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Combined monitoring of aerobic and anaerobic contaminant degradation genes and microbial populations as a tool to assess natural attenuation

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Abstract

Groundwater is one of the most important (drinking) water resources and therefore must be protected from anthropogenic pollution. Microorganisms are the most important driving forces for the degradation of BTEX and other hydrocarbon contaminants in aquifers under different redox settings. It was previously shown that the distribution of anaerobic toluene degraders as traced via functional marker is correlated to 'hot spots' of increased anaerobic degradation at the lower fringe of an actual contaminant plume and that natural attenuation (NA) can be affected by external hydraulic 'disturbance' of the system. In this thesis, evidence for the competition between and the sinchronicity of distinct aerobic and anaerobic toluene degrader populations and respective processes for net contaminant removal at plume redox gradients is provided. Moreover, the immediate response of a pristine aquifer system towards contamination, which is defining the development and succession of degraders within the evolving contaminant plume and its dynamic redox gradients is elucidated.

First, at a former gasworks site in Düsseldorf, Germany, qualitative and quantitative molecular monitoring strategies based on ribosomal (16S rRNA) and functional marker genes were applied over the upper fringe of a contaminant plume. Well-defined small scale distribution patterns, in intervals of 3-10 cm, of typical aerobic degrader lineages within the *Pseudomonadaceae, Comamonadaceae*, and *Burkholderiaceae* (and also the anaerobes) were revealed, but were only partly correlated to redox strata. An unexpected stratification of toluene monooxygenase (*tmoA*) genes was observed, having maximal abundances of 9.2×10^6 tmoA genes per g of sediment in the strongly reduced plume core, and not increasing towards the more oxidised upper plume fringe. This provides primary field evidence that the competition between aerobic and anaerobic degraders and degradation processes may be controlled by more than just electron acceptor availability, and that some typically aerobic degraders may be physiologically more flexible than currently perceived.

As a second milestone, for the first time, the initial impact of toluene contamination on natural microbial communities of a pristine aquifer system, and the succession of distinct degraders and processes during the onset of natural attenuation were traced in an indoor aquifer mesocosm system. A rapid development of abundant aerobic toluene degraders as shown by *tmoA* genes (up to 2.1×10^6 genes mL^{-1} groundwater and 1.5×10^6 genes g^{-1} sediment) was found already 16 days after initially moderate contamination (max. 20 mg L^{-1}) over the whole plume. Highly significant differences in the microbial communities between the sediment and the groundwater ($p = 0.0001$) were observed. A selection of *Pseudomonas* spp. populations as the primary aerobic degraders, with successional dominances also of *Zoogloea* spp., *Dechloromonas* spp., unclassified *Comamonadaceae*, were revealed as immediate response towards toluene contamination. Under secondary high contamination (max. 100 mg L^{-1}), profound and immediate changes of overall microbiota and specific degrader communities were observed, with denitrifying

hydrocarbon degraders related to *Azoarcus* spp. appearing in the highly-contaminated plume core sediment, coupled to spatial shifts of the *Pseudomonadaceae* towards the plume fringes. These findings provided unique insights into the initial reaction of pristine aquifer microbiota to contamination, something never investigated in the field to date. It is show how ongoing reactive processes and involved degrader populations evolve simultaneously, adapting spatially to local contaminant and redox scenarios but staying highly variable in groundwater.

These findings provide relevant novel insights into the ecology of contaminant plumes, as well the spatial and temporal succession of degrader populations and respective processes. These are prerequisites for developing more integrated monitoring strategies for NA. The generation of congruent lines of evidence between different monitoring methods, including molecular approaches, certainly contribute to an enhanced design and implementation of site-specific remediation strategies.

Zusammenfassung

Grundwasser ist eine der wichtigsten (Trink)Wasser Ressourcen und muss deshalb vor anthropogenen Verschmutzungen geschützt werden. Mikroorganismen sind hierbei die treibende Kraft für den Abbau von BTEX und allen anderen verunreinigenden Kohlenwasserstoffen in Aquiferen, unter unterschiedlichen Redoxbedingungen. Durch die Verfolgung von funktionellen Markern wurde unverzüglich gezeigt, dass die Verteilung von anaeroben Toluolabbauern mit erhöhter anoxischer Aktivität am unteren Fahnenrand stattfindet und dass die natürliche Selbstreinigung (NA) durch hydraulische „Störungen" bestimmt wird. In vorliegender Arbeit konnte ich die Konkurrenz und Simultanität von aeroben und anaeroben Prozessen und entsprechenden mikrobiellen Populationen zum gesamten Schadstoffabbau sowie auch die bisher unerkannten ersten Reaktionen auf eine Kontamination untersuchen, welche richtungsweisend sind für den weiteren Verlauf/Sukzession unter sich ändernden Schadstoff –und Redoxbedingungen.

