. From 87 clones, 1 was identified as chimera and excluded from further analysis. Phylogenetic trees were reconstructed from sequence data as described (Winderl et al., 2007c; Winderl et al., 2007a). All sequences generated in this study have been deposited with GenBank under the Accession Numbers EFxxx to EFxxx (t.b.a.).

3.3. Results

3.3.1. Exposure of aquifer sediments to ^{13}C-toluene

Freshly sampled aquifer sediments were incubated with fully labelled ^{13}C-toluene under close-to-*in situ* conditions (at 16°C, with less than ~100 µM effective toluene concentrations, in a low-ionic strength artificial groundwater medium) in closed microcosms. For the first 57 days of incubation, a significant increase $^{13}CO_2$ or sulfide was not observed (Fig. 3.1.). Within this phase, isotopic ratios of CO_2 and concentrations of sulfide remained at 1.1 AT% (atom percent, AT% = ^{13}C/(^{12}C + ^{13}C) * 100, see also experimental procedures) and ~0.5 mM, respectively, which were initially contained in the artificial groundwater medium. Between 64 and 100 days of incubation, these values increased to ~10 AT% and 2.7 mM, respectively, and remained at about these levels until the end of the experiment (day 133). Free toluene concentrations in the medium were followed via GC-MS. From the initially added reservoir of ~960 µM toluene, only between ~50 µM and ~100 µM were actually freely available during the first 50 d of incubation, due to adsorption to the Amberlite XAD7 carrier resin. Therefore, it was not possible to quantitatively monitor toluene decrease during the time course of incubation, but it was possible to detect depletion after ~100 d, at the time when sulfide and $^{13}CO_2$ concentrations ceased to increase. Toluene was below GC-MS detection limit (~0.3 µM) after day 121 (Fig. 3.1.). A similar increase of sulfide was observed in the ^{12}C-toluene control incubations, while no depletion of toluene or increases of sulfide were observed in sediment-unamended controls (data not shown).

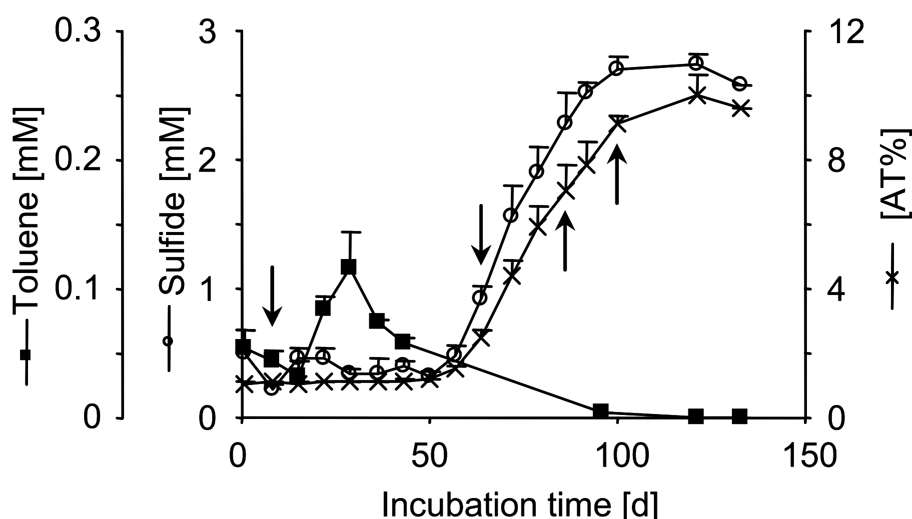

Figure 3.1.: Transformation of ^{13}C-labelled toluene coupled to the production of sulfide and $^{13}CO_2$ in SIP microcosms. Carbon isotopic compositions of CO_2 are indicated in atom percent (AT%). Arrows indicate time points selected for DNA-SIP analysis.

Interestingly, although BTEX measurements indicated a complete degradation of the reservoir of ~960 µM toluene with time, only ~50% of the transferrable electrons were recovered in produced sulfide. In contrast, the ~10 AT% of ^{13}CO2 measured towards the end of degradation reached ~72% of the maximal ~14 AT% expected if all carbon from the added toluene was oxidised to CO_2.

3.3.2. Analysis of DNA-SIP gradient fractions

Based on the process measurements, several successive time points of the experiment were selected for DNA extraction (Fig. 3.1.). These were at the beginning of the experiment (day 8), and at the apparent onset, approximately at midpoint, and towards the end of substrate transformation activities (days 64, 86, 100). SIP gradients of all respective DNA extracts from the ^{12}C- and ^{13}C-incubations were conducted. qPCR analysis of fractionated gradients detected maximal DNA banding in the light fractions (at buoyant densities (BD) between 1.68 and 1.69 g ml^{-1}) for all analysed gradients. In Fig. 3.2., the DNA distribution profiles of two exemplary time points (days 8 and 86) are displayed. Increased amounts of bacterial DNA could not be detect clearly in any of the heavy gradient fractions of the ^{13}C-toluene gradients (i.e. BD > 1.72 g ml^{-1}). Only minor tailings of DNA into intermediate fractions (1.70 - 1.71 mg l^{-1}) of the ^{13}C gradients of later time points were apparent (Fig. 3.2.B), but based purely on qPCR, these small BD shifts cannot be considered as significant label incorporation.

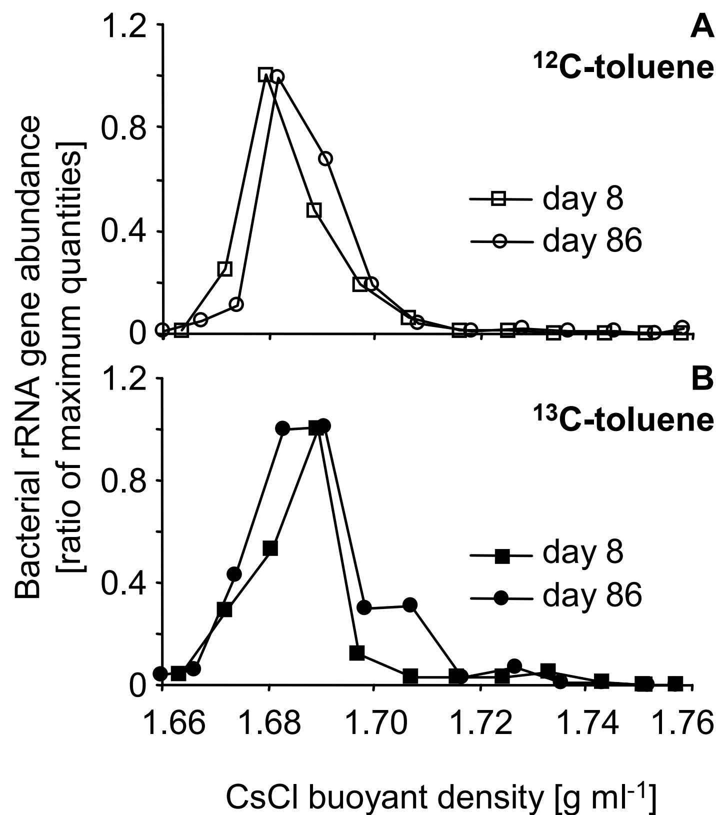

Figure 3.2.: Quantitative profiles of DNA distribution in SIP gradients after 8 and 86 days of incubation with either unlabelled (A) or fully ^{13}C-labelled (B) toluene. Gene distribution was measured by qPCR of gradient fractions.

However, bacterial 16S rRNA gene targeted T-RFLP analysis revealed over time clear distinctions between the ^{12}C and ^{13}C-toluene incubations (Fig. 3.3.). Strong label-dependent community shifts were observed especially within the intermediate fractions (BD 1.70 - 1.71 mg l^{-1}) after 64 days, and also within intermediate and heavy fractions (~ 1.74 mg l^{-1} BD) after 86, and 100 days. In contrast to results after 8 days, the community structure within 'heavier' fractions retrieved from ^{13}C-gradients differed significantly from both the light

fractions from ^{13}C-gradients and the heavy and light fractions from the ^{12}C control gradients. At the same time, light fractions from the ^{12}C- and ^{13}C-gradients remained highly similar over time. These findings strongly indicate that ^{13}C-label from the added toluene was in fact incorporated by specific members of the microbial community, although barely detectable by quantitative BD shifts of DNA in qPCR. As identified by T-RFLP, especially the 146, 159, and 177 bp T-RFs were of increased abundance in the 'heavier' fractions of the ^{13}C-gradients (Fig. 3.3.). In the light fractions, especially the 89, 141, 470, 486, and 490 bp T-RFs were important constituents.

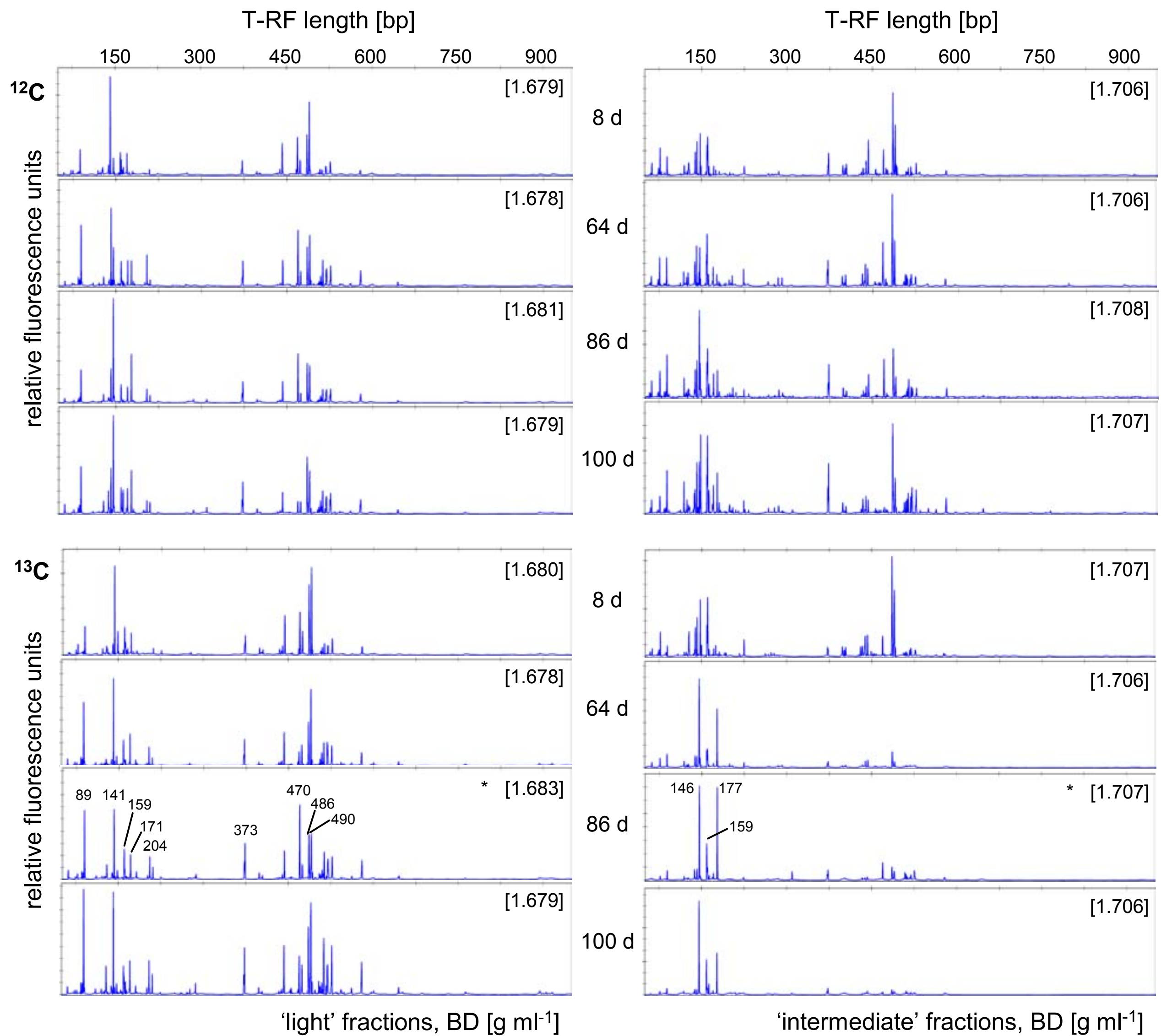

Figure 3.3.: Bacterial 16S rRNA gene based T-RFLP fingerprints of density-resolved gradient fractions of SIP gradients after 8, 64, 86, 100 days of incubation with unlabelled or ^{13}C-labelled toluene. Fractions selected for cloning and sequencing are marked with an asterisk (*).

To further unravel specific label allocation to defined community members (i.e. T-RFs), relative total bacterial 16S rRNA gene abundance (from qPCR) were multiplied by the relative peak height abundance of selected T-RFs for all fractions (Kunapuli et al., 2007), thus facilitating a comparative specific T-RF abundance distribution between selected ^{12}C- and ^{13}C-gradients (Fig. 3.4.). This clearly showed that especially the microbes represented by the

146 and 177 bp T-RFs shifted their maxima of distribution from ~1.685 to ~1.705 g ml^{-1}. This BD shift of ~0.02 g ml^{-1} corresponds to 50% of the BD shift expect for full ^{13}C-labelling (Lueders et al., 2004b). To a lesser extent, also the 159 bp T-RF shifted in BD, but was distributed in two peaks, one unlabelled, and one also ~50% labelled. All other T-RFs did not shift in distribution maxima, as exemplified for the 160 and 490 bp T-RFs in Fig. 3.4..

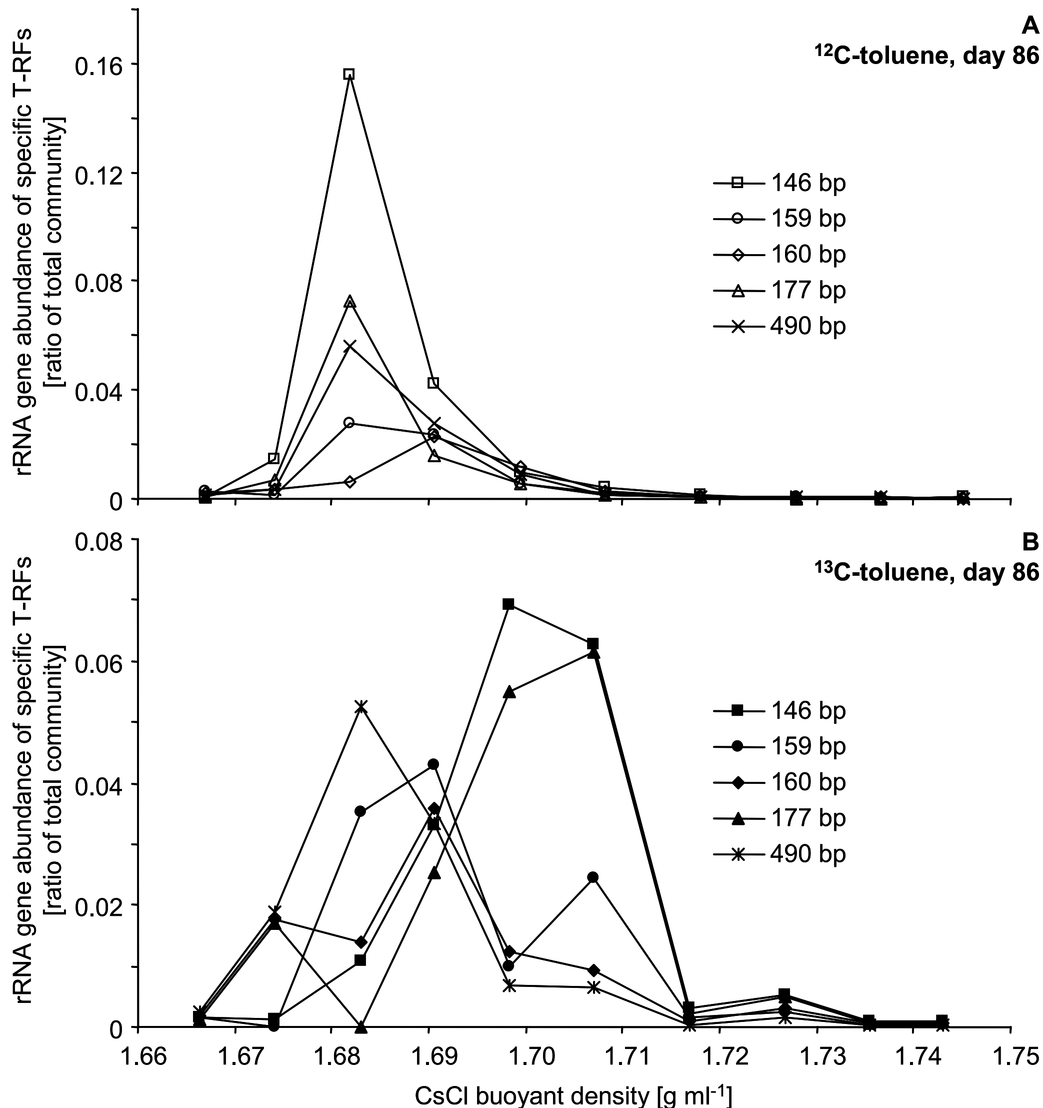

Figure 3.4.: Comparative abundance distribution profiles of specific T-RFs in ^{12}C- (A) and ^{13}C-toluene (B) SIP gradients. Abundances were calculated by multiplying total gene abundances (see Fig. 3.2.) by specific T-RF peak height abundances for all fractions of given gradients.