Als erstes wurden durch einen BTEX-Gradienten an einem früheren Gaswerk in Düsseldorf, Deutschland, genetische Monitoring-Strategien basierend auf ribosomalen (16S rRNA) -und funktionellen Genen qualitativ und quantitativ eingesetzt. Kleinräumige, hochdefinierte Verteilungsmuster (in 3-10 cm Intervallen) von typischen aeroben Abbauern, v.a. *Pseudomonadaceae, Comamonadaceae* und *Burkholderiaceae* wurden festgestellt (selbes auch für die anaeroben); diese korrelierten aber nur teilweise mit den entsprechenden Redoxzonen. In der gleichen Art wurde eine unvorhergesehene Stratifikation von Toluol-Monooxygenase (*tmoA*) Genen beobachtet, mit höchsten Abundanzen von 9.2 x 10^6 *tmoA* Gene g^{-1} Sediment in Zonen die als absolut reduziert gelten. Auch im oxidierteren oberen Fahnenrand kam es nicht zu einem deutlichen Anstieg dieser Gene. Dies ergibt eine erste Evidenz dass die Konkurrenz zwischen aeroben und anaeroben Abbauern und Prozessen von weitaus mehr als nur den vorhandenen Elektronenakzeptoren kontrolliert wird und dass einige typische aerobe Abbauer physiologisch viel flexibler sind als bisher angenommen.

Als ein zweiter Meilenstein wurde, zum ersten Mal, in einem experimentalen Modellaquifer die Reaktion natürlicher mikrobieller Gemeinschaften in reinem Grundwasser auf Toluol verfolgt, sowie die Sukzession von Abbauern und Prozessen mit fortschreitender NA. Sofort 16 Tage nach moderater Toluol-Kontamination (max. 20 mg L^{-1}) zeigte sich die ganze Fahne als aktive aerobe Zone mit hohen Mengen an *tmoA* Genen (bis zu 2,1 x 10^6 Gene mL^{-1} Grundwasser und 1.5 x 10^6 Gene g^{-1} Sediment). Signifikante Unterschiede zwischen den mikrobiellen Gemeinschaften in Sediment und Grundwasser ($p = 0{,}0001$) wurden festgestellt und eine Selektion von *Pseudomonas* spp.-Populationen als primäre aerobe Abbauer, mit zusätzlichen Dominanzen an *Zoogloea spp., Dechloromonas spp.,* unklassifizierten *Comamonadaceae* wurden als sofortige Reaktion gegen Toluol ausgemacht. Bei sekundärer, hoher Kontamination (max. 100 mg L^{-1}) erfolgte ein kompletter Umschwung in der Zusammensetzung der gesamten- und Abbauer-Bakteriengemeinschaft, bemerkbar durch das Hervorkommen von denitrifizierenden

Kohlenwasserstoff-Abbauern mit v.a. *Azoarcus* in sehr stark kontaminierten Sedimentschichten. Daran gekoppelt schien ein Verschieben von *Pseudomonadaceae* hin zu den Fahnenrändern. Diese Ergebnisse erlauben einen einmaligen Einblick in der Reaktion von reiner Grundwasser-Mikrobiota auf Kontaminationen und zeigen wie reaktive Prozesse und beteiligte Abbauer-Populationen simultan ablaufen und sich räumlich an lokale Kontaminations- und Redox-Szenarien anpassen können, aber im freien Grundwasser trotzdem hoch variabel bleibt.

Die hier erzielten Befunde können wertvoll sein um die Ökologie von Schadstofffahnen sowie die beinhaltende Sukzession von Prozessen besser zu verstehen. Dies sind wichtige Voraussetzungen für die bessere Entwicklung und Anwendung von integrierten, standort-spezifischen Monitoring-Strategien für NA-Konzepte.

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1. Introduction

1.1. Relevance of microbial hydrocarbon degradation in groundwater

Groundwater is one of the most important sources for drinking water on Earth and represents approximately 30 % of the global freshwater reservoir (Foster and Chilton, 2003). In Germany, the considerable water use of at least 34 % of the total renewable water resources per year (~171,000 Mio. m^3) has already been reached on the turn of the 2nd millennium (Prokop et al., 2000). Here, amongst the sources of public freshwater, groundwater provides more than 72 % (EEA, 1999). Therefore, groundwater has to be protected from anthropogenic perturbation, or relieved from past defilements. Aquifers should be 'holistically' regarded as systems in a labile equilibrium, very susceptible to impacts from outside. Hence, aquifers need to be comprehensively understood in their hydrological, geochemical, and biological processes as well as nutrient cycles. In such a new perspective, groundwater has also to be considered as a living ecosystem (Griebler et al., 2010). In subsurface ecosystems prokaryotes are the most abundant and widespread organisms, in particular *Bacteria*.

Hydrocarbons are notorious groundwater contaminants. After chlorinated hydrocarbons, the second major class of groundwater contaminants is found within the aromatic hydrocarbons (Eljarrat and Barcelo, 2003). Monoaromatic hydrocarbons, especially benzene, toluene, ethylbenzene, and xylenes (BTEX) are widespread, toxic, groundwater pollutants originating from petroleum derivatives. Due to their large-scale production and use, the release of BTEX into the environment, even accidentally, is inevitable. To date, there are potentially more than 200,000 sites contaminated by hydrocarbons like monoaromatic BTEX and polycyclic aromatic hydrocarbons (PAH) in Germany (abandoned waste sites, abandoned industrial sites, and military sites) with a risk of affecting the groundwater in surrounding areas (Prokop et al., 2000). BTEX are relatively water soluble and light organic substances which form contamination plumes in upper groundwater zones and thus can migrate over long distances in aquifers. Microorganisms naturally occurring in groundwater are important agents in the metabolisation of such toxic pollutants, and are capable to degrade BTEX and to utilize them as carbon and energy source under both aerobic and anaerobic conditions (Andreoni

and Gianfreda, 2007; Nicolaou et al., 2010). In fact, biodegradation results in the only sustainable mass reduction of contaminants *in situ* (Roling and van Verseveld, 2002).

Natural attenuation (NA) is of increasing interest for the remediation of hydrocarbon-contaminated aquifers due to the reduced costs, land disruption, and human exposure compared to *ex situ* bioremediation (Stapleton et al., 2000). However, NA should be considered as a discrete strategy and 'applied' under targeted and well documented circumstances (Alvarez and Illman, 2006). Therefore, an accurate and comprehensive monitoring system for NA is important, especially at heterogeneous contaminated sites. Considering the important role of microbial processes in the protection and remediation of groundwater resources, a better integration of microbial processes in current management schemes is needed. Detailed knowledge on the bacteria that are most effective metabolizers of a certain pollutant at a given site, as well as their population densities and controls of activities will contribute to optimize bioremediation strategies. Monitoring of bioremediation usually includes measurements of distinctive metabolites, residual contaminants, contaminant stable isotopes, or the capacity of the indigenous microbial community for biodegradation (Andreoni and Gianfreda, 2007). In the latter approach, focusing on catabolic genes that encode specific pollutant degrading enzymes avoids the classical cultivation of bacteria. Indeed, prokaryotic microorganisms from aquatic environments demonstrate a rather poor average cultivability of <1 % (Hugenholtz, 2002; Goldscheider et al., 2006).

Currently, compound-specific stable isotope analysis (CSIA) of contaminants (monitoring of natural isotope ratios in contaminants) and monitoring of oxidized or reduced electron acceptors inside and outside of contaminant plumes are established methods to monitor and quantify contaminant biodegradation in groundwater systems. CSIA can even be applied to assess biodegradation rates in the field (Morasch et al., 2001; Richnow et al., 2003). Stable isotopes are present in given ratios in nature, i.e. the abundance of ^{13}C in the biosphere is ~1%. When biodegradation of hydrocarbons occurs, contaminants within lighter isotopes are preferentially metabolized due to the lower dissociation energies, causing an accumulation of the heavier isotope in the residual substrate. This shift is called isotope fractionation (Morasch et al., 2001; Richnow et al., 2003; Steinbach et al., 2004). In contrast, abiotic processes usually do not change the stable isotope ratio within routine analytical detection limits (Steinbach et al., 2004). The fractionation (shift) of isotope ratios, usually C^{13}:C^{12}-ratios can be measured by very sensitive Gas Chromatography-Isotope Ratio Mass Spectrometry (GC-IRMS). This thus

allows for the detection and quantification of microbiological contaminant degradation on site (Morasch et al., 2001; Richnow et al., 2003).

However, especially in heterogeneous (hydrologically and geochemically) settings, monitoring results are often not easy to interpret due to different and/or changing fractionation factors. Not all aerobic and anaerobic biodegradation pathways result in comparable isotope enrichment (Vogt et al., 2008). The first step in anaerobic toluene degradation (fumarate addition) is the major mechanism causing isotope fractionation. It includes a C-H-bond - relocation where a heavy isotope can be discriminated (Morasch et al., 2001; Steinbach et al., 2004). These effects are by far not as pronounced in certain aerobic toluene degradation pathways. Hence, at heterogeneous sites with altering oxic/anoxic redox, despite biodegradation can be substantial, only weal isotopic fractionation may be measurable. In such cases, the correlation between microbial degradation and isotopic fractionation is not robust.