3.3.3. Identification of labelled 16S rRNA genes

The specific microbial community members represented by the detected T-RFs were identified by cloning and sequencing. Two 16S rRNA gene targeted clone libraries were established from a light (BD = 1.683 g ml^{-1}) and an intermediate fraction (1.707) of the 86 days ^{13}C–toluene gradients. 41 and 45 clones were sequenced, respectively (Tab. 3.1.). Members of the *Chloroflexi* (29%), *Deltaproteobacteria* (14%), and *Betaproteobacteria* (10%) were dominating the 'light library', whereas *Clostridia* (24%), *Actinobacteria* (16%),

and *Chloroflexi* (11%) were found to dominate within the library of the intermediate fraction. Specifically, a cluster of uncultured relatives of the Gram-positive *Desulfosporosinus* and *Desulfotomaculum* genera were identified to be represented by both the 146 and 177 bp T-RFs (Fig. 3.5.), which were shown to incorporate ~50% of ^{13}C label.

Table 3.1.: Composition of bacterial 16S rRNA gene clone libraries from 'intermediate' (LA07Ba clones) and 'light' fractions (LA10Ba clones) of the 86 days ^{13}C-toluene SIP gradient. Characteristic T-RFs [bp] predicted for all or a major fraction of clones of given affiliation are indicated together with T-RFs actually measured in the T-RFLP fingerprints.

	No. of clones		**T-RF [bp]**	
Phylogenetic affiliation	'intermediate' 1.707 g ml^{-1}	'light' 1.683 g ml^{-1}	predicted	measured
Alphaproteobacteria	**4**	**2**	–	n.a.
Betaproteobacteria	**1**	**4**	–	n.a.
- *uncultured* (*Thauera*-related)	–	3	490	490
Gammaproteobacteria	**1**	**1**	490	490
Deltaproteobacteria	**4**	**6**	–	n.a.
- TRM1/*Desulfocapsa*-related	3	1	162	159
- *uncultured Desulfobacteriaceae*	1	3	166,513	163,511
Epsilonproteobacteria	–	**2**	468	469
***Bacteroidetes* (*uncultured*)**	**1**	–	–	n.a.
Mollicutes	–	**2**	–	n.a.
Clostridia	**11**	**2**	–	n.a.
- *Desulfosporosinus*-related	10	–	148,179	146,177
Actinobacteria	**7**	**3**	–	n.a.
- *Microbacteriaceae*-related	3	1	–	n.a.
- *Coriobacteriaceae*-related	2	1	163	160
***Acidobacteria* (*uncultured*)**	2	–	–	n.a.
Chloroflexi	**5**	**12**	–	n.a.
- *uncultured I*	2	2	372,373	373
- *uncultured II*	3	7	518,519	520
- *uncultured III*	–	3	579	579
OP5	**3**	–	82,230	n.a.
OP8	**1**	**1**	142	138
OP11	–	**3**	–	n.a.
Other diverse uncultured	**5**	**3**	–	n.a.

Also other important T-RFs could be identified via the clone libraries (Tab. 3.1., Fig. 3.5.). These were uncultured members of the *Bacteroidetes* (89 bp), relatives of the *Desulfocapsa*-related toluene-degrading sulfate reducer TRM1 (Meckenstock, 1999a) (159 bp), *Coriobacteriaceae* (160 bp), uncultured *Chloroflexi* (373 and 520 bp), and *Thauera*-related *Betaproteobacteria* (490 bp, together with some members of the *Gammaproteobacteria*).

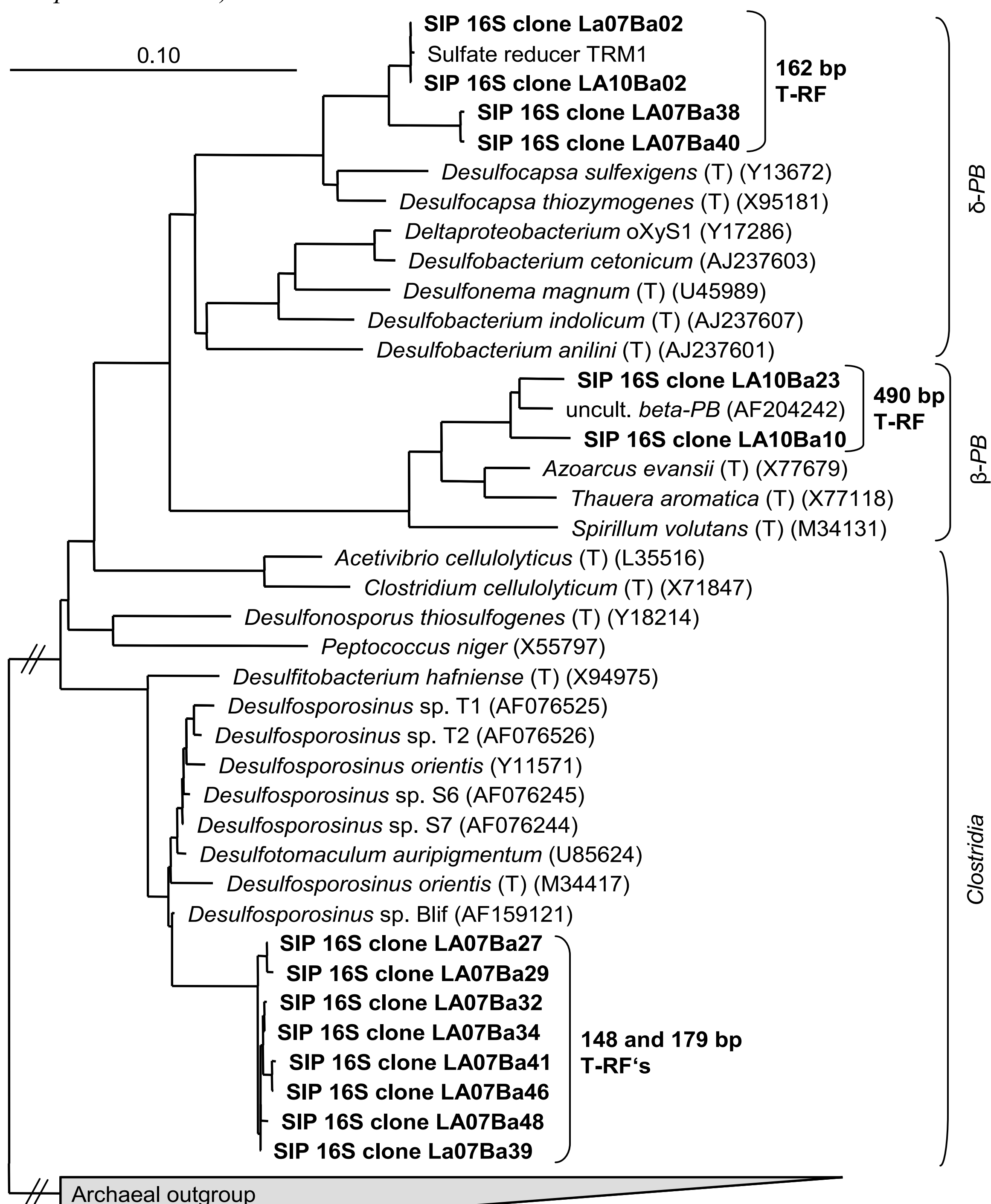

Figure 3.5.: Phylogenetic tree showing the affiliation of representative bacterial 16S rRNA clones from density-resolved DNA fractions of the day 86 ^{13}C-toluene gradient (see Fig. 3.3.). Clones retrieved from the 'intermediate fraction' library (BD 1.707) are named LA07Ba and those from the 'light' library (BD 1.683) LA10Ba. Predicted T-RF's and major lineages of affiliation are indicated, PB=*Proteobacteria*. The scale bar represents 10% sequence divergence, branch lengths were scaled down by a factor of 6.5.

3.3.4. *bssA* genes detected in labelled DNA fractions

The DNA fraction at 1.707 g ml^{-1} of the 86 d ^{13}C-toluene gradient, for which it was possible to substantiate a ^{13}C-label dependent dominance of the detected *Desulfosporosinus*-relatives, was also used to generate a *bssA*-targeted clone library. Here 20 putative *bssA* clones were sequenced (Fig. 3.6.). Surprisingly, the library was dominated by a cluster of clones (15 of all sequences) closely related to the previously described D12_31 clone of the 'F2-lineage' of unidentified environmental *bssA* genes (Winderl et al., 2007a). Furthermore, 3 of the clones were of *Thauera*-like *bssA* sequence types, and 1 was distantly related to the *bssA* gene of strain TRM1 (Winderl et al., 2007a).

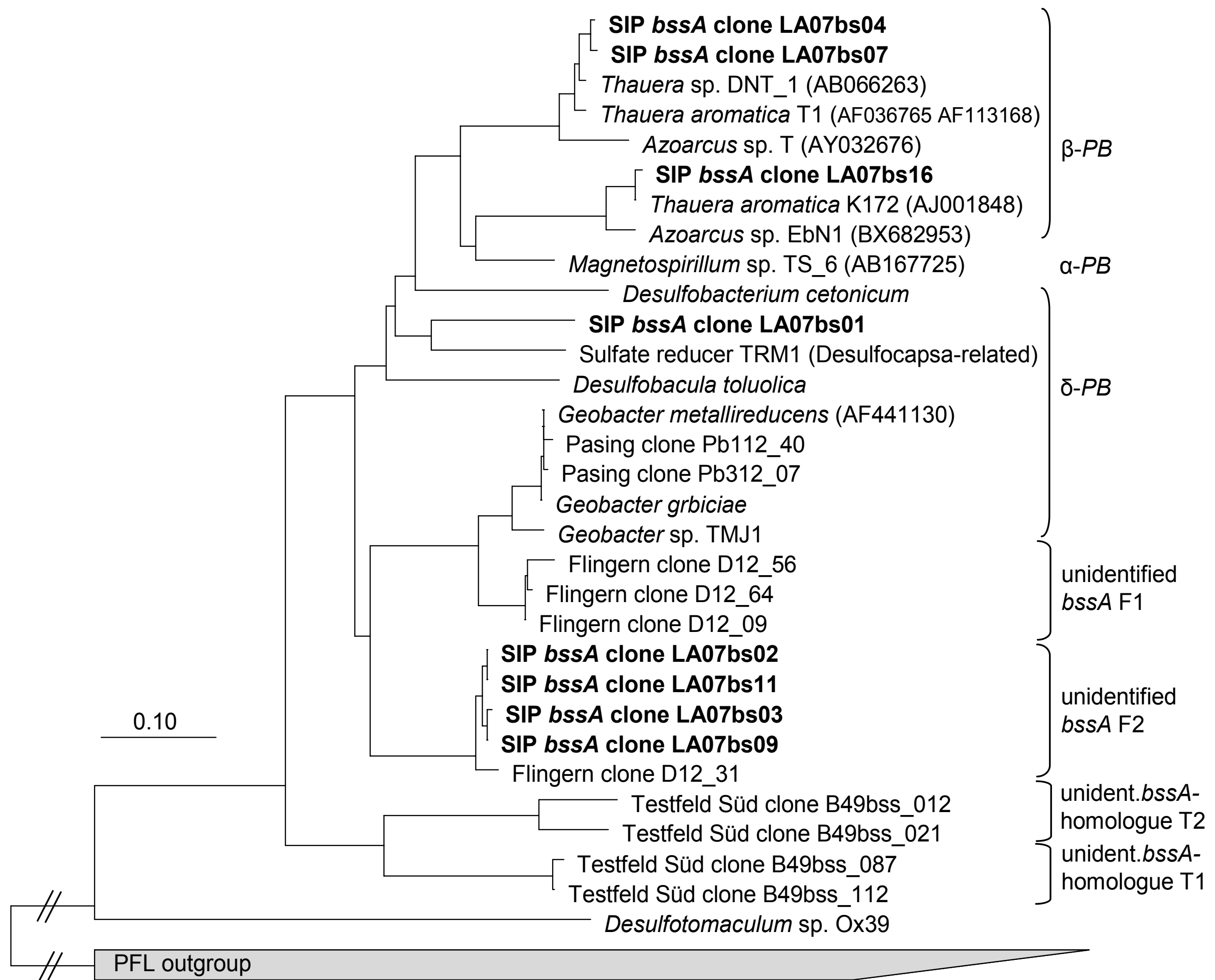

Figure 3.6.: Phylogenetic tree showing the affiliation of partial *bssA* genes retrieved at a BD of 1.707 g ml^{-1} from the day 86 ^{13}C-toluene SIP gradient and named LA07bs. Naming of environmental *bssA* sequences is in accordance to (Winderl et al., 2007a) (see Fig. 2.2.). Major lineages of affiliation are indicated, PB=*Proteobacteria*. The scale bar represents 10% sequence divergence, branch lengths were scaled down by a factor of 4.

3.4. Discussion

3.4.1. SIP under close-to-*in situ* conditions

One objective in this study was to mimic *in situ* growth conditions for the sampled degrader community as closely as possible. The microcosms were incubated at 16°C in the dark, which is reasonable regarding the usual on site groundwater temperature of ~10-16°C. All components except for the bicarbonate buffer of the freshwater medium as described by (Widdel and Bak, 1992) were diluted 1:10 in order to better mimic groundwater low ionic strength. However, 5 µM cAMP were added, as this has been described to significantly increase growth efficiency in mineral media (Bruns et al., 2002). Due to the known long incubation periods of several months for sulfate-reducing organisms enriched from Testfeld Süd sediments (Morasch et al., 2004b), the addition of this growth stimulant was chosen for practical reasons.

In order to ensure constant ^{13}C-substrate supply at low concentrations, Amberlite XAD7 adsorber resin was added to the bottles, which lowered initial concentrations from ~960 µM to an average of ~66 µM over the initial 50 d of incubation, an ~15-fold reduction of effective concentrations described before (Morasch et al., 2001). This concentration was only about 2 times higher than maximal on-site toluene concentrations of ~33 µM reported close to the sampled well earlier (Griebler et al., 2004). Despite these close-to-*in situ* conditions, degradation activity in the microcosms required a considerable time span to pick up momentum. Significant increases of $^{13}CO_2$ and sulfide with concomitant shifts in the density-resolved bacterial communities were only observed after 64 days of incubation and thereafter. This obviously highlights that indigenous toluene-degrading communities either were not active *in situ* at the time point of sampling, or had to undergo substantial adaptation and lag phases within the SIP microcosms.

Compared to RNA-SIP, DNA-SIP requires longer incubation times due to DNA replication and ^{13}C label incorporation into the newly synthesised DNA (Manefield et al., 2002a; Manefield et al., 2002b), and especially the combination of RNA-SIP and DNA-SIP can be a powerful tool to unravel substrate fluxes within and interactions amongst environmental microbiota (Lueders et al., 2004c). Unfortunately, sufficient amounts of high quality rRNA were not extractable from the samples. Still it remains questionable, whether the application of rRNA-SIP would have provided more sensitive insights into a system as low in temporal dynamics as the one presented here.

3.4.2. Key players in ^{13}C-toluene degradation

It was possible to demonstrate clearly that toluene was quantitatively degraded and converted to CO_2 coupled to sulfate-reduction within the microcosms. However, significant amounts of ^{13}C-labelled DNA were not detectable by qPCR. Nevertheless, ^{13}C-label incorporation of ~50% into the DNA of the involved microbial key players could be shown. These organisms were identified as uncultured relatives of the spore-forming *Desulfosporosinus* and *Desulfotomaculum* spp.. Members of these lineages have been previously described as important constituents of contaminated aquifer microbial communities (Robertson et al., 2001; Franzmann et al., 2002), and one strain is available that oxidises toluene (Liu et al., 2004a). However, the electron balance revealed that only ~50% of the electrons from the added toluene were transferred to sulfate, while more than 70% of the ^{13}C was recovered as

$^{13}CO_2$. These findings seem to be directly connected to each other, which led us to consider the following hypotheses:

If the active microorganisms would have used energy from the added toluene mainly for maintenance, no significant effects of ^{13}C-labelling would have been detectable. This was not the case, and also the typical growth-curve like time course of $^{13}CO_2$ and sulfide production seems to exclude this scenario. Another possible explanation for the weak labelling of degraders could be a dilution of ^{13}C label with ^{12}C originating from carbon sources other than the added toluene, i.e. acetate or carbonate simultaneously available in the surrounding medium. Unfortunately, acetate was not measured during incubation, but may well have been produced by other ongoing degradation processes in the sampled aquifer material. Co-assimilation of acetate is well known for sulfate-reducing microbes, but if abundant acetate had been available, sulfate reduction would have been expected independent of toluene degradation, which was not observed. Also, the lack of electrons recovered in the produced sulfide seems to suggest a different mechanism:

Recently, the phenomenon of heterotrophic CO_2 assimilation has been shown to occur for a number of model microbes under both aerobic and anaerobic conditions. It could be demonstrated that microbes incorporated ^{14}C-labelled bicarbonate buffer into their biomass during growth on various organic substrates (Hesselsoe et al., 2005). This can be explained by the need of CO_2 for intermediary metabolism of cells, where carboxylase reactions are involved (Dijkhuizen and Harder, 1985). In some heterotrophs, up to 8% of the biomass carbon are assumed to originate from heterotrophic CO_2 assimilation (Romanenko, 1964). Unfortunately, nothing is known about heterotrophic CO_2 assimilation capacities of *Desulfosporosinus* spp. and also other sulfate reducers while degrading BTEX compounds. CO_2 assimilation is always costly, and thus could well explain the lack of electrons recovered in the microcosms in the form of sulfide. However, the mechanisms behind this putative CO_2 fixation and especially the question why the microbes may require additional carbon while degrading toluene remains to be elucidated. Also, it remains questionable, whether such mechanisms alone could result in a 1:1 dilution of the ^{13}C label originating from the added toluene with ^{12}C from carbonate in degrader biomass. Nevertheless, these mechanisms may be more relevant in anaerobic aromatics degradation than previously recognised, because a comparable ~50% label incorporation efficiency has been reported recently for key players in iron(III)-dependent benzene degradation (Kunapuli et al., 2007).