At the same time, it has been shown that local microbial community patterns and especially the distribution of specific degraders can provide valuable insights on the importance of ongoing degradation processes. Microorganisms have developed distinct contaminant degradation pathways adapted to aerobic and anaerobic conditions (Gulensoy and Alvarez, 1999; Junca and Pieper, 2004). Some bacteria even have adopted different degradation pathways which can be expressed in dependence of oxygen availability or absence (Lang, 1996; Cavalca et al., 2004). The genetic information of some degradation enzymes, especially those located on plasmids, is subjected to high mutation rates and can eventually be transferred to other phylotypes (Junca and Pieper, 2004). Thus, a targeted detection of such catabolic markers, either in their genetic (DNA) or expressed (mRNA) form offers a unique strategy to monitor specific microbial contaminant degradation potentials (or activities) at a given site. In fact, genetic monitoring strategies based on the detection of catabolic genes may be very attractive especially in monitoring of synchronous aerobic and anaerobic degradation processes at heterogeneous sites, which may be hard to unravel by some of the other established monitoring strategies.

1.2. Biogeochemistry of hydrocarbon contaminated aquifers

For the complete mineralization of aromatic contaminants, microorganisms need a sufficient supply of electron acceptors. Due to the relatively low rates of oxygen

replenishment to groundwater ecosystems, oxygen as electron acceptor is rapidly depleted by respiration under contamination (Anderson and Lovley, 1997). Microbes capable of coupling the degradation of the contaminant to anaerobic terminal electron acceptors thus are of particular relevance especially under high hydrocarbon loads (Coates and Anderson, 2000; Lovley, 2000). For the different anaerobic electron-accepting processes, thermodynamics predicts a decreasing possible energy gain for the involved microorganism compared to oxygen (Bradley and Chapelle, 1998; Christensen et al., 2000). The highest energy gain is resulting from aerobic respiration, followed by denitrification, manganese- and iron reduction (common elements in many aquifers), sulphate reduction, and finally fermentation coupled to methanogenesis with the lowest energy yield (Table 1.1). The most favourable electron acceptors will be utilised and therefore depleted first in space and time. The different reaction stoichiometries and Gibb's free energy gains ($\Delta G^{0'}$) are compared in Table 1.1.

Table 1.1. Gibb's free energy yield ($\Delta G^{0'}$) resulting from oxidation of toluene by the most important electron acceptors present in groundwater aquifers and by methanogenesis, under standard conditions (25°C, 1 M, 101,325 Pa, pH 7). Modified after (Stumm and Morgan, 1981).

Process	Reaction	$\Delta G^{0'}$ [kJ electron equivalent^{-1}]
Aerobic respiration	$C_7H_8 + 9\ O_2 \rightarrow 7\ CO_2 + 4\ H_2O$	-106.3
Denitrification	$5\ C_7H_8 + 36\ NO_3^- + 36\ H^+ \rightarrow 18\ N_2 + 35\ CO_2 + 38\ H_2O$	-99.8
Manganese reduction	$C_7H_8 + 18\ MnO_2 + 36\ H^+ \rightarrow 18\ Mn^{2+} + 7\ CO_2 + 22\ H_2O$	-93.3
Iron reduction	$C_7H_8 + 36\ Fe(OH)_3 + 72\ H^+ \rightarrow 36\ Fe^{2+} + 7\ CO_2 + 32\ H_2O$	-40.1
Sulphate reduction	$8\ C_7H_8 + 36\ SO_4^{2-} + 72\ H^+ \rightarrow 36\ H_2S + 56\ CO_2 + 32\ H_2O$	-6.8
Methane fermentation/CO_2 reduction	$8\ C_7H_8 + 40\ H_2O \rightarrow 36\ CH_4 + 20\ CO_2$	-2.1

The limited supply of favourable electron acceptors results in a typical redox zonation of contaminant plumes. In the classic perspective, redox zones are seen as a longitudinal succession of more or less equal redox compartments. The plume zone most

distant from the contaminant source is thought to sustain aerobic biodegradation, preceded by a nitrate –and manganese reducing zone, an iron reduction zone, a sulphate reducing zone, and initially a methanogenic zone directly surrounding the contaminant source (Fig. 1.1.A). In a more recent perspectives, the plume core is represented by zone depleted in most electron acceptors, considered as nearly inactive, with very slow methanogenic, sulphate- and iron-reducing biodegradation occurring. Instead, only in defined geochemical gradient zones surrounding the plume, where electron donors and acceptors meet by mixing,, sustainable degradation activities are possible. These can be relatively small areas with a succession of sulphate-reducing, denitrifying and aerobic degradation (Fig. 1.1.B). In fact, the availability of electron acceptors and their supply by mixing is now recognised as one of the key controls of pollutant degradation (Leahy and Olsen, 1997; Bauer et al., 2008; Winderl et al., 2008; Anneser et al., 2010). Consistently, the upper and lower plume fringes are now known as important hot spots of microbial activity and biodegradation (Winderl et al., 2008). However, specific stratification of distinct aerobic and anaerobic degrader populations at such plume fringes, their competition and interaction, as well as their respective contribution to overall contaminant breakdown are still poorly understood.