The only further T-RF indicative of label incorporation into other microbes was the 159 bp fragment, representing relatives of the *Desulfocapsa*-related toluene-degrading sulfate reducer TRM1 (Meckenstock, 1999a). This fragment however, was hardly separable in T-RFLP analysis from the 160 bp fragment characteristic for certain detected *Actinobacteria*. However, due to the high resolution and reproducibility of the utilised advanced capillary electrophoresis DNA analyzer, these could be consistently discriminated in T-RFLP analysis. Both T-RFs were found together with the dominating *Desulfosporosinus* spp. T-RFs within the 'heavier' fractions of the ^{13}C gradients, while the *actinobacterial* 160 bp T-RF was already of increased relative abundance in intermediate fractions of the ^{12}C gradients, due to the intrinsically higher G+C content of actinobacterial genomes (see. Fig. 3.4.). Thus minor additional labelling effects could be inferred for the TRM1-relatives only. From these findings it can be concluded that these *Deltaproteobacteria* are also frequent members of the Testfeld Süd sediment communities and may also have consumed small amounts of the labelled substrate, but were by far not as relevant for quantitative toluene turnover as their *clostridial* key players in the microcosms. Secondary cross-feeding of label to other microbes can be

excluded, since no fragments other than the 146, 177, and also the 159 bp T-RFs were identified to appear in intermediate and heavy fractions also for the latest time points.

3.4.3. Catabolic genes of clostridial key players

SIP data indicates the *Desulfosporosinus*-relatives to be most relevant for toluene degradation in the SIP microcosms and that their genomes were preferentially allocated to intermediate gradient fractions after ^{13}C-labelling. Thus it is a fair assumption that by amplifying not only 16S rRNA, but also *bssA* genes from the respective DNA fractions, the *bssA* sequence type of these clostridial contaminant degraders can be identified. The cloning and sequencing of *bssA* amplicons revealed a clear dominance of one *bssA* sequence type (79%), which was, however, directly related to the previously described F2–lineage of unaffiliated environmental *bssA* sequences (Winderl et al., 2007a), and not to the more deeply branching putative *bssA* homologues previously amplified from Testfeld Süd sediments (T1 & T2-clusters, see Fig. 3.6.). This indicates that the F2-cluster *bssA* belongs to the identified *Desulfosporosinus*-relatives, and that these genes were actively involved in anaerobic toluene degradation. Thus the Flingern F2 cluster, which is branching inbetween the known beta- and deltaproteobacterial reference sequences (Fig. 3.6.) may well represent a typical clostridial *bssA* sequence type. At the same time, however, this indicates that the T-cluster *bssA* homologues (and probably also the putative bssA fragment retrieved from *Desulfotomaculum* sp. Ox39 (Winderl et al., 2007a) are not involved in anaerobic toluene degradation, but may well be active in the initial attack of other hydrocarbons (Kniemeyer et al., 2003a; Cravo-Laureau et al., 2005b). Alternatively, it could indicate that intrinsic toluene degrading microbial communities have changed between the two time points of sampling (Aug. 2004 and Dec. 2006).

Furthermore, 3 of the cloned *bssA* sequences were closely related to betaproteobacterial *Thauera/Azoarcus bssA* genes, and 1 to TRM1 *bssA*. While a contribution of TRM1 to net toluene degradation can well be interpreted from 16S rRNA gene fingerprinting and clone libraries (see above), this can clearly be excluded for the denitrifying *Thauera*-relatives. Here, an increased abundance of neither the clones nor the 490 bp T-RF was inferable for fractions of increased BD (Tab. 3.1., Fig. 3.4.). Thus, while the employed 16S rRNA and *bssA* gene targeted PCR assays detected these microbes, SIP analysis can clearly exclude any functional importance in toluene degradation for these community members in the applied biogeochemical setting.

3.5. References

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4. Depth-resolved microbial community structure and quantitative localisation of anaerobic toluene degraders in distinct redox zones of a tar-oil contaminant plume

4.1. Introduction

The fate of organic contaminants in groundwater environments is controlled by a multitude of abiotic and biotic processes such as transport, dilution, dispersion, and chemical or microbial degradation. The latter is frequently considered as the only sustainable component of natural attenuation processes (Christensen et al., 2000; Röling and van Verseveld, 2002). Due to high carbon loads and the slow rates of oxygen replenishment to groundwater systems, oxygen is usually depleted quite rapidly upon contaminant impact (Anderson and Lovley, 1997). Therefore, within hydrocarbon contaminated aquifers, anoxic contaminant plumes with distinct redox compartments are formed, where microbial guilds capable of using locally available electron donors and acceptors are active (Christensen et al., 2000). Biodegradation processes occur at different rates in these redox zones (Röling and van Verseveld, 2002), but it is still poorly understood which plume compartments are most relevant for net contaminant removal. Especially overlapping counter-gradients of electron donors and acceptors are assumed to be hot spots of biodegradation activities (Cozzarelli et al., 2001; van Breukelen and Griffioen, 2004; Tuxen et al., 2006; Anneser et al., 2007b), which has been recently been summarised as the "plume fringe concept" (Bauer et al., 2007). Such redox gradients may be extant on very fine spatial scales, thus a detailed characterisation of redox species and indigenous microbiota at appropriate spatial resolution is a prerequisite for the better understanding of biodegradation processes.

It is especially relevant to ask how the spatial distribution of plume compartments and degradation processes is correlated to local microbial communities in general, and whether the distribution of specific contaminant degraders may give information on the occurrence and localisation of the respective processes. Local microbial community composition has been shown to yield important insights on the microbes characteristic for different contaminated zones, as well as on their putative involvement in specific transformation processes (Dojka et al., 1998; Lin et al., 2005; Mouser et al., 2005; Allen et al., 2007). However, the monitoring of microbial capacities and their distribution at contaminated sites as a basis to assess natural attenuation and to promote biota-based site management options is still in its infancy. This may be partially attributed to the fact that conventional multi-level groundwater sampling occurs with a depth-resolution of meters (Smith et al., 1991; Lerner et al., 2000). This spatial resolution has been suggested inadequate to truly assess ongoing natural attenuation processes (Wilson et al., 2004).

In this study, to address these questions, the microbial community distribution across a high-resolution depth profile of a tar-oil impacted aquifer was characterised. Investigations were done at a well characterised contaminated former gasworks site where degradation has been reported to depend mainly on sulfate reduction (Wisotzky and Eckert, 1997; Eckert and al., 2000). As a model system, intrinsic populations of anaerobic toluene degraders were specifically traced using their benzylsuccinate synthase (*bssA*) genes as a specific catabolic marker (Winderl et al., 2007). The benzylsuccinate synthase is the key enzyme of anaerobic toluene oxidation and has been repeatedly proven as a valuable functional marker gene for unknown anaerobic toluene degraders (Washer and Edwards, 2007; Winderl et al., 2007), and

applied also in quantitative PCR (Beller et al., 2002; Da Silva and Alvarez, 2004). Thus it could be shown for the Flingern site that local anaerobic toluene degraders are dominated by an as-yet unaffiliated lineage of environmental *bssA* genes, tentatively termed "F1-cluster" *bssA*, which has ~90% amino acid similarity to known geobacterial *bssA* genes (Winderl et al., 2007). However, the identity of the degraders carrying these *bssA* genes, as well as their localisation and relevance for net BTEX degradation in the contaminant plume remain to be addressed.

At the site, undisturbed sediment cores were collected and subsequently installed a unique high-resolution multilevel monitoring well (HR-MLW (Anneser et al., 2007b)). Using fine-scale hydrogeochemical analyses (with a depth-resolution in intervals between 5 and 33 cm) as well as spatially resolved qualitative and quantitative molecular microbial community analytics it could be demonstrated that a highly specialised community of microbes related to known iron- and sulfate reducers, as well as a surprisingly high abundance of specific anaerobic toluene degraders resides within the biogeochemical gradient zone underneath the highly contaminated plume core. These findings show that the distribution of specific aquifer microbiota as well as redox and degradation processes in contaminated aquifers are tightly coupled.

4.2. Experimental procedures

4.2.1. Site description, sediment sampling and biogeochemical measurements

Subsurface samples from a tar-oil contaminated former gasworks site in Düsseldorf-Flingern (Wisotzky and Eckert, 1997; Eckert and al., 2000; Liebich et al., 2000) were obtained from fresh sediment cores taken during the installation of HR-MLW 19222 in June 2005. A precise description of the well and its installation is detailed elsewhere (Anneser et al., 2007b; Anneser et al., 2007a). Sediment liners were flushed with argon during drilling and sampling in order to prevent oxidation of oxygen sensitive analytes. Sediment sub-cores were taken from the liners between ~6 and ~13 m below ground surface (bgs, the groundwater table was at ~6.40 m bgs) using sterile 50 ml plastic tubes. Sub-samples were immediately frozen on dry ice and stored at –20°C until DNA extraction. Groundwater samples for the analysis of contaminants and redox species were sampled from the HR-MLW in September 2005. Dissolved toluene, sulfide and ferrous iron in depth-resolved groundwater samples as well as sedimentary PAHs and ferrous iron were measured as previously described (Anneser et al., 2007b; Anneser et al., 2007a).

4.2.2. Nucleic acid extraction

DNA was extracted in triplicate from sediments of 19 different depths between 5.7 and 12.7 m bgs. This covered all major redox compartments of the Flingern aquifer as well as one sample from the unsaturated zone (5.7 m) and one from the capillary fringe (6.3 m). DNA was extracted from freshly thawed ~1 g (~500 µl) aliquots of sediment material using a modification of a previously described protocol (Lueders et al., 2004). Samples were suspended in 650µl PTN buffer (120mM NaPO4, 125 mM Tris, 0.25 mM NaCl, pH8) and incubated at 37°C for 30 min with 40 µl Lysozym (50 mg ml^{-1}) and 10 µl Proteinase K (10 mg ml^{-1}). After addition of 200 µl 20% SDS, incubation was continued for 30 min at 65°C. Subsequently, the sediments were bead beaten (45 s at 6.5 ms^{-1}) with ~0.2 ml of Zirconia/Silica beads (a 1:1 mix of 0.1 and 0.7 mm diameter, Roth) in 2 ml screw cap vials. Afterwards, nucleic acids were sequentially purified by extraction with 1 vol. of phenol-chloroform-isoamylalcohol (25:24:1) and 1 vol. of chloroform-isoamylalcohol (24:1), and precipitated with 2 volumes of 30% polyethylene glycol (Griffiths et al., 2000) by incubation at 4°C for 2 h, and by centrifugation at 20,000 g and 20°C for 30 min. All reagents were from Sigma, if not otherwise stated. For each single extract, 2 replicate extractions were pooled in 25 µl of elution buffer (Qiagen) and stored frozen (-20°C) until further analyses.

4.2.3. Quantitative PCR

Real-time quantitative PCR (qPCR) measurements were performed on a MX3000P qPCR cycler (Stratagene). For each sediment depth, three independent DNA extracts were quantified in three different dilutions (1:5, 1:10, 1:20) to account for the possibility of PCR inhibition in less diluted templates (Ochsenreiter et al., 2003). Total bacterial 16S rRNA gene quantities were measured using a previously described SYBR Green PCR (Stubner, 2002) with minor modifications. Standard *Taq* polymerase (Fermentas) assays were used in the presence of 0.1 x SYBR Green (FMC Bioproducts). Initial denaturation (94°C, 3 min) was followed by 50 cycles of denaturation (94°C, 15 s), annealing (52°C, 15 s), and elongation

(70°C, 30 s). Subsequently, a melting curve was recorded between 55°C and 94°C to discriminate between specific and unspecific amplification products. An almost full length bacterial 16S rRNA gene amplicon of *Azoarcus sp.* T (Krieger et al., 1999) genomic DNA was quantified using the PicoGreen dsDNA quantification kit (Molecular Probes) and utilised as standard DNA for qPCR in a concentration range between 10^7 and 10^1 copies μl^{-1}.

For *bssA* qPCR, a TaqMan system for the previously identified F1-cluster of unidentified *bssA* genes (Winderl et al., 2007) was developed (Tab. 4.1.). An analogous assay for the *bssA* gene of "*Aromatoleum aromaticum*" strain EbN1 (Kube et al., 2004; Wöhlbrand et al., 2007) was also developed. Primer and probe design was conducted with the primer3 software v.0.3.0. (http://frodo.wi.mit.edu/). *bssA* qPCR was performed with the TaqMan Universal Master Mix Kit (Applied Biosystems) as specified by the manufacturer, using an annealing temperature of 55°C and 5'-FAM, 3'-TAMRA dual-labeled probes (Biomers). A PicoGreen quantified M13 amplicon of the F1-cluster partial *bssA* gene clone D12-03 (Accession # EF123678) previously retrieved from Flingern sediments and an almost full-length *bssA* amplicon of "*A. aromaticum*" strain EbN1 were taken for standardisation in concentrations between 10^7 and 10^1 copies μl^{-1}.

Table 4.1.: qPCR primer and TaqMan probe sets used in this study for the quantification of defined *bssA* gene copy numbers in sediment DNA extracts.

Primer/Probe	5' – 3' sequence	Location	Specificity
bssApd2f	CCT ATG CGA CGA GTA AGG TT	8235-8254	F1-cluster *bssA* (Winderl et al., 2007)
bssApd2r	TGA TAG CAA CCA TGG AAT TG	8416-8435	
bssApd2h	TCC TGC AAA TGC CTT TTG TCT CAA	8295-8318	
BssN2f	GGC TAT CCG TCG ATC AAG AA	7904-7923	strain EbN1 *bssA* (Kube et al., 2004)
BssN2r	GTT GCT GAG CGT GAT TTC AA	8109-8128	
BssN2h	CTA CTG GGT CAA TGT GCT ATG CAT G	8005-8029	

Primer and probe location are given according to *Thauera aromatica* K172 *bss* operon nucleotide numbering (Leuthner et al., 1998).

To correct for potentially distinct amplification/detection efficiencies of the different qPCR assays for 16S rRNA and F1 *bssA* genes (i.e. SYBR Green vs. TaqMan PCR assays) and also for putative extraction/detection efficiencies of the general DNA-workflow in the labratory, defined biomass amendments were evaluated for the Flingern sediments. For this, sediments were sterilised over night at 180°C to eliminate all intrinsic nucleic acids. Then, sediments were re-wetted with sterile water and amended with defined cell numbers of a freshly grown, SYBR Green-counted liquid culture of "*A. aromaticum*" EbN1 between 8 x 10^5 and 8 x 10^6 g^{-1} wet weight of sediment. Care was taken to adjust the sediments to original water content. Strain EbN1 carries 4 *rrn*- and 1 *bss*-operon per genome (Kube et al., 2004; Rabus et al., 2005). Nucleic acids were re-extracted as described above, and ribosomal rRNA genes and EbN1-specific *bssA* genes were quantified using the respective SYBR Green and TaqMan assays. From the detected vs. expected gene quantities, correction factors for 16S and *bssA* gene counts directly obtained from Flingern sediments were inferred.

4.2.4. 16S rRNA and *bssA* gene targeted T-RFLP fingerprinting

Terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA gene amplicons was done as previously described (Lueders et al., 2006) with primers Ba27f-FAM / 907r and *Msp*I digestion. For analysis of *bssA* gene fragments the primer set 7772f / 8546r-FAM was used followed by a *Taq I* restriction treatment for 2h at 65°C. Primary electropherogram analysis was performed using the GeneMapper5.1 software (Applied Biosystems) and community T-RF frequencies were inferred from peak heights (Lueders and Friedrich, 2003). Signals with a peak height below 100 relative fluorescence units (Osborn et al., 2000) or with a peak area contribution below 1% (Lukow et al., 2000) were considered as background noise and excluded from further analysis. The Shannon-Wiener index *H'* was calculated as $H' = -\sum pi \ln pi$, whereas *pi* is the relative abundance of single OTUs in a given fingerprint (Hill et al., 2003).

For statistics, T-RFLP data were evaluated as previously described (Ramakrishnan et al., 2001; Lueders and Friedrich, 2002) using the SYSTAT 10 software (SPSS Inc.). Principal component analysis (PCA) was performed on T-RFLP profiles with samples (sediment depths) as rows and relative fluorescence intensities of the binned T-RF data set as columns. A covariance data matrix was extracted with pairwise deletion and no factor rotation. Data reduction provided a two-factorial PC ordination of the overall variance of the T-RFLP profiles. Additionally, a loading plot of inferred PC factors on specific T-RFs was generated to identify the T-RFs especially correlated to the discrimination of depth-resolved microbial community fingerprints in PCA ordination.

4.2.5. Cloning, sequencing, and phylogenetic analyses

Almost full length bacterial 16S rRNA gene amplicons were generated from DNA extracts of four different depths (6.3 m, 6.8 m, 7.6 m, 11.7 m) using the primer set Ba27f and 1492r (Weisburg et al., 1991). Amplicons were cloned and sequenced as previously described (Winderl et al., 2007). Sequencing reads were manually assembled and checked for quality using the SeqMan II software (DNAstar). All clones were subsequently screened for similarities to published sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and integrated into an ARB database (Ludwig et al., 2004). Alignment was done using the Fast Aligner tool as implemented in ARB and corrected manually, if necessary. All clone sequences were checked for chimeric nature with CHIMERA_CHECK 2.7 of RDP-II version 8.1 (http://rdp8.cme.msu.edu/html/) and by manual inspection of the alignment. From 151 clones, 6 were identified as chimera and excluded from further analysis. For phylogenetic affiliation, trees including clones and closely related representative sequences (> 1400 bp) of cultivated and as yet uncultivated species of major bacterial phyla were constructed using neighbor-joining algorithms. Highly variable regions within the 16S rRNA sequences were omitted from the analysis by application of a 50% base frequency filter. T-RFs of cloned sequences were predicted using ARB_EDIT4. For a representative set of clones, T-RFs predicted *in silico* were verified by direct T-RFLP analysis of cloned amplicons to more precisely assign observed environmental T-RFs to cloned lineages. Rarefaction analysis of 16S rRNA clone libraries was performed using the Analytical Rarefaction software (version 1.3; S. M. Holland, University of Georgia, Athens,

http://www.uga.edu/~strata/software/Software.html). All clone sequences of this study were deposited with GenBank under the accession numbers XXX to XXX (to be added in review).

4.3. Results

4.3.1. Depth distribution of contaminants and redox species in the plume

Sediment cores were taken at the tar-oil contaminated sandy aquifer in Düsseldorf-Flingern in the course of the drilling and installation of a HR-MLW in June 2005 (Anneser et al., 2007b; Anneser et al., 2007a). After installation, the well and the surrounding contaminated aquifer were allowed to equilibrate, before the first depth-resolved sampling of groundwater was conducted in September 2005. In contrast to conventional low-resolution sampling, the HR-MLW revealed steep gradients of contaminants and redox species over the depth transect (Anneser et al., 2007b; Anneser et al., 2007a). In Fig. 4.1., the depth-resolved distribution the most prominent BTEX compound at the site, toluene, is displayed together with that of the reduced electron acceptors sulfide and ferrous iron in groundwater, as well as sedimentary PAHs and ferrous iron.

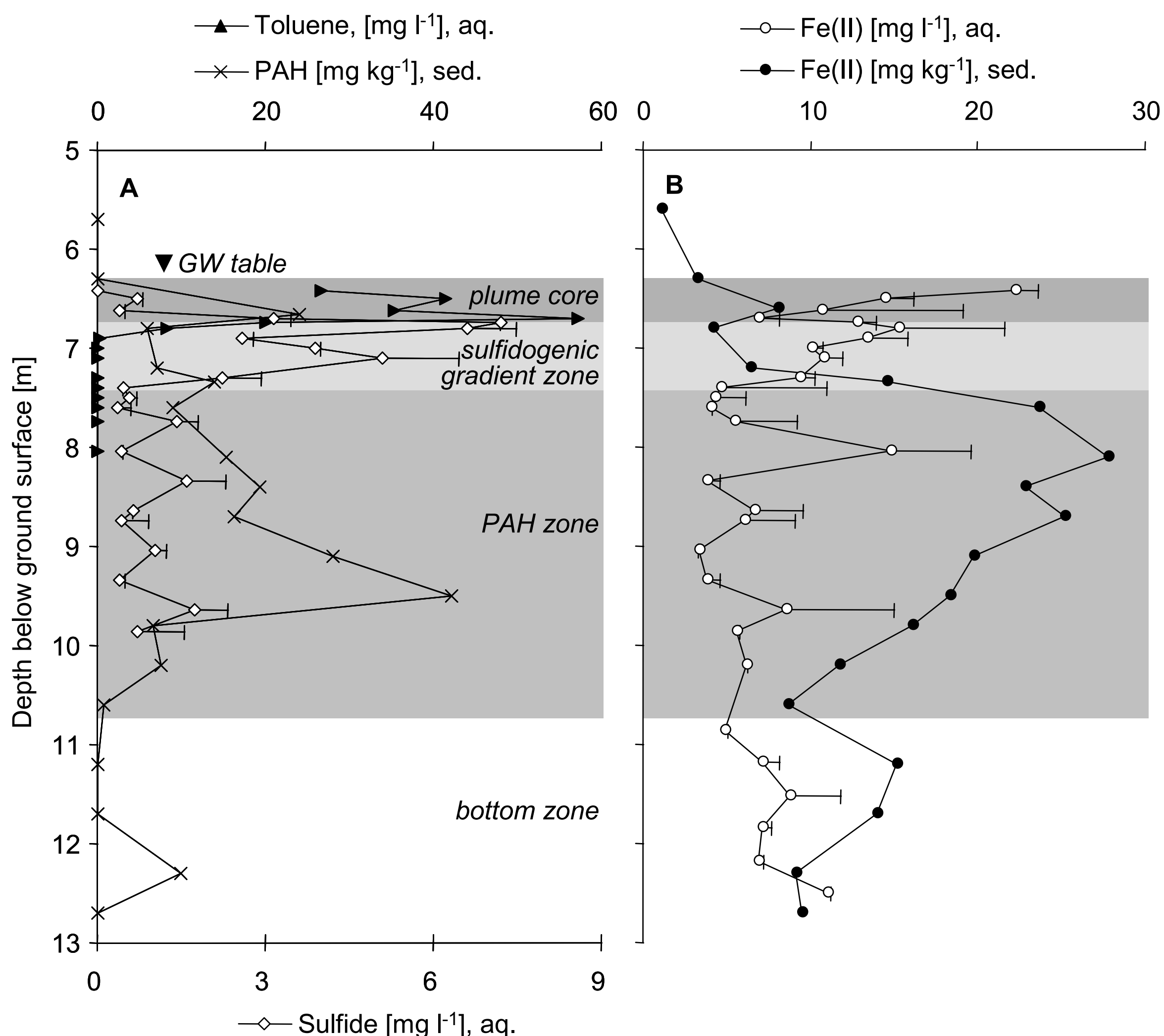

Figure 4.1.: Depth profiles of representative aromatic contaminants (A) and reduced electron acceptors (A, B) in sediment samples (sed.) and groundwater (aq.) of the Flingern aquifer. Samples were from June (sed.) and September (aq.) 2005, respectively. Error bars indicate means of duplicate measurements, the absence of error bars indicates single measurements. Plume and redox compartments identified in accordance to the biogeochemical data are specified.

In the upper 30 centimeters of the saturated zone (~6.4 m to ~6.7 m bgs), toluene concentrations in groundwater averaged at ~40 mg l^{-1} and thus defined a highly contaminated plume core directly beneath the groundwater table. Toluene contributed ~2/3 of total BTEX concentrations detected within these depths. Underneath this plume core, a ~65 cm wide gradient zone characterised by a strong decrease in contaminant concentrations and elevated levels of dissolved ferrous iron and especially sulfide was identified between 6.75 and 7.4 m bgs. Biogeochemical data indicated this “sulfidogenic gradient zone” (Fig. 4.1.) to be the “hot spot” of BTEX degradation within the Flingern plume at time of sampling. Previous investigations indicate sulfate reduction to dominate contaminant degradation at this site (Wisotzky and Eckert, 1997; Anneser et al., 2007a) and groundwater sulfide clearly peaked here. All BTEX compounds were below detection limit underneath depths of ~8 m.

Beneath the strongly sulfidogenic gradient zone, increased microbial activities were still inferable from free sulfide and especially sedimentary ferrous iron concentrations (Fig. 4.1.B). Contaminants other than BTEX, i.e. PAHs, were sorbed to the sediments down to ~10 m bgs, but also below (Fig. 4.1.A). This points towards a distinct, mainly PAH contaminated zone (~7.5 m to ~10.7 m). Naphthalene and flourene contributed most of the PAHs identified by EPA standards. Free sulfide was below detection limit in depths beneath 10 m. The remaining sampling depth to the underlying aquitard (~10.7 m to 12.7 m) was defined as a still reduced, but less contaminated bottom zone of the Flingern aquifer (Fig. 4.1.).

4.3.2. Quantitative distribution of bacteria and anaerobic toluene degraders

Previously detected microbes carrying an as-yet unaffiliated type of putative deltaproteobacterial *bssA* genes (termed the F1-cluster) were found to dominate the community of anaerobic toluene degraders in the Flingern aquifer, which led to the hypothesis that the respective microbes are involved in on-site toluene degradation under sulfate-reducing conditions (Winderl et al., 2007). To correlate the quantitative distribution of the F1-cluster microbes to that of contaminant and redox species and total bacterial populations, depth-resolved qPCR quantifications were conducted over the various plume compartments.
First, however, it was necessary to validate the comparative extraction/detection efficiencies of the distinct employed qPCR assays (16S rRNA gene SYBR Green PCR vs. *bssA* TaqMan PCR). For this, defined cell numbers of the denitrifying anaerobic toluene-degrading “*Aromatoleum aromaticum*” strain EbN1 were added to sterilised Flingern sediments. Strain EbN1 carries 4 *rrn*- and 1 *bss*-operon per genome (Kube et al., 2004; Rabus et al., 2005). While expected *bssA* abundances were almost absolutely recovered at ~8 x 10^6 added cells g^{-1} sediment, 16S rRNA gene copy numbers were significantly underrepresented by a factor of ~6. The inferred correction factor was 5.5 ± 0.6 for the expected vs. detected 16S/*bssA* gene frequencies over several orders of magnitude of cell biomass amendment. This factor was used to deduce corrected gene quantities from field qPCR measurements (Fig. 4.2.).

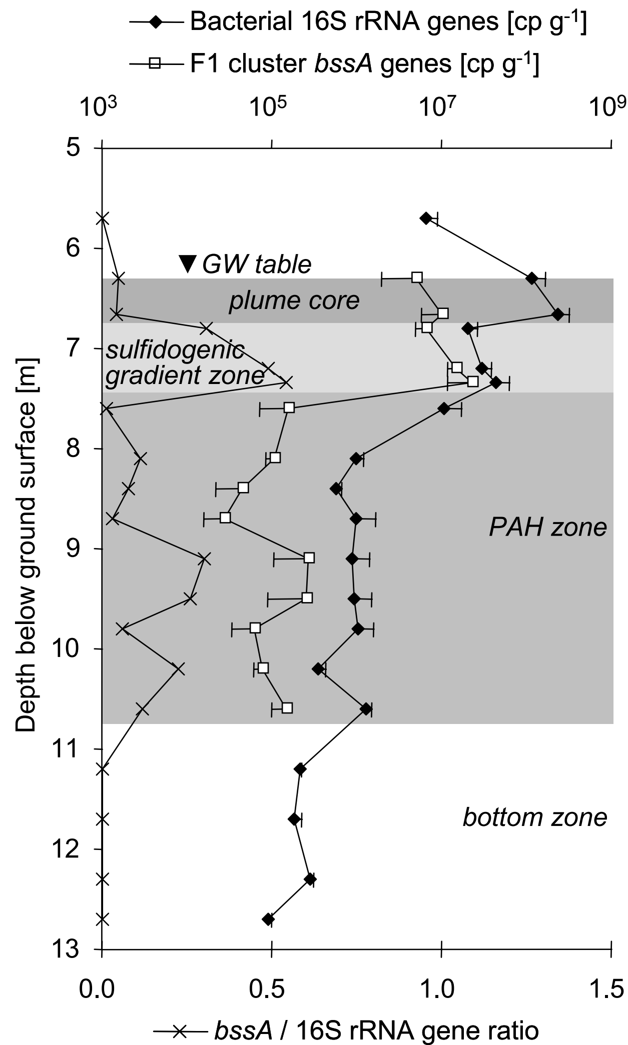

Figure 4.2.: Depth distribution of bacterial 16S rRNA genes and specific *bssA* genes of the F1-cluster of unknown anaerobic toluene degraders (Winderl et al., 2007) as measured via qPCR. Shown are means of copies per g wet weight of sediment (cp g^{-1}) + or – StdErr of three independent DNA extractions for each depth. The ratio of *bssA* vs. 16S rRNA genes is plotted on the secondary axis.

Bacterial rRNA genes were detected in all depths and peaked at a maximum of 2.4 ± 0.8 x 10^8 copies per g sediment wet weight (cp g^{-1}) directly within the plume core (6.65 m bgs). 16S quantities were generally above 10^7 cp g^{-1} in the upper zones of the aquifer (plume core and sulfidogenic zone), and dropped drastically to ~9 x 10^5 in the PAH contaminated zone, and to 1 – 3 x 10^5 in the bottom zone. The most stable abundance distribution was within the PAH zone at a depth between 8.7 m and 9.8 m.

In contrast to bacterial rRNA genes, the quantitative distribution of *bssA* genes peaked at a maximum of 2.3 ± 0.1 x 10^7 cp g^{-1} within the sulfidogenic gradient zone, and not within the plume core. The sulfidogenic zone was generally characterised by high *bssA*/16S rRNA gene ratios, ranging between 0.3 and 0.5 (Fig. 4.2.). This points towards the establishment of a highly specialised degrader community in these sediment depths. Moreover, F1-cluster *bssA* genes were not detectable by TaqMan PCR (detection limit ~5 x 10^3 cp g^{-1}) in sediments deeper than 11 m or above 6 m. Thus, their detection was highly correlated to saturated anaerobic contaminated sediments. Furthermore, by *bssA*-targeted T-RFLP fingerprinting with a previously published general primer set (Winderl et al., 2007), it could be shown that

the F1-cluster of *bssA* genes strongly dominated all depth-resolved *bssA* populations detected in the Flingern aquifer (Fig. 4.3.A).

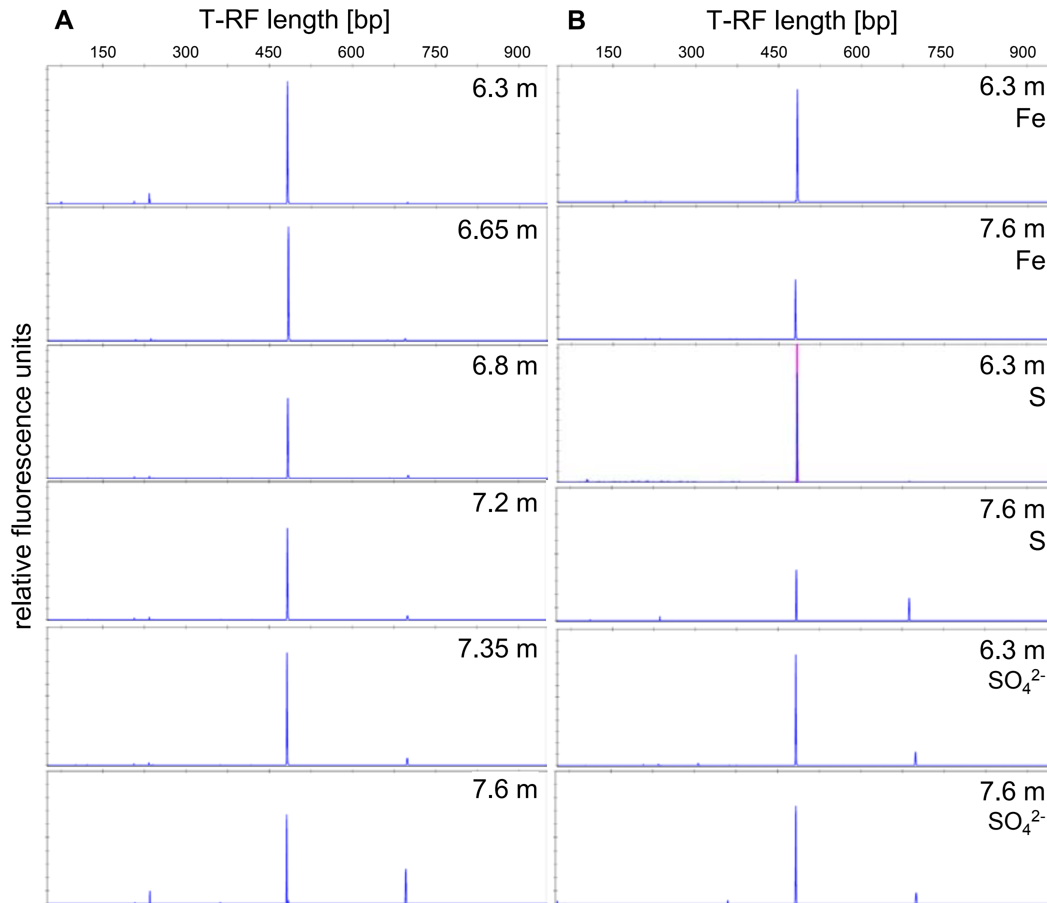

Figure 4.3.: T-RFLP fingerprinting targeting *bssA* genes retrieved from the Flingern sediments (A) and from enrichment cultures (B) of different depths. The enrichments were grown anaerobically with iron, sulfur, and sulfate, respectively, as electron acceptors (as indicated) and kindly provided by Umakanth Kunapuli (GSF-IGOE, Neuherberg).

4.3.3. Depth-resolved shifts in bacterial communities

Structural shifts in depth-resolved microbial communities were assessed via T-RFLP fingerprinting (Fig. 4.4.). Fingerprinting data indicated strong changes in the diversity and composition of bacterial communities established within the different depths. The Shannon-Wiener diversity *H'* as inferred from relative T-RF abundances dropped to local minima within the highly contaminated plume core (6.6 m bgs), in the sulfidogenic gradient zone (7.2 m), and at the transition of the PAH and bottom zones (10.6 m) (Fig. 4.4.A).