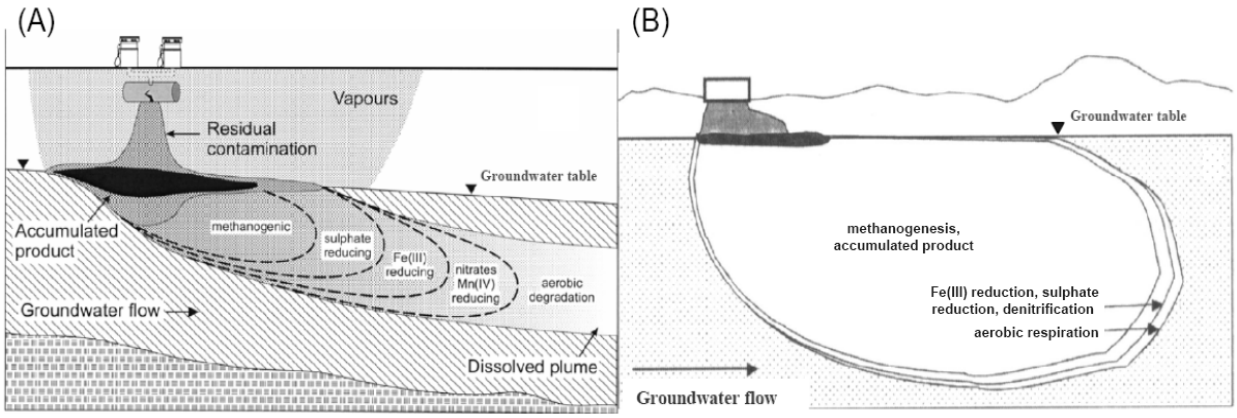

Figure 1.1. Classical (A) and updated (B) perspective of redox zonation in and around hydrocarbon plumes in aquifers (modified after Christensen et al., 2000). The most important available electron acceptors and redox processes per compartment are indicated.

1.3. Pathways of microbial toluene degradation

In this thesis, focus is to be laid on toluene degradation as a case study. Toluene can be considered as a central model substance for microbial aromatics degradation and numerous studies on BTEX degradation in aerobic as well as in anaerobic groundwater include toluene. Toluene represents also a 'basic structure' of all BTEX compounds, having a benzene-nucleus and an alkyl-side chain. Additionally, the mineralization of toluene starts immediately after contamination of an aquifer and biodegradation is relatively fast (Piskonen et al., 2008). Likewise, other BTEX compounds are often degraded in the presence of toluene by microorganisms with a relatively broad substrate use. Similar compounds like xylenes can be degraded via the same catabolic pathways, catalysed by the same enzymes (Colombo et al., 2004). Hence, aerobic toluene degradation can enhance the degradation of other compounds, especially xylenes, due to the high affinity of *xyl*-monooxygenase for toluene (Colombo et al., 2004). Decreasing degradation activity has also been noted for xylene or ethylbenzene instead of toluene as substrate (Bertoni et al., 1998). Hence, toluene is frequently regarded as model compound for the investigation of aerobic as well as anaerobic monoaromatic hydrocarbon degradation.

The first step in the anaerobic metabolism of toluene (i.e. under nitrate-, iron(III)-, sulphate-reducing or methanogenic conditions) is mediated by the addition of fumarate to the methyl group to form benzylsuccinate (Fig. 1.2). This is catalysed by the enzyme benzylsuccinate synthase (*bss,* with *Thaurea aromatica* T1 and K172 and *Azoarcus* sp. T as reference strains (Achong et al., 2001)). Benzylsuccinate is then further oxidized to benzoyl-CoA, the central intermediate in aromatic hydrocarbon metabolism. Besides nitrate-reducers, *bss* has been found in reducers of ferric iron (*Geobacter metallireducens*) (Achong et al., 2001) and also in many sulphate-reducing strains (Andreoni and Gianfreda, 2007; Winderl et al., 2007). Thus, *bss* activity is reported across a wide range of phylogenetically and physiologically diverse bacteria, and even for anoxygenic phototrophic bacteria (Achong et al., 2001; Beller et al., 2002; Kane et al., 2002). So far, *bss* is the only described enzyme that catalyzes the first activation step of anaerobic toluene degradation (Beller et al., 2002). *bssA*, which encodes for the α-subunit of *bss*, is used as a catabolic marker gene (Beller et al., 2002; Winderl et al., 2007). *bssA* gene quantity can be well related to contaminant concentrations and biodegradation (Beller et al., 2008); i.e. a higher *bssA* gene abundance is reported for systems and zones with more active toluene degradation (Achong et al., 2001; Winderl et al., 2008). Anaerobic toluene degradation