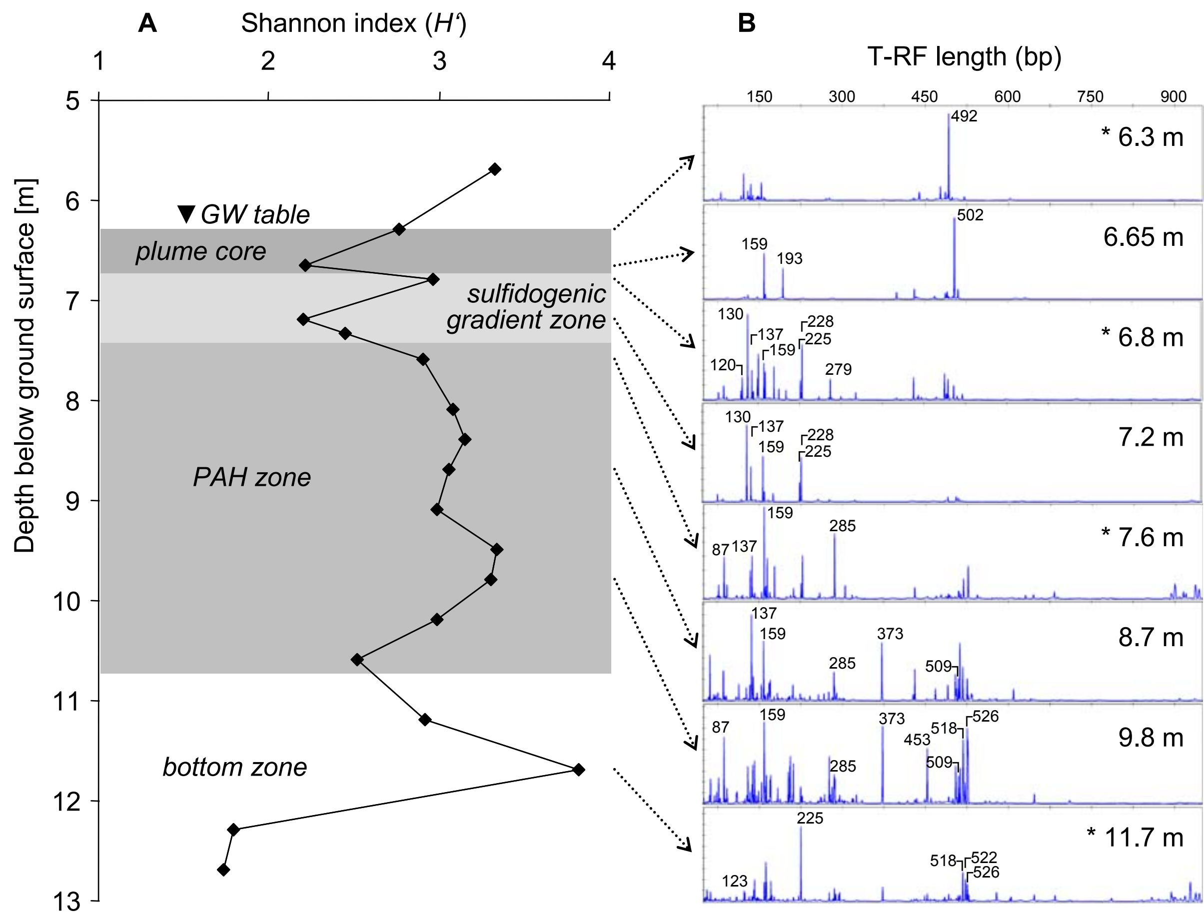

Figure 4.4.: Depth-resolved 16S rRNA gene T-RFLP fingerprinting of bacterial community structures in plume compartments. (A) Shannon-Wiener diversity index *H'* calculated for the entire T-RFLP data set. (B) Representative T-RFLP electropherograms of selected depths. Community patterns marked by an asterisk [*] were subsequently selected for cloning. Selected characteristic T-RFs [bp] mentioned in the text are indicated.

Similar as for absolute gene quantities, diversity was relatively stable throughout the PAH contaminated zone. Surprisingly, *H'* reached both absolute maximum and minimum within the bottom zone.

Not only the diversity, but also the structural composition (*i.e.* the identity [bp] and relative abundance of detected T-RFs) of depth-resolved fingerprints indicated significant community shifts to occur with depth. To better visualise these depth- and zone-related population shifts and to identify specific T-RFs characteristic for respective depths or plume zones, principal component analysis (PCA) of the combined T-RFLP data set was conducted. This data reduction clearly grouped fingerprints from different plume zones into distinct clusters (Fig. 4.5.A). Especially, populations from the sulfidogenic zone differed significantly from other samples, indicating distinct populations in these strata. Communities from the PAH and bottom zones were quite similar to each other and formed two adjacent clusters in PCA ordination. Outliers were observed for the capillary fringe and especially the plume core. In contrast, the vadose zone community clustered closely together with the PAH and bottom zone samples.

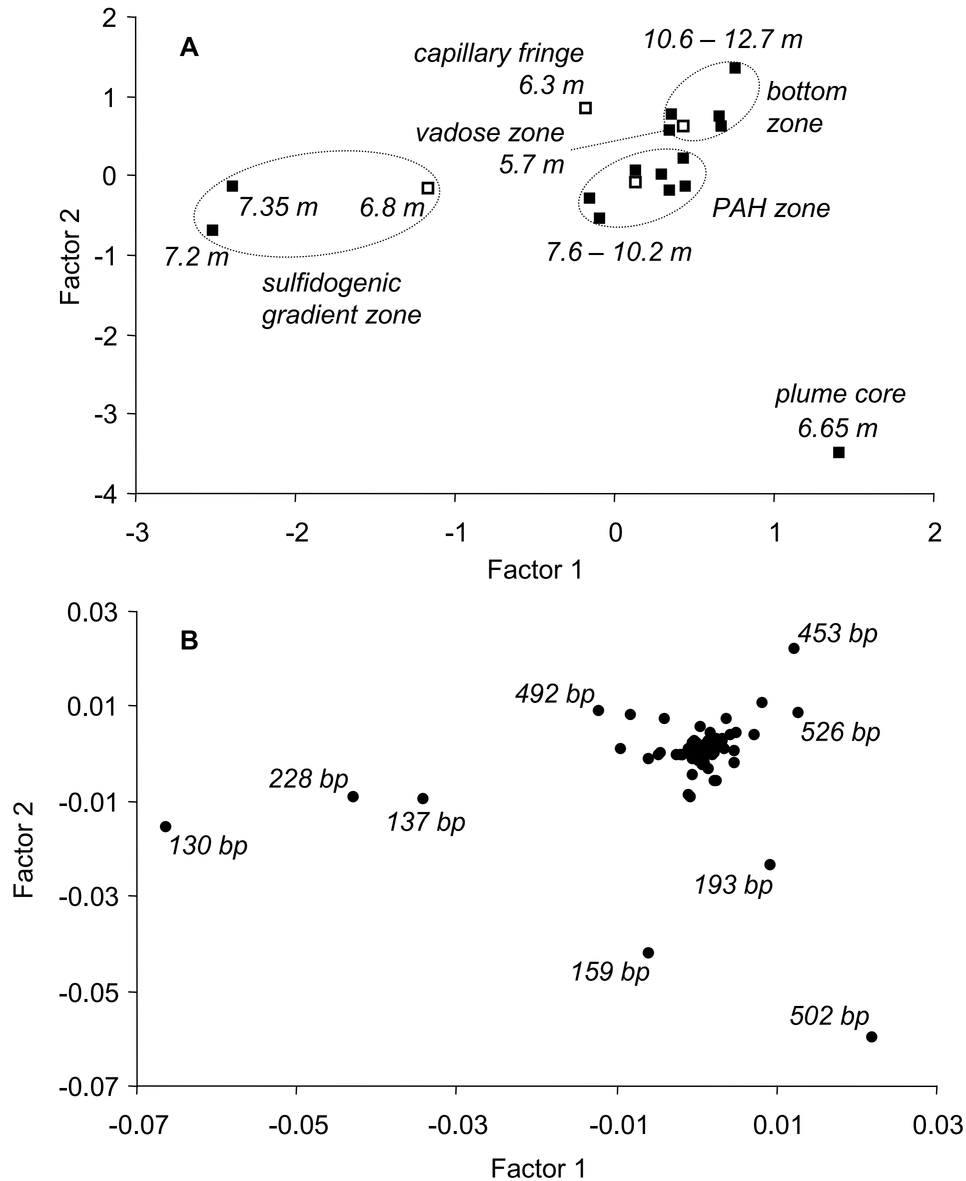

Figure 4.5.: (A) Principal component ordination of the overall variance in depth-resolved bacterial community composition as analysed T-RFLP fingerprinting. The depth at which specific fingerprints were retrieved is indicated in italics next to ordination points. Communities marked by an open square where subsequently selected for cloning. Inferred principal component factors 1 and 2 accounted for 21.3 and 18% of total variance, respectively. (B) Loading plot of inferred principal component factors on specific T-RFs. The identity [bp] of selected T-RFs with characteristic factor loading is indicated in italics.

The loading plot of inferred principal component factors (Fig. 4.5.B) helped to identify the specific T-RFs responsible for the distinct PCA ordination of depth-resolved community fingerprints. Hence, the sulfidogenic zone samples were characterised by high relative abundances of especially the 130, but also the 137 and 228 bp T-RFs. Also the 225 and 279 bp fragments were abundant in these communities (Fig. 4.4.B), but not PCA discriminators from other depths. The distinct ordination of the plume core fingerprint was correlated to a high abundance of the 502 and 193 bp, but also the 159 bp T-RFs. The latter, however, was ordinated in-between the plume core and sulfidogenic zone samples and thus can be interpreted as an important constituent of all communities, as confirmed also by visual inspection of electropherograms (Fig. 4.4.B). The capillary fringe community was

discriminated by the 492 bp fragment, and the bottom zone samples by increased abundances of the 453 and 526 bp T-RFs. Characteristic fragments of the PAH zone were not detectable in PCA ordination, since all remaining T-RFs clustered near zero in ordination space. Nevertheless, their high relative abundance allowed for the identification of the 87, 137, 159, 285, 373, 509, 518, and 526 bp fragments being important within this zone (Fig. 4.4.B).

4.3.4. Bacterial 16S rRNA clone libraries from four depths

To phylogenetically characterise the distinct microbial assemblages intrinsic to the different plume compartments and to identify putative community members represented by the identified characteristic T-RFs, four clone libraries were constructed. Overall, 145 full-length bacterial 16S rRNA clones from the depths of 6.3 m bgs (capillary fringe), 6.8 m (sulfidogenic zone), 7.6 m (PAH zone), and 11.7 m (bottom zone) were sequenced. The relative phylum-level composition of the clone libraries revealed surprisingly significant distinctions in local community assembly, as summarised in Tab. 4.2. *Alphaproteobacteria* were found almost exclusively at the capillary fringe (6.3 m), where they dominated the library together with members of the *Betaproteobacteria*. *Deltaproteobacteria*, which were frequent in all other libraries, were not detected at 6.3 m. In contrast, members of the *Beta-* and *Deltaproteobacteria*, and the *Clostridia* appeared especially frequent in the library of the sulfidogenic gradient zone (6.8 m). Members of the *Deltaproteobacteria*, *Actinobacteria*, and *Chloroflexi* were abundant in the PAH zone library, while *Betaproteobacteria* were missing here. The bottom zone clone library (11.7 m) contained the most even distribution of major bacterial phyla, and almost all major groups detected in one of the other depths were also found in this library (Tab. 4.2.).

Table 4.2.: Relative phylum-level composition of depth-resolved bacterial 16S rRNA gene clone libraries. Selected genus- or lineage-specific clone frequencies are also reported. Characteristic T-RF lengths [bp] predicted from sequence data for all or a major fraction of clones of a given affiliation are indicated together with T-RFs actually measured in T-RFLP analysis. Naming of phyla without cultivated representatives is in accordance to (Rappé and Giovannoni, 2003). Libraries at 6.3, 6.8, 7.6, and 11.7 m contained 35, 31, 43, and 36 clones, respectively. Division-level percentages (given in bold) include the genus- or lineage-specific percentages (non-bold).

	% of clones in library Name of library, depth bgs				T-RF [bp]	
Phylogenetic affiliation	**D10, 6.3 m**	**D12, 6.8 m**	**D15, 7.6 m**	**D25, 11.7 m**	pred.	meas.
Alphaproteobacteria	**31**	–	–	**3**	–	n.a.
- *Methylocystis*-related	3	–	–	–	–	n.a.
Betaproteobacteria	**26**	**19**	–	**14**	123,492	120,492
- *Thiobacillus*-related	6	–	–	6	–	n.a.
- *Gallionella*-related	6	6	–	–	123,125	120,122
Gammaproteobacteria	**9**	–	–	–	–	n.a.
- *Beggiatoa*-related	6	–	–	–	138	135
Deltaproteobacteria	–	**26**	**16**	**11**	–	n.a.
- TRM1/*Desulfocapsa*-rel.	–	6	–	–	162	159
- *Geobacter*-related	–	19	–	–	132	130
- *Syntrophus*-related	–	–	7	6	127,509	123,509
- *Desulfobacterium*-related	–	–	–	6	166	164
Bacteroidetes	–	**6**	–	**14**	91	87
Nitrospirae	**3**	–	**7**	**8**	–	n.a.
- *Magnetobacterium*-related	–	–	5	8	290	285
Bacilli	–	**10**	–	**8**	137	133
Clostridia	**14**	**29**	**2**	**3**	–	n.a.
- *Sedimentibacter*-related	–	19	–	–	280	279
- *uncult. Peptococcaceae*	6	–	–	–	282	281
- *Desulfosporosinus*-related	–	3	–	–	227	225
- *Desulfotomaculum*-related	–	–	–	3	214	n.d.
Actinobacteria	**3**	**3**	**16**	**11**	–	n.a.
- *Rubrobacter*-related	–	–	7	6	131,162	128,160
Chloroflexi	**3**	–	**23**	**17**	–	n.a.
- *uncultured I*	–	–	–	14	518	518
- *uncultured II*	3	–	21	3	373,454,523	373,453,522
- *Dehalococcoides*-related	–	–	2	–	–	n.a.
TM6	**9**	–	–	–	–	n.a.
OP5	–	–	**7**	–	230	224
OP10	–	–	**7**	**3**	–	n.a.
TM7 / OP11	–	**3**	**9**	–	–	n.a.
Others	**3**	**3**	**12**	**8**	–	n.a.

n.a. – not applicable; n.d. – not determined

A considerable diversity of clones closely related to defined genera or lineages well-known to be capable of characteristic catabolic or respiratory activities was detected. Sequences related to known methylotrophs, sulfur- and ferrous iron oxidisers were especially frequent in the capillary fringe library. The sulfidogenic zone library contained high frequencies of clones that were between 95 – 97% related to *Geobacter chapellei*, a well-known deltaproteobacterial iron reducer (Coates et al., 2001). Furthermore, clones affiliated to the *Desulfocapsa*-related sulfate-reducing strain TRM1 (Meckenstock, 1999) were found in this library. For these two lineages, signature T-RFs of 130 and 159 bp were inferrable from the clone libraries (Tab. 4.2.). The library was also characterised by a high ratio of *Clostridia*, related to *Sedimentibacter* and *Desulfosporosinus* spp.. These where represented within the 279 and 225 bp T-RFs, respectively. On the other hand, *Syntrophus*- (123 & 509 bp) and *Desulfobacterium*-related clones (164 bp T-RF) were detected only in the libraries from the two deepest samples, respectively. Clones closely related to unusual iron reducers of the *Magnetobacterium* candidate genus (285 bp) within the *Nitrospirae* (Spring et al., 1993) were also detected only in samples deeper than the sulfidogenic zone. Other observed signature T-RFs for the sampled metacommunity were 87 bp (different members of the *Bacteroidetes*), 120 and 492 bp (different *Betaproteobacteria*), 133 bp (different Bacilli), 224 bp (OP5 candidate division), and 373, 453, 518 and 522 bp (different uncultured *Chloroflexi*).
Rarefaction analyses of the bacterial communities retrieved within the clone libraries and that of the combined metacommunity showed that coverage of the libraries was by far insufficient, at both 95% sequence similarity (~ genus-level) and at phylum-level diversity cut-off (Fig. 4.6.). This was expected, but the aim of this study was not to fully elucidate the diversity of the distinct microbial communities retrieved from the different plume compartments, but to highlight most significant distinctions. Nevertheless, rarefaction curves revealed that the sampled diversity at 95% sequence similarity was highest and equal to that of the combined metacommunity in the PAH zone library (Fig. 4.6.A).

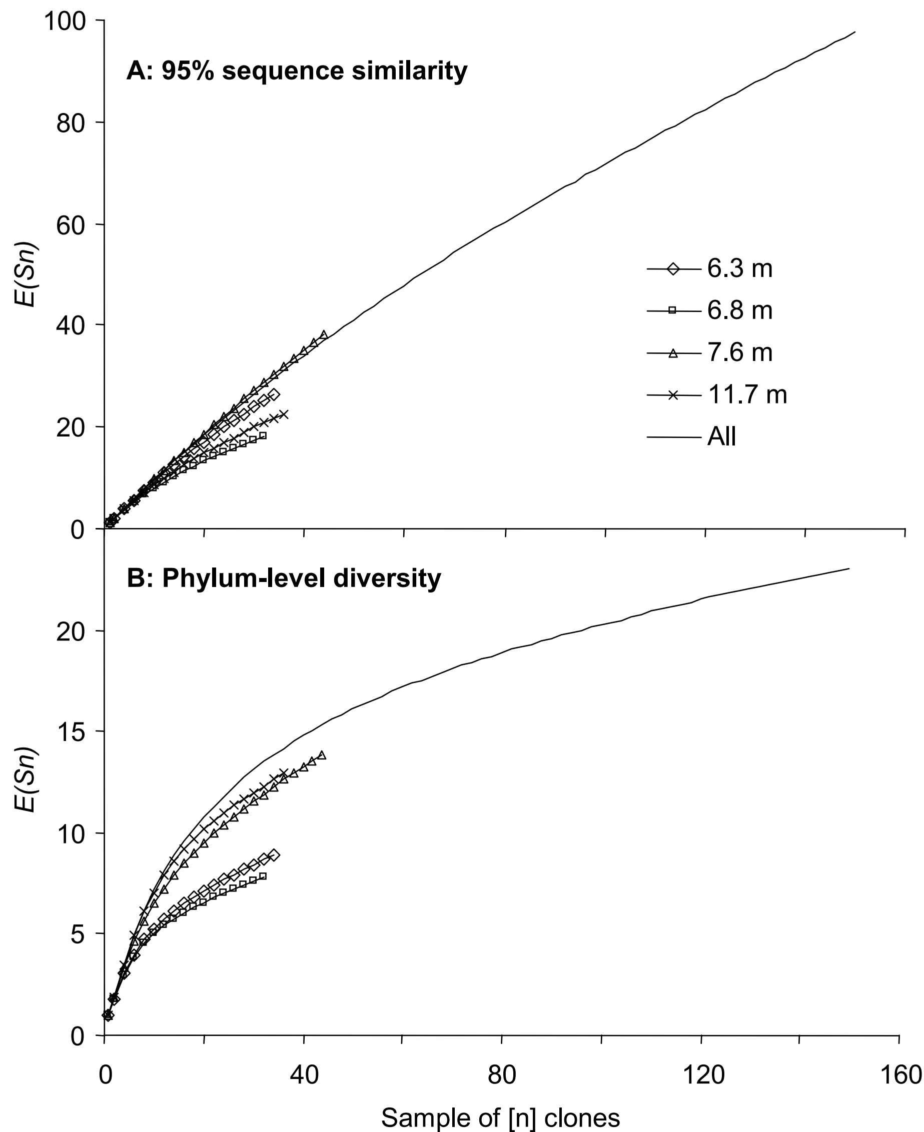

Figure 4.6.: Rarefaction analysis of the bacterial diversity retrieved in the four depth-resolved 16S rRNA gene clone libraries. Calculations were performed using (A) a 95% sequence similarity (~genus-level) cut-off and (B) a bacterial phylum-level cut-off consistent to Tab. 4.2. "All" indicates the expected number of taxonomic units [*E(Sn)*] for the summarised metacommunity.

Phylum-level diversity appeared significantly reduced in the two upper libraries, generated from heavily BTEX contaminated sediment samples (Fig. 4.6.B). And finally, the sulfidogenic zone library contained the lowest sequence diversity on both levels of resolution.

4.4. Discussion

4.4.1. Geochemical and microbial zonation in the Flingern plume

In this study, a fine-scale in-depth molecular characterisation of the distinct sediment microbial assemblages is provided. These organisms are found within defined contaminant and redox compartments of a BTEX contaminant plume. Microbial data based on nucleic acid extracts of aquifer sediments could be linked successfully to groundwater and sediment geochemical data obtained from the same site, an approach which has previously been shown to provide valuable insides on aquifer microbe/process correlations (Dojka et al., 1998; Bekins et al., 2001; Haack et al., 2004). A chief novelty of the present study is the fine spatial resolution of the microbiological and especially of the hydrogeochemical analyses, with intervals ranging between 5 -15 cm in zones of special interest, and between 30 - 60 cm in deeper zones. Conventional multi-level wells usually have a spatial resolution in the range of meters (Smith et al., 1991; Dojka et al., 1998; Lerner et al., 2000; Wilson et al., 2004), but geochemical redox gradients formed by microbial degradation processes can be expected to prevail at much smaller scales within contaminated aquifers (Cozzarelli et al., 2001; Tuxen et al., 2006; Anneser et al., 2007b). Thus, low-resolution groundwater sampling may fail to identify the defined strata where electron donors and acceptors meet, and provide only insufficient information on the biogeochemical and microbiological characteristics of such hot spots of degradation.

It was possible to identify a zone of increased sulfidogenic activities underneath the actual BTEX plume core, a zone of ~55 cm width in which sulfate-dependent contaminant degradation activities in the Flingern aquifer are assumed to be of special relevance (Anneser et al., 2007b; Anneser et al., 2007a). qPCR analysis (Fig. 4.2.) showed that this zone was characterised by significantly increased absolute and relative abundances of the yet unidentified microbes represented by the F1-cluster of environmental *bssA* genes, which was previously detected at the site (Winderl et al., 2007). Via a *bssA* clone library (Winderl et al., 2007) and also depth-resolved T-RFLP fingerprinting of *bssA* genes (Fig. 4.3.) it could be demonstrated that the F1-cluster constitutes the only major population of *bssA* genes detectable in the different depths of the Flingern aquifer. Moreover, enrichment cultures from Flingern sediments grown under different redox conditions show the same 483 bp T-RF as detected directly within the original sediments (Fig. 4.3.B), indicating either highly conserved *bssA* sequence types carried by different members of the Flingern community or pointing to one clade of metabolically versatile organisms capable of couple different electron accepting processes to BTEX degradation. Interestingly, the quantitative distribution of this catabolic gene marker with depth did not correspond to that of rRNA genes. In the core of the plume, where absolute rRNA gene abundance was highest, the inferred *bssA*/16S rRNA gene ratio was only ~0.04. Within the next few dm, this ratio increased dramatically to values between ~0.3 and ~0.5, concomitant to the observed increase in dissolved sulfide. This points towards the establishment of a highly specialised degrader community specifically adapted to the local biogeochemical setting not within, but underneath the highly contaminated plume core. These findings provide unprecedented field data to support the presumed “plume fringe concept”, which predicts that biodegradation of groundwater contaminants occurs mostly within surrounding biogeochemical gradients, and not directly within contaminant plumes (Cozzarelli et al., 2001; van Breukelen and Griffioen, 2004; Tuxen et al., 2006; Anneser et al., 2007b; Bauer et al., 2007).

It must be cautioned, however, that although the measured quantitative distribution profiles of both rRNA and *bssA* genes can be expected to be reliable, the absolute gene counts and hence also the specific *bssA*/rRNA gene ratios may still be biased. The attempt was made to correct for relative extraction/detection biases of the two distinct assays by analysing defined biomass amendments of strain EbN1. However, while the inferred correction factor may hold true for populations of strain EbN1, it may not be fully appropriate for the organisms of the F1-cluster. In fact, the corrected relative *bssA*/rRNA gene ratios between 0.3 and 0.5 in the sulfidogenic zone still seem quite high, assuming that typical oligotrophic groundwater microbiota will have 1 or 2 *rrn* operons, and degraders will carry 1 *bss* operon. Nevertheless, independent of absolute gene ratios the depth-resolved differences of both ribosomal and functional marker gene distribution will remain constant, clearly emphasising the increased degrader abundance within the sulfidogenic zone.

4.4.2. Depth-resolved microbial community distribution

Complementary fingerprinting and sequencing strategies were applied to unravel the correlations between local microbial community patterns and biogeochemical processes in different compartments of the Flingern BTEX plume. Within the upper parts of the contaminated aquifer (capillary fringe, plume core and sulfidogenic zone), where bacteria were generally more abundant than in the deeper zones, repeated significant shifts and drops in community diversity were observed on very small scales. This corroborates the existence of distinct local communities in these strata which are specifically selected for by the local contaminant and redox regime and highly adapted to the processes feasible in their small-scale habitat. It is currently not known on what time scales such specialised assemblages establish, especially considering the generally slow doubling times of aquifer microbiota, and how reactive they are to hydraulic dynamics or inputs of distinct electron acceptors (Haack et al., 2004; McGuire et al., 2005). Nevertheless, this spatial selection of distinct communities may be very relevant for the efficiency of net contaminant degradation processes.

In contrast, communities within the lower PAH zone were less abundant, but more equally distributed in terms of abundance and diversity over several meters of sediment depth. Thus here, selective pressures and ecological niches seem to be more equally distributed. Surprisingly, detected T-RF diversity as well as phylum-level clone library diversity was highest in the bottom zone sediments sampled at 11.7 m bgs. Unfortunately it was not possible to infer from the available data whether contamination actually caused a loss in total biodiversity (Gans et al., 2005) in the upper zones, or whether established degrader communities simply outgrow “baseline” diversity, thus shifting less active microbes below the detection limit of the applied assays (Goldscheider et al., 2006).

4.4.3. Identification of zone-specific microbiota

Direct spatial correlations between aquifer microbial community structure and contaminant scenarios have been previously recognised (Dojka et al., 1998; Franzmann et al., 2002; Lin et al., 2005; Allen et al., 2007). However, correlations between aquifer geochemistry and microbial communities can be extremely complex, and powerful statistical tools are needed to unravel these relations (Mouser et al., 2005; Schryver et al., 2006; Allen et al., 2007). Here, by a combination of T-RF statistics and cloning it was possible to actually identify distinct aquifer microbiota characteristic for the resolved plume compartments. This

identification however, relies on a satisfactory precision in linking T-RFs observed in environmental fingerprints to T-RFs of cloned sequences. Generally, incongruencies of ± 2 – 3 bp between predicted and measured T-RFs have to be taken into account (Liu et al., 1997; Kitts, 2001; Lueders and Friedrich, 2003) even if T-RFLP electrophoresis and T-RF sizing conditions are optimised with utmost caution (Thies, 2007). Thus, predicted T-RFs for representative clone sequences were verified in order to precisely affiliate detected environmental T-RFs to defined phylogenetic lineages. The average difference between characteristic predicted and measured T-RFs was -2.6 ± 1.7 bp in the analyses (Tab. 4.2.).

The sulfidogenic zone sediments where characterised by high relative abundances of especially the 130, but also the 137, 159, 225 and 228 bp T-RFs (Figs. 4.4. & 4.5.). Via sequence data from the respective clone libraries, the 130 bp T-RF could be clearly affiliated to the *Geobacter*-relatives detected only in the sulfidogenic zone library. It is striking that these iron reducers were not retrieved from other zones of the Flingern aquifer, where biogeochemical data seems to suggest a special importance of iron reduction (i.e. the capillary fringe, the PAH zone). The 130 bp T-RF, however, was detected also in some fingerprints of the PAH zone, albeit at low frequencies. Furthermore, microbes affiliated to the *Desulfocapsa*-related sulfate-reducing contaminant degrader TRM1 (Meckenstock, 1999) (159 bp T-RF), as well as clostridial sulfate reducers (Robertson et al., 2001) and fermenters (Breitenstein et al., 2002) (225 & 279 bp) were abundant in this zone.

Unfortunately, no clear affiliation of the 137 and 228 bp fragments within the sulfidogenic zone was possible. Although these T-RFs were predicted for different *Bacilli* and *Clostridia* within the clone libraries (Tab. 4.2.), they could not be verified by *in vivo* T-RFs. Thus these apparent constituents of the Flingern community remain unidentified at present, potentially due to undersampling, and may possibly even represent fingerprinting artifacts (Egert and Friedrich, 2003). Along the same line it must be stated that not all important T-RFs observed for the depth-resolved communities were recovered within respective clone libraries. Again, this may be an effect of the small clone libraries or the different primer sets used for both approaches (Ba27f / 907r for T-RFLP, and Ba27f / 1492r for cloning). Certainly, this is also attributed to the fact that unfortunately communities not from all relevant sediment depths, such as the plume core, were cloned and sequenced.

Nevertheless, the specific microbes characteristic for the sulfidogenic zone (deltaproteobacterial *Geobacter*- and *Desulfocapsa*-relatives, sulfate-reducing and fermenting *Clostridia*) were in clear contrast to those characteristic for e.g. the capillary fringe (*Alpha*- and *Betaproteobacteria*, the latter especially represented by the 493 bp T-RF) and for deeper zones of the Flingern aquifer (e.g. *Bacteroidetes*, 87 bp; *Magnetobacterium* relatives, 285 bp; *Chloroflexi*, 373, 454, 518, 526 bp T-RFs). High frequencies of deltaproteobacterial 16S rRNA genes within contaminated aquifer sediments have been previously described, especially for the known iron-reducing contaminant degraders of the *Geobacter* genus (Lovley et al., 1993; Coates et al., 1996; Lovley, 1997; Lin et al., 2005). Here, however, it could be demonstrated that close relatives of these known iron reducers co-dominate the sulfidogenic gradient zone together with relatives of deltaproteobacterial and clostridial sulfate reducers, which may point towards a co-localisation or an overlap of the respective redox processes in the Flingern aquifer. This hypothesis is supported by the apparently highly specific allocation of these distinct microbes to the gradient zone underneath the plume core, and the increased concentrations of both dissolved sulfide and Fe(II) in the sulfidogenic zone (Anneser et al., 2007b; Anneser et al., 2007a).

4.4.4. Affiliation of the F1-cluster *bssA* genes

Here, it could be shown that the yet-unaffiliated *bssA* genes of the F1-cluster (Winderl et al., 2007) relatively and absolutely dominate in abundance in the sulfidogenic gradient zone. Thus, it is a fair assumption that the corresponding microbes will have been covered also in 16S rRNA gene analyses. The F1-cluster *bssA* genes were previously identified to be ~90% related on amino acid level to known geobacterial *bssA*, and only ~75% related to the TRM1 *bssA* gene (Winderl et al., 2007). At the same time, it was shown for a distinct site that the employed general *bssA* primer set is well capable of detecting typical geobacterial *bssA* genes when present (Winderl et al., 2007). These facts allow formulating the following working hypotheses:

1.) Either the F1-cluster *bssA* genes belong to the detected *Geobacter*-relatives. This would then imply that these local populations carry *bssA* genes more distinct to typical geobacterial *bssA* (Kane et al., 2002; Winderl et al., 2007). Concomitantly, the quantitative dominance of these specific *bssA* genes especially in the sulfidogenic gradient zone could imply that toluene degradation in these strata is mostly dependent on iron reduction, and not on sulfate reduction as previously assumed (Wisotzky and Eckert, 1997; Eckert and al., 2000; Anneser et al., 2007b). Alternatively, the Flingern *Geobacteriaceae* may have means of transferring electrons from degraded BTEX compounds not only to ferric iron, but also to sulfate and to elemental sulfur, which may discriminate them from established *Geobacter* spp. The latter is supported by ongoing enrichment experiments in the Meckenstock laboratories, where Flingern sediment toluene degraders carrying the F1-type *bssA* gene were detected in both sulfate-reducing as well as iron-reducing enrichment microcosms (work in progress, Fig. 4.3.).

2.) Alternatively, the F1 *bssA* genes could belong to the deltaproteobacterial sulfate reducers (i.e. the TRM1-relatives) that were also highly characteristic for the gradient zone. Hence, these would then carry *bssA* genes distinct from that of strain TRM1 (Winderl et al., 2007), which would be acceptable. At the same time, though, it would imply that none of the detected *Geobacter*-relatives carry *bssA* genes, which otherwise would have been detected (see above), and that these are thus not involved in toluene degradation in the sulfidogenic zone. Regarding the specific allocation of the microbes to the BTEX degradation hot spot, and also the closer phylogenetic affiliation of the F1 *bssA* genes to known geobacterial sequences, the second scenario seems rather unlikely.

4.5. References

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5. General conclusion and outlook

The work presented in this thesis allows for a detailed insight into the structure of microbial communities present within hydrocarbon impacted aquifer ecosystems. Specific degraders were investigated at different tar-oil contaminated sites, where monoaromatic hydrocarbons e.g. benzene, toluene, ethylbenzene, and xylenes (BTEX) as well as polyaromatic hydrocarbons (PAH) are found as typical pollutants within the groundwater or the aquifer sediments (Zamfirescu and Grathwohl, 2001). The mainly anoxic contaminant plumes accommodate microorganisms capable of utilising the contaminant compounds anaerobically as substrate (Anderson and Lovley, 1997; Christensen et al., 2000). While aerobic degradation of BTEX compounds was studied for decades in various laboratory and field studies (Worsey and Williams, 1975; Zylstra et al., 1988; Zylstra and Gibson, 1989; Whited and Gibson, 1991a, b; Fries et al., 1997; Baldwin et al., 2003; Bagneris et al., 2005; Hendrickx et al., 2006a; Hendrickx et al., 2006b; Witzig et al., 2006), knowledge about anaerobic BTEX degradation was based mainly on cultivated bacteria (Beller and Edwards, 2000; Van Hamme et al., 2003; Kube et al., 2004; Meckenstock et al., 2004; Jahn et al., 2005). Within this thesis, one of the first comprehensive studies of natural communities of anaerobic toluene degraders generated by culture independent methods is provided.