potentials in the field can be quantified by quantitative polymerase chain reaction (qPCR) of the *bssA* gene in the field (Winderl et al., 2008). Additionally, similar anaerobic degradation pathways activated by enzymes analogous to *bss*, are known (or assumed) for a variety of other hydrocarbons, like methylnaphthalene, alkanes, cresols, and also linear alkylbenzenesulfonate detergents (Kane et al., 2002).

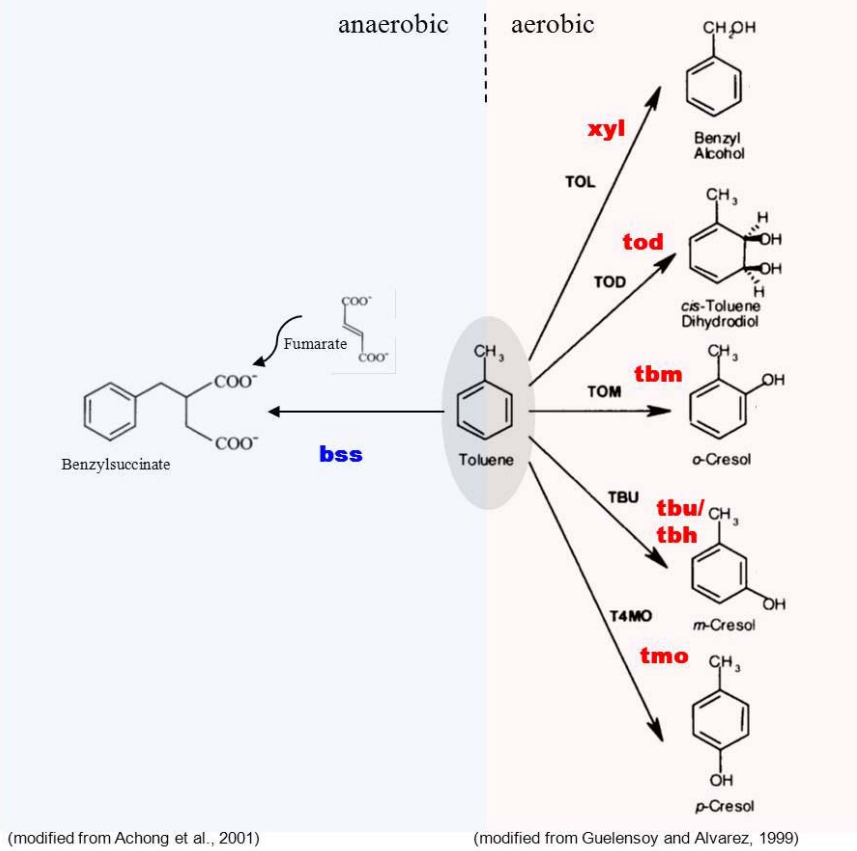

Figure 1.2. Left: First step of anaerobic toluene degradation catalyzed by *benzylsuccinate-synthase (bss)* (modified from (Achong et al., 2001)). Right: Initial reactions of different aerobic toluene degradation pathways. For gene names see text (modified from (Gulensoy and Alvarez, 1999)).

The aerobic degradation of toluene, and generally monoaromatic hydrocarbons, occurs via three successive main steps. The degradation is first initiated by introduction of oxygen and formation of hydroxyl-groups, i.e. activated by the conversion of the BTEX compound into a (alkylated) catechol structure by different initial attack mechanisms involving either mono- or dioxygenases (Fig. 1.2). Oxygenases of very different microorganisms can catalyse similar reactions, share similar features or structures, and have very similar (or almost identical) reaction mechanisms. Genes encoding these key

enzymes are used as targets to detect the presence of aerobic hydrocarbon degraders (Andreoni and Gianfreda, 2007), also to determine their activity by mRNA detection (Lovley, 2003). To differentiate extant aerobic degradation activities in a given system, the initial oxidative attack is relevant. Five different aerobic activation mechanisms and catabolic pathways catalyzed by an equivalent number of distinct key-enzyme are known (Fig. 1.2). The respective aerobic toluene degradation genes are divided, first, into mono- and dioxygenases arising from the capacity to catalyze the addition of one oxygen atom or a dioxygen molecule. This direct oxidation of the aromatic ring is encoded by monooxygenase (TMO-pathway) or dioxygenase genes (TOD-pathway). A second classification of toluene-monooxygenases refers to the position at which the aromatic ring is hydroxylated, affecting further ring-fission and hence metabolite formation. The oxidation of the alkyl side-chain (i.e. the methyl group of the toluene and the o-, m-, p-xylene) is encoded by a monooxygenase whose genes are located on the TOL-plasmid (Baldwin et al., 2003). Key genes for respective reference strains are shown in Table 1.2.