The first objective of this work was to elucidate, which bacteria capable of degrading BTEX compounds are detectable at contaminated sites and whether site-specific differences can be identified regarding the diversity of these organisms. Due to the fact that anaerobic toluene degraders are widespread amongst different phylogenetic groups of organisms, a specific detection via ribosomal genes was not possible, and therefore a specific catabolic (=functional) marker assay had to be developed. This was realised with benzylsuccinate synthase (*bssA*) genes, encoding the key enzyme of anaerobic toluene and xylene degradation (Biegert et al., 1996; Kube et al., 2004). The assay was applied to different tar-oil contaminated sites (Pasing, Flingern, and Testfeld Süd) and different site-specific clusters of sequences were obtained. At the Pasing site, where dominant redox conditions are unknown, *geobacterial bssA* sequence types were exclusively detected, indicating a potential role of iron-reduction for on-site contaminant breakdown. At BTEX contaminated aquifers dominated by sulfate-reduction (Flingern and Testfeld Süd), it was possible to show that geobacterial, clostridial, and yet unidentified anaerobic toluene degraders are present (clusters F1, F2 and T1, T2), and thus potentially relevant for biodegradation processes. The novel deeply branching sequences (cluster T1 and T2) retrieved from highly contaminated Testfeld Süd sediments indicated at first glance that degraders affiliated outside the known proteobacterial lineages may be involved in toluene breakdown at Testfeld Süd. With this the evidence was given, that both so far known as well as novel contaminant-degrading microorganisms are of detectable at contaminated sites.

With this in mind, the next step was to identify some of the key players carrying these novel and deeply branching *bssA* sequence types, and to elucidate whether they are actively responsible for toluene degradation within the contaminated aquifers. To clarify this, stable isotope probing (SIP) was applied to the Testfeld Süd sediments. With SIP the key players in toluene degradation at this site could be identified as *Desulfosporosinus* related Gram-positive *Clostridia* (via their 16S rRNA genes), but no T1 or T2 cluster *bssA* sequences were found in these experiments. Surprisingly, these organisms were carrying *bssA* genes closely related to previously detected F2 cluster *bssA* sequence types retrieved from the Flingern site. Thus the deeply branching *bssA* sequences previously detected at Testfeld Süd and also the sequence

type retrieved from the Gram positive *Desulfotomaculum* sp. Ox39, which was isolated from the same site (Morasch et al., 2004), have to be regarded with caution. At present it seems more likely that these are *bssA* homologues and not involved in anaerobic toluene degradation, but may well be active in the initial attack of other hydrocarbons (Kniemeyer et al., 2003; Cravo-Laureau et al., 2005). With the SIP data presented here, it is more likely that the Flingern F2 cluster may represent a "true" *clostridial bssA* sequence type. Since these deeply branching sequence types were not detected within the SIP microcosms, it also has to be assumed that these enzymes are not involved in toluene breakdown at Testfeld Süd. Moreover, the distinct Flingern F2 cluster *bssA* sequences could be more directly linked to *clostridial* organisms by the amplification of the *bssA* genes of cultured toluene degrading *Desulfosporosinus* spp. (Liu et al., 2004), which was unfortunately not completed within the present thesis, but may help to further clarify the identity of clostridial *bssA* genes in the future.

Nevertheless, with the developed *bssA* assay it was possible for the first time, to detect toluene degrading microorganisms outside the known proteobacterial lineages, particularly the Flingern "F2" cluster, which was detected by applying the *bssA* assay directly to environmental samples as well as within the SIP approach, respectively. This was an important step, because non-proteobacterial clades could be identified as key players responsible for anaerobic BTEX breakdown in the environment. Another aspect revealed by the SIP experiment, was: why does the key player DNA under sulfate-reducing conditions not become fully ^{13}C-labelled? This should be expected, if the respective microbes exclusively utilise ^{13}C-labelled toluene, and has been observed for aromatic degraders under aerobic and denitrifying conditions. This highlights that the flow of carbon and energy during toluene degradation under sulfate-reducing conditions is still poorly understood. The "dilution" of the ^{13}C-label incorporated into nucleic acids by ^{12}C originating from carbon sources other than toluene is assumed to be observed due to "heterotrophic CO_2 assimilation". Electrons originating from the toluene would be used for CO_2 assimilation resulting in "missing" energy for growth of the degraders. If this is true, it would mean that the growth of degraders under certain anaerobic conditions may be limited due to the described mechanisms, which may account for reduced degradation rates and efficiency. It is of special importance to further investigate these metabolic pathways and physiologies in order to gain more knowledge about the limitations of biodegradation in the environment.

Further objectives to be accomplished in the present work were how microbial communities are structured within the different plume compartments, and how degrader abundances and distribution are correlated to ongoing degradation processes. Therefore, a fine-scale characterisation of the microbial community of the Flingern site was performed and abundances of total bacteria as well as specific degraders were quantified in spatial resolution. For the first time it could be demonstrated in the environment that specialised anaerobic toluene degraders are localised in highest relative and absolute abundances within the sulfidogenic gradient zone underneath the plume. This contributes to the hypothesis that the hot spot of degradation activity can be found where electron donors and electron acceptors meet: at the fringes of contaminant plumes. Within two-dimensional (2-D) flow-through systems (tanks), this plume fringe hypothesis could be proven in the laboratory (Bauer et al., 2007). The present thesis provides unprecedented field data to support this hypothesis. Both, laboratory experiments and field data provide essential information for the prediction of plume development and degradation processes e.g. with numerical models, and thus for risk assessment.

Via specific gene distributions at the Flingern site, the F1 cluster *bssA* genes were identified to be affiliated to novel *Geobacter* related organisms, which were dominantly present within the sulfidogenic zone and which play a presumed major role in BTEX degradation at the Flingern site. Although these *bssA* sequences show ~90% amino acid sequence similarity to known *geobacterial* sequence types, they are not closely related to more typical *bssA* genes of *Geobacter* spp. as they are detected at the Pasing site with the same assay. The revealed data also suggests that different anaerobic processes (e.g. iron- and sulfate-reduction, fermentation), may co-occur within the same redox zone. The sulfidogenic gradient zone shows also increased reduced iron concentrations in groundwater. Interestingly, ongoing enrichment efforts support the hypothesis that the organisms which carry the F1 cluster *bssA* genes may be even able to couple toluene degradation to both sulfate and iron reduction, and thus different relevant electron accepting processes (Kunapuli, unpublished). *Bacillus* and *Clostridia* spp., which were also dominantly present within the sulfidogenic gradient zone, were predicted in silico to generate the 137 and 228 bp T-RF's also dominating the sulfidogenic zone, respectively. Unfortunately, *in vivo* T-RF's could not be linked to these organisms within this study. It has to be kept in mind that further, yet-unidentified organisms besides the detected *Geobacter* relatives may also be important for biodegradation in the sulfidogenic gradient zone at the Flingern site. Further insights into the key players involved in BTEX degradation at the Flingern site should be gained e.g. with stable isotope probing, as demonstrated here for Testfeld Süd. With SIP the active degraders and also the *bssA* genes they carry can clearly be identified.

The fine-scale microbial community data combined with high-resolution biogeochemical measurements yielded valuable information about the complex transformation processes occurring on very small scales, mediated by specialised local microbial assemblages in close coupling to the plume redox scenario. For the detailed characterisation of the microbial community within the steep gradients of the Flingern plume the high-resolution sampling approach was especially important. Sampling in meter-scales would miss important plume compartments und thus provide only an insufficient assessment of ongoing natural attenuation processes.

The *bssA* assay in general was suitable to detect different site-specific clades of contaminant-degrading microorganisms within and also outside the known proteobacterial lineages. Unfortunately, also putative *bssA*-homologues were detected at Testfeld Süd. Therefore, with the assay as presented within this thesis it may not be possible to strictly detect only anaerobic toluene degraders in a generic manner. Also other genes coding for closely related fumarate-adding homologues may be covered by the assay. Further *bssA* sequence information retrieved from environmental samples as well as from cultures especially from non-proteobacterial strains will help to further optimise the assay. Nevertheless, the lineage-specific *bssA* qPCR assay developed for the Flingern site made it possible to detect the highest relative abundance of specific anaerobic toluene degraders within the sulfidogenic gradient zone of the Flingern plume. In this case, the *bssA* assay was of essential importance to obtain the finding that the spatial distribution of degraders is in strong correlation to redox conditions and contaminant degrading processes at the site.

In summary, the presented data provides "snapshots" of environmental microbiota-process couplings. In the future, the investigation of temporal dynamics of degrader communities within contaminant plumes may be of special interest, because in the environment microbes are permanently exposed to temporal fluctuations and changes in hydrogeochemical parameters. Thus, changing contaminant concentrations (pulses) or

fluctuating groundwater tables may result in dramatic changes of local redox conditions. By analysing successive “snapshots” in time, both within laboratory systems and in field experiments, the response of the microbial communities to these fluctuations may be observable, and allow for an even more refined assessment of microbial community functions in contaminated aquifers. Without doubt, the assays and approaches developed in this thesis will be helpful tools to accomplish these tasks.

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Appendix

Table A.1.: Transformation of ^{13}C-labelled toluene coupled to the production of sulfide and $^{13}CO_2$ in SIP microcosms as shown in **Fig. 3.1.** Stdev = standard deviation, n.a. = not applicable, n.d. = not determined

day	AT%	Stdev	Sulfid [mM]	Stdev	Toluene [mM]	Stdev
1	1,078	0,003	0,509	0,173	0,054	0,013
8	1,081	0,011	0,217	0,033	0,044	0,008
15	1,078	0,002	0,463	0,072	0,032	0,011
22	1,084	0,001	0,462	0,071	0,084	0,011
29	1,089	0,002	0,331	0,040	0,116	0,028
36	1,101	0,011	0,330	0,133	0,074	0,003
43	1,143	0,038	0,400	0,048	0,058	0,004
50	1,214	0,019	0,321	0,038	n.d.	n.d.
57	1,547	0,093	0,472	0,086	n.d.	n.d.
64	2,465	0,286	0,922	0,107	n.d.	n.d.
72	4,372	0,537	1,554	0,243	n.d.	n.d.
79	5,894	0,644	1,890	0,204	n.d.	n.d.
86	7,015	0,795	2,275	0,237	n.d.	n.d.
92	7,829	0,720	2,517	0,083	n.d.	n.d.
96	n.d.	n.d.	n.d.	n.d.	0,003	0,001
100	9,081	0,310	2,698	0,101	n.d.	n.d.
121	9,978	0,664	2,731	0,095	0,000	0,000
133	9,586	n.a.	2,574	n.a.	0,000	0,000

Table A.2.: Transformation of ^{13}C-labelled toluene coupled to the production of sulfide and $^{13}CO_2$ in SIP microcosms as shown in **Fig. 3.2.**

day 8, 12C BD	ratio	day 86, 12C BD	ratio
1,757	0,000	1,758	0,016
1,751	0,001	1,753	0,003
1,743	0,001	1,745	0,007
1,733	0,002	1,737	0,013
1,725	0,013	1,728	0,016
1,716	0,015	1,718	0,014
1,706	0,059	1,708	0,043
1,697	0,189	1,699	0,190
1,688	0,478	1,691	0,679
1,680	1,000	1,682	1,000
1,672	0,249	1,674	0,114
1,664	0,012	1,667	0,047
1,656	0,034	1,660	0,013

day 8, 13C BD	ratio	day 86, 13C BD	ratio
1,757	0,001	1,757	0,000
1,751	0,004	1,752	0,003
1,743	0,009	1,743	0,010
1,733	0,055	1,735	0,010
1,725	0,034	1,727	0,069
1,716	0,030	1,717	0,025
1,707	0,027	1,707	0,306
1,697	0,122	1,698	0,293
1,690	1,000	1,691	1,000
1,681	0,527	1,683	0,992
1,672	0,285	1,674	0,427
1,663	0,042	1,666	0,055
1,654	0,028	1,660	0,042

Table A.3.: Comparative abundance distribution profiles of specific T-RFs in 12C (A) and 13C (B) SIP gradients as shown in **Fig. 3.4.**

A	T-RF [bp]				
BD	146	159	160	177	490
1,67	0,0007	0,0024	0,0018	0,0005	0,0011
1,67	0,0142	0,0014	0,0036	0,0070	0,0033
1,68	0,1555	0,0277	0,0060	0,0728	0,0560
1,69	0,0421	0,0232	0,0232	0,0157	0,0279
1,70	0,0100	0,0056	0,0115	0,0057	0,0089
1,71	0,0044	0,0018	0,0024	0,0014	0,0011
1,72	0,0012	0,0004	0,0006	0,0005	0,0005
1,73	0,0002	0,0005	0,0007	0,0002	0,0008
1,74	0,0002	0,0003	0,0008	0,0002	0,0006
1,75	0,0007	0,0002	0,0002	0,0004	0,0002

B	T-RF [bp]				
BD	146	159	160	177	490
1,67	0,0016	0,0014	0,0019	0,0014	0,0025
1,67	0,0013	0,0000	0,0176	0,0171	0,0189
1,68	0,0109	0,0352	0,0139	0,0000	0,0525
1,69	0,0331	0,0428	0,0360	0,0253	0,0334
1,70	0,0692	0,0099	0,0124	0,0550	0,0069
1,71	0,0626	0,0245	0,0092	0,0616	0,0065
1,72	0,0031	0,0017	0,0010	0,0023	0,0003
1,73	0,0053	0,0023	0,0032	0,0051	0,0014
1,74	0,0008	0,0003	0,0005	0,0008	0,0002
1,74	0,0011	0,0003	0,0006	0,0009	0,0003

Table A.4.: Depth profiles of representative contaminants and reduced electron acceptors in groundwater (aq.) of the Flingern aquifer as shown in **Fig. 4.1.** All measurements were done by Bettina Anneser (GSF-IGOE, Neuherberg). Stdev = standard deviation, n.a. = not applicable, n.d. = not determined

Depth [m] (aq.)	Toluene [mg/l]	Stdev	Fe(II) [mg/l]	Stdev	Sulfide [mg/l]	Stdev
6,41	26,606	n.a.	22,223	1,458	n.d.	n.a.
6,51	41,310	n.a.	14,455	1,729	-0,032	0,046
6,61	35,349	n.a.	10,620	8,509	0,714	0,092
6,70	57,106	n.a.	6,808	1,288	0,390	0,092
6,75	19,959	0,940	12,729	1,187	3,150	0,321
6,81	8,161	0,581	15,295	6,306	7,209	0,000
6,91	0,314	0,005	13,377	2,441	6,592	0,873
7,01	0,046	0,005	9,997	0,780	2,565	0,230
7,11	0,038	0,001	10,692	1,288	3,897	0,092
7,31	0,016	0,001	9,301	1,017	5,066	1,378
7,41	0,019	0,001	4,555	6,441	2,241	0,689
7,51	0,019	0,002	4,219	1,899	0,455	0,000
7,61	0,016	0,000	4,003	0,102	0,552	0,138
7,75	0,015	0,000	5,466	3,797	0,357	0,230
8,05	0,007	0,001	14,767	4,814	1,429	0,367
8,35	0,000	0,000	3,764	0,848	0,422	0,046
8,65	0,000	0,000	6,592	2,950	1,591	0,689
8,75	0,000	0,000	5,969	3,085	0,649	n.a.
9,05	0,000	0,000	3,284	0,034	0,422	0,505
9,35	0,000	0,000	3,812	0,780	1,039	0,184
9,65	0,000	0,000	8,510	6,543	0,390	0,092
9,87	0,000	0,000	5,562	0,203	1,721	0,597
10,20	0,000	0,000	6,137	0,136	0,714	0,827
10,86	0,000	0,000	4,842	0,271	-0,260	0,000
11,19	0,000	0,000	7,144	1,017	-0,195	0,092
11,52	0,000	0,000	8,702	3,153	-0,195	0,092
11,85	0,000	0,000	7,120	0,576	-0,260	0,000
12,18	0,000	0,000	6,880	0,373	-0,260	0,000
12,51	n.d.	n.a.	11,027	0,136	-0,227	0,046

Table A.5.: Depth profiles of representative contaminants and reduced electron acceptors in sediment samples (sed.) of the Flingern aquifer as shown in **Fig. 4.1.** Fe(II) measurements were done by Bettina Anneser (GSF-IGOE, Neuherberg). n.d. = not determined

Depth [m] (sed.)	Fe(II) [mg/kg] (ww)	PAH [mg/kg] (ww)
0,15	0,06	n.d.
0,90	1,45	n.d.
1,70	1,07	n.d.
2,20	0,50	n.d.
2,50	1,13	n.d.
3,20	1,23	n.d.
3,90	1,35	n.d.
4,65	2,17	n.d.
5,70	1,07	0,000
6,30	3,24	0,000
6,65	8,06	24,023
6,80	4,13	5,964
7,20	6,43	6,984
7,35	14,55	13,910
7,60	23,59	8,961
8,10	27,72	15,378
8,40	22,74	19,324
8,70	25,11	16,217
9,10	19,78	28,072
9,50	18,30	42,127
9,80	16,07	6,546
10,20	11,72	7,506
10,60	8,60	0,750
11,20	15,15	0,000
11,70	13,99	0,000
12,30	9,14	9,968
12,70	9,48	0,000

Table A.6.: Depth distribution of bacterial 16S rRNA genes and specific *bssA* genes of the F1-cluster of unknown anaerobic toluene degraders measured via qPCR and as shown in **Fig. 4.2.** Stdev = standard deviation, n.a. = not applicable, n.d. = not determined

Depth [m]	16S x 5,5 [cp/g]	Stdev x 5,5	bssA [cp/g]	Stdev	16S/bssA ratio
5,70	6,63E+06	4,27E+06	n.d.	n.a.	n.a.
6,30	1,12E+08	9,74E+07	5,00E+06	5,20E+06	0,04
6,65	2,36E+08	1,40E+08	9,86E+06	7,00E+06	0,04
6,80	2,08E+07	1,10E+07	6,40E+06	2,30E+06	0,31
7,20	3,03E+07	1,61E+07	1,48E+07	5,05E+06	0,49
7,35	4,26E+07	3,47E+07	2,31E+07	1,95E+07	0,54
7,60	1,06E+07	1,17E+07	1,55E+05	1,44E+05	0,01
8,10	9,73E+05	3,99E+05	1,08E+05	4,25E+04	0,11
8,40	5,75E+05	1,73E+05	4,50E+04	3,94E+04	0,08
8,70	9,80E+05	1,12E+06	2,67E+04	1,83E+04	0,03
9,10	8,90E+05	9,50E+05	2,66E+05	2,73E+05	0,30
9,50	9,40E+05	1,01E+06	2,44E+05	2,70E+05	0,26
9,80	1,03E+06	9,59E+05	6,05E+04	4,75E+04	0,06
10,20	3,48E+05	1,29E+05	7,81E+04	2,95E+04	0,22
10,60	1,26E+06	4,74E+05	1,46E+05	8,40E+04	0,12
11,20	2,12E+05	3,35E+04	n.d.	n.a.	n.a.
11,70	1,79E+05	8,17E+04	n.d.	n.a.	n.a.
12,30	2,83E+05	6,12E+04	n.d.	n.a.	n.a.
12,70	9,07E+04	1,78E+04	n.d.	n.a.	n.a.

Table A.7.: Depth-resolved 16S rRNA gene T-RFLP fingerprinting of the Flingern plume: Shannon-Wiener index *H'* calculated for the entire data set as shown in **Fig. 4.4.A**.

Depth [m]	Shannon H'
5,70	3,324
6,30	2,758
6,65	2,206
6,80	2,956
7,20	2,196
7,35	2,441
7,60	2,902
8,10	3,079
8,40	3,149
8,70	3,048
9,10	2,976
9,50	3,329
9,80	3,292
10,2	2,977
10,6	2,513
11,2	2,915
11,7	3,814
12,3	1,789
12,7	1,723

Table A.8.: Principal component ordination of the overall variance in depth-resolved bacterial community composition retrieved from T-RFLP fingerprinting as shown in **Fig. 4.5.A**

Depth [m]	F1	F2
5,70	0,346	0,569
6,30	-0,175	0,829
6,65	1,414	-3,491
6,80	-1,166	-0,180
7,20	-2,507	-0,707
7,35	-2,393	-0,148
7,60	0,134	-0,099
8,10	0,356	-0,193
8,40	0,450	-0,141
8,70	0,301	0,010
9,10	-0,087	-0,546
9,50	0,140	0,063
9,80	0,436	0,218
10,20	-0,151	-0,289
10,60	0,672	0,612
11,20	0,659	0,750
11,70	0,443	0,612
12,30	0,767	1,355
12,70	0,362	0,773

Table A.9.: Loading plot of inferred principal component factors on specific T-RFs as shown in **Fig. 4.5.B**

T-RF	F1	F2
56	0,000	0,001
62	0,004	0,001
66	0,001	0,001
69	0,000	0,000
73	0,004	0,007
77	-0,006	-0,001
81	0,000	0,002
87	0,000	-0,002
91	0,001	0,000
109	0,000	0,000
114	0,000	0,000
117	-0,001	0,001
120	-0,005	0,000
122	0,001	0,006
124	0,002	0,002
128	0,001	0,001
130	-0,066	-0,016
133	0,003	0,002
135	0,000	0,002
137	-0,034	-0,010
139	-0,001	0,000
140	0,002	0,003
142	0,002	0,003
145	0,000	0,000
146	0,000	0,001
147	0,001	-0,003
148	-0,002	0,000
149	-0,004	0,000
151	0,000	0,000
154	0,002	0,004
159	-0,006	-0,042
161	-0,004	0,007
162	0,008	0,010

Table A.9. (continued): Loading plot of inferred principal component factors on specific T-RFs as shown in **Fig. 4.5.B**

T-RF	F1	F2
163	0,003	0,002
169	-0,002	0,000
170	0,005	0,004
171	0,003	0,003
177	-0,010	0,001
178	0,001	0,001
180	0,001	0,002
183	0,001	0,001
187	-0,001	0,000
193	0,009	-0,024
201	-0,001	0,000
205	0,001	0,000
206	0,002	0,000
212	0,002	0,000
225	-0,008	0,008
228	-0,043	-0,009
259	-0,003	-0,001
271	0,000	0,000
277	0,001	0,000
280	-0,002	0,000
286	0,005	0,000
287	0,001	-0,001
293	0,001	0,001
295	0,001	0,002
305	0,001	-0,001
317	0,000	0,000
325	-0,001	0,000
372	0,005	-0,002
373	0,003	0,002
398	0,002	-0,006
404	0,000	0,000
421	0,001	0,001
431	-0,001	-0,009

Table A.9. (continued): Loading plot of inferred principal component factors on specific T-RFs as shown in **Fig. 4.5.B**

T-RF	F1	F2
440	0,000	0,001
453	0,004	0,004
454	0,012	0,022
468	0,001	-0,002
477	0,000	0,003
486	0,000	-0,005
489	0,002	-0,006
491	0,001	0,001
493	-0,012	0,009
499	0,001	-0,003
502	0,022	-0,060
505	0,002	0,000
506	0,000	0,000
509	-0,001	-0,009
513	0,000	-0,001
514	0,000	0,000
518	0,007	0,004
522	0,002	0,002
526	0,013	0,009
528	0,002	0,002
533	0,001	0,000
535	0,000	0,000
559	0,000	0,001
578	0,001	0,001
609	0,000	0,000
646	0,002	0,003
670	0,000	0,001
895	0,002	0,002
928	0,001	0,001
937	0,000	0,000
943	0,000	0,000

Table A.10.: Rarefaction analysis of the bacterial diversity retrieved in the four depth-resolved 16S rRNA gene clone libraries as shown in **Fig. 4.6.**

	A	Depth [m]				**B**	Depth [m]			
n	all	6,3	6,8	7,6	11,7	all	6,3	6,8	7,6	11,7
2	2	2	1,9	2	2	1,9	1,8	1,8	1,9	1,9
4	3,9	3,9	3,7	4	3,8	3,5	3,1	3	3,4	3,5
6	5,9	5,7	5,2	5,9	5,5	4,9	4	3,9	4,6	4,9
8	7,7	7,5	6,6	7,8	7,1	6,1	4,7	4,5	5,6	6,1
10	9,6	9,2	7,9	9,7	8,5	7,2	5,2	5	6,5	7
12	11,4	10,9	9,1	11,5	9,9	8,1	5,7	5,4	7,2	7,9
14	13,2	12,5	10,2	13,3	11,2	8,9	6,1	5,7	7,9	8,6
16	15	14,1	11,3	15,1	12,5	9,6	6,5	6	8,5	9,2
18	16,7	15,6	12,3	16,9	13,7	10,2	6,8	6,3	9	9,7
20	18,4	17,1	13,2	18,6	14,8	10,8	7,1	6,5	9,5	10,2
22	20,1	18,5	14,2	20,4	15,9	11,3	7,4	6,8	10	10,6
24	21,7	19,9	15,1	22,1	16,9	11,8	7,7	7	10,4	11
26	23,3	21,3	15,9	23,7	17,9	12,3	7,9	7,2	10,8	11,4
28	24,9	22,6	16,7	25,4	18,9	12,7	8,2	7,4	11,2	11,7
30	26,5	23,9	17,5	27,1	19,9	13,1	8,4	7,6	11,6	12
32	28	25,2	18,3	28,7	20,8	13,5	8,7	7,8	11,9	12,3
34	29,6	26,4		30,3	21,7	13,8	8,9		12,3	12,6
36	31,1			31,9	22,6	14,1			12,6	12,9
38	32,6			33,5		14,5			12,9	
40	34			35,1		14,8			13,2	
42	35,5			36,7		15			13,5	
44	36,9			38,2		15,3			13,8	
46	38,3					15,6				
48	39,7					15,8				
50	41,1					16,1				
52	42,5					16,3				
54	43,8					16,5				
56	45,2					16,7				
58	46,5					17				
60	47,8					17,2				
62	49,1					17,4				
64	50,4					17,5				
66	51,7					17,7				
68	52,9					17,9				
70	54,2					18,1				
72	55,4					18,3				
74	56,6					18,4				

Table A.10. (continued): Rarefaction analysis of the bacterial diversity retrieved in the four depth-resolved 16S rRNA gene clone libraries as shown in **Fig. 4.6.**

	A	Depth [m]				**B**	Depth [m]			
n	all	6,3	6,8	7,6	11,7	all	6,3	6,8	7,6	11,7
76	57,8					18,6				
78	59					18,7				
80	60,2					18,9				
82	61,4					19,1				
84	62,6					19,2				
86	63,8					19,3				
88	64,9					19,5				
90	66,1					19,6				
92	67,2					19,8				
94	68,3					19,9				
96	69,5					20				
98	70,6					20,2				
100	71,7					20,3				
102	72,8					20,4				
104	73,9					20,5				
106	75					20,7				
108	76,1					20,8				
110	77,2					20,9				
112	78,2					21				
114	79,3					21,1				
116	80,3					21,2				
118	81,4					21,3				
120	82,4					21,5				
122	83,5					21,6				
124	84,5					21,7				
126	85,6					21,8				
128	86,6					21,9				
130	87,6					22				
132	88,6					22,1				
134	89,6					22,2				
136	90,6					22,3				
138	91,6					22,4				
140	92,6					22,5				
142	93,6					22,6				
144	94,6					22,7				
146	95,6					22,8				
148	96,5					22,9				
150	97,5					23				

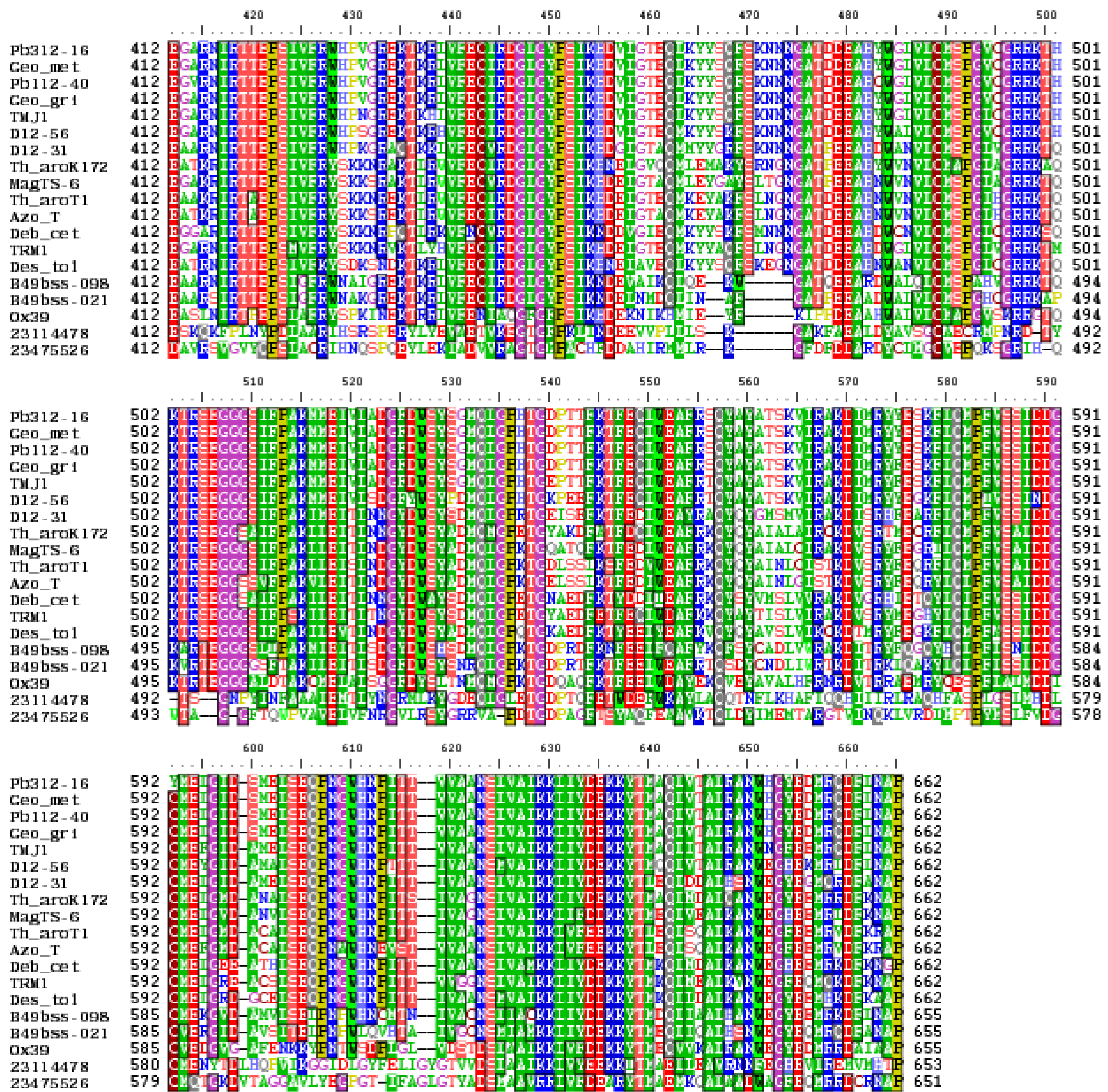

Figure A.1.: Alignment of amino acid sequences of representative BssA and homologous enzymes as described in Fig. 2. The threshold for conservation was 70% for identities and similarities. Columns with conserved identical amino acids are shaded in colour and outlined in black. Columns with similar amino acids were identified according to Blosum62 matrix (Henikoff, S., and Henikoff, J.G. (1992) *PNAS* 89: 10915-10919) and are marked in colour. The numbering is according to *T. aromatica* K172 BssA (Leuthner, B., *et al.* (1998) *Mol Microbiol* 28: 615-628). Closely related non-BssA PFL paralogues are from *Desulfitobacterium hafniense* (NCBI protein identifier 23114478) and *Desulfovibrio desulfuricans* (NCBI protein identifier 23475526). The alignment was generated using BioEdit version 5.0.9 (Hall, T.A. (1999) *Nucl Acids Symp Ser* 41: 95-98).

Curriculum vitae

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Education	
1982 – 1986	Grundschule Grainau
College education	
1986 – 1996	Werdenfelsgymnasium Garmisch-Partenkirchen Abitur Jun. 1996
Studies	
Nov. 1997 – Dec. 2003	Biology Technical University Munich Major subject: Microbiology subsidiary subjects: Genetics, Pharmacology and Toxicology Diploma in Biology Dec. 2003
Diploma thesis	„Genetische Untersuchung von Typ I-Pili innerhalb des *Burkholderia cepacia* Komplexes“ The thesis was done at the Department of Microbiology (head: Prof. Karl-Heinz Schleifer) of the Technical University Munich, supervisor: Dr. Leo Eberl
Ph.D.	
May 2004 – Aug. 2007	The work was done at the Institute of Groundwater Ecology (Director: Prof. Rainer U. Meckenstock) of the GSF – National Research Center for Environment and Health (Helmholtz Association), Neuherberg, supervisor: Dr. Tillmann Lueders

Publications in peer reviewed journals:

Winderl, C., S. Schaefer, and T. Lueders. (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.

Winderl, C., B. Anneser, C. Griebler, R. U. Meckenstock, and T. Lueders. Depth-resolved microbial community structure and quantitative localization of anaerobic toluene degraders in distinct redox zones of a tar-oil contaminant plume. *Appl. Environ. Microbiol.* (submitted)

Winderl, C., H. Penning, S. Schaefer, and T. Lueders. Detection of sulfate-reducing microbes responsible for toluene degradation at contaminated sites using stable isotope probing. *FEMS Microbiol. Ecol.* (in preparation)

Selected contributions to national and international scientific meetings (only first authorship):

Winderl, C., S. Schaefer, C. Griebler, and T. Lueders. Tracing toluene-degrading bacterial communities in a tar-oil contaminated aquifer. *UNESCO Workshop on transport and fate of diffuse contaminants in catchments with special emphasis on stable isotope applications*. November 2004, Neuherberg. (Poster presentation)

Winderl, C., and T. Lueders. Benzylsuccinate synthase as a specific functional marker gene for anaerobic toluene degraders in contaminated aquifers. *VAAM annual meeting*. September 2005, Göttingen. (Poster presentation)

Winderl, C., S. Schaefer, and T. Lueders. Benzylsuccinate synthase genes as a tool to target anaerobic toluene-degrading microbial communities at BTEX contaminated aquifer sites. *Munich Environmental Microbiology Meeting 2005*,
November 2005, Neuherberg. (contributed oral presentation)

Winderl, C., S. Schaefer, and T. Lueders. Fine-scale depth resolved analysis of the fringe of a petroleum hydrocarbon plume – II: Microbial community shifts and specific localization of anaerobic toluene degraders. *VAAM annual meeting*,
March 2006, Jena. (invited oral presentation)

Winderl, C., B. Anneser, C. Griebler, and T. Lueders. Depth resolved quantification of anaerobic toluene degraders across a contaminant plume using benzylsuccinate synthase and ribosomal marker genes. *Eleventh International Symposium on Microbial Ecology*,
August 2006, Vienna. (Poster presentation)

Winderl, C., B. Anneser, U. Kunapuli, R. U. Meckenstock, C. Griebler, and T. Lueders. Characterization of anaerobic hydrocarbon degrading microbial communities in a tar-oil contaminated aquifer with tools of molecular microbiology. *VAAM annual meeting*,
April 2007, Osnabrück. (contributed oral presentation)