Table 1.2. Genes encoding the first step in the aerobic degradation (oxidation) of toluene.

Gene	Gene name	Reference organism	Pathway	Reference
Tod	toluene-2,3-dioxygenase	*P. putida* F1	TOD	(Zylstra and Gibson, 1989)
tom/tbm/tbc	toluene-ortho(2)-monooxygenase	*B. cepacia* G4, *P. sp.* JS150	TOM/T2MO	(Johnson and Olsen, 1995)
tbu/tbh	toluene-meta(3)-monooxygenase	*B. pickettii* PKO1, *B. cepacia* AA1	TBU/T3MO	(Byrne et al., 1995)
Tmo	toluene-para(4)-monooxygenase	*P. mendocina* KR1	T4MO	(Yen et al., 1991)
Tou	toluene and ortho-xylene utilization	*P. stutzeri* OX1		(Bertoni et al., 1998)
Xyl	xylene/toluene monooxygenase	*P. putida* mt-2	TOL	(Suzuki et al., 1991)

Following, the second step of the aerobic BTEX degradation is the cleavage of the aromatic ring of the catechol, catalyzed only by a dioxygenase. Almost all bacterial degradation pathways for aromatic hydrocarbons convert the initial substrates into a catechol- or protocatechuate-structure. The fission of aromatic rings can occur at two different positions of the ring (Harayama et al., 1992): between the two hydroxyl groups

(therefore called ‘intradiol cleavage or ortho-cleavage’) or between a hydroxylated carbon atom and one of the neighbouring non-hydroxylated carbon atoms (‘extradiol cleavage or meta-cleavage’). After this ring fission, the resulting metabolite is further catabolised by a series of enzymes to pyruvate and acetaldehyde, which are the finally converted to the end products CO_2 and H_2O, or assimilated.

A consumption of oxygen as co-substrate is linked to the activity of mono- and dioxygenase enzymes. Populations with different oxygen demands for BTEX degradation can be expected to develop in different zones of redox gradients (Hendrickx et al., 2006b). In some microorganisms, different pathways of toluene degradation operate simultaneously (Lang, 1996; Cavalca et al., 2004). Multiple catabolic genotypes with a similar catabolic activity in a single bacterial strain are not unusual (e.g. *xyl* genes in different *P. putida* strains). Nevertheless, changes in the BTEX-catabolic genotypes detected at a given site over space and time can be expected to reflect changes in degrader community structure (Cavalca et al., 2000). Under limiting oxygen concentrations, strains with monooxygenases were found to be more successful than those with dioxygenase activity, while at high oxygen availability, dioxygenase systems are more competitive in degrading toluene (Leahy and Olsen, 1997). Bacterial strains that hydroxylate the methyl group of aromatic hydrocarbons are energetically less competitive in comparison to strains which start by direct oxidation of the aromatic nucleus (Duetz et al., 1994).

1.4. Monitoring approaches for biodegradation based on catabolic genes

A molecular method for monitoring pollutant degradation should target a functional gene associated with the contaminant, such as to produce a positive correlation between the number of gene copies and degrading activity (Beller et al., 2002). And indeed, the density of established degrader populations is assumed as a further key limiting factor for net biodegradation *in situ*. Thus, local microbial community patterns and especially the distribution of specific degraders can provide valuable insights on the importance of on-going degradation processes. Here, molecular analysis can provide vital information on the presence (and abundance) of a specific catabolic capacities, and inform on natural attenuation potentials *in situ*.

Until recently, the knowledge on contaminant degrading microbes in groundwater was still largely limited to what was demonstrated by enrichment and cultivation of

microorganisms. However, it is generally accepted that these can differ substantially from degraders active in natural habitats. Also there is a lack of knowledge on the microorganisms that are the most effective pollutant metabolizers at specific sites, also under changing or heterogeneous environmental conditions. Their distribution, diversity, dynamics and succession, activity, and exact physiology under *in situ* conditions are still poorly understood. Therefore, there is an increasing interest in methods capable to detect and quantify such degraders in the field under natural conditions. Here, alternative monitoring approaches for NA complementary to established biogeochemical and isotopic monitoring tools are needed. Molecular approaches hold the potential to provide more quantitative insights on the presence of a certain degradation potentials and on-going *in situ* bioremediation.

Proteins involved in the initial oxidative attack of BTEX show significant sequence homology, which allows the design of group-specific primer sets for detection of respective genes by PCR (Baldwin et al., 2003; Hendrickx et al., 2006a; Andreoni and Gianfreda, 2007). In the literature, the development of several respective primers has indeed allowed to detect and quantify, in environmental samples, the presence of specific genes encoding key steps in aerobic BTEX degradation and to measure their activity (Baldwin et al., 2003; Junca and Pieper, 2004; Hendrickx et al., 2006a). Degrader communities show two main properties which are crucial in terms of molecular monitoring. First, they are often spatially restricted to specific zones and geochemical conditions, showing characteristic local abundances; secondly, diversity and functional redundancy within these communities ensures the resistance and resilience of biodegradation under habitat dynamics and fluctuations (Winderl et al., 2007; Winderl et al., 2008; Anneser et al., 2010). In sum, the greater the number of catabolic genes detectable within a contaminated zone, the greater the established potential for NA that is to be expected (Andreoni and Gianfreda, 2007).

The typical molecular work flow starts with a selection of appropriate monitoring target, mostly characteristic key genes (key enzymes) such as for the initial activation of toluene. The development or adaptation of existing PCR assays may be needed for the application of selected degradation markers in the field, and for the requirements of quantitative (q)PCR. The development of general, but also specific primers for the selected marker gene is necessary to detect the desired diversity of degraders possibly involved in biodegradation at a given site. Primer design and optimization should be guided by

continuously curated alignments and databases, e.g. using the phylogeny program package ARB (Ludwig et al., 2004).

The application of marker assays to characterize the quantitative distribution of intrinsic degradation genes in sediments and groundwater at selected contaminated field sites can be based on qPCR or functional gene microarrays (Andreoni and Gianfreda, 2007). Potentially, even the expression of degradation genes can be monitored by quantification of their transcription directly as mRNA (Alfreider et al., 2003). As example, the expression of *chlororcatechol 1,2*-and *2,3-dioxygenase* was detected for contaminated subsurface samples, but could not be quantified (Alfreider et al., 2003). Here, additional adaptations for reverse-transcription qPCR (RT-qPCR) may be needed. There are existing assays for the quantification of anaerobic toluene degradation genes of sulphate –and nitrate reducing bacteria. As alternative, DNA- and mRNA-quantification by microarrays can be used to monitor biodegradation potentials and activity for numerous samples in parallel. DNA microarrays have been used for the detection, but not quantification, of benzene monooxygenase gene diversity in contaminated soils (Iwai et al., 2007). Optimally, multiple lines of evidence including biogeochemical, isotopical, and hydrological parameters have to be integrated with molecular monitoring data for a comprehensive assessment of NA.

1.5. Monitoring degrader succession upon contamination of an indoor aquifer

Monitoring of natural attenuation in the field usually starts a long time after a contamination event. Thus, insights on early microbial community adaptations and initial degrader successions are completely missing, but vital to identify driving forces of ecosystem adaptation to contamination. To address this knowledge gap, an indoor aquifer mesocosm (Fig. 1.3) was established at the Institute of Groundwater Ecology, Helmholtz Zentrum München in the frame of the GOODWATER ITN, by which also this thesis was funded, to investigate the initiation and succession of toluene degradation processes under controlled conditions in a ‘close-to-natural’ setting. This indoor aquifer model system was intended as a reference experiment to dissect and extrapolate back to nature all processes and factors important for the understanding of plume establishment. The main benefits of such a model aquifer system compared to field sites are: (i) monitoring of NA can start

immediately after contamination event; (ii) a multidisciplinary approach and integrated monitoring to elucidate biodegradation is easily possible; (iii) ease of access allows a high resolution of biotic and abiotic parameters in space and time. Thus, new concepts and feedback mechanisms for contaminant degradation in groundwater can potentially be uncovered. The intentional contamination of pristine aquifers in nature is generally not an acceptable procedure. Thus to date, several microscale experiments (in sediment columns or 2D-tanks) have been used for respective investigations.

The most innovative aspect of the IGÖ indoor aquifer was the multidisciplinary approach towards process understanding, including microbiological, hydro-geological, chemical, and stable isotope analytics. The team working with the indoor aquifer consisted of several PhD students, all with their own specific projects and aims, as well as most of the technical assistants of the IGÖ helping with experimental maintenance, routine measurements, and general technical support. The project of A. Herzyk (Microbial Ecology Group) aimed to understand how groundwater biodiversity initially reacts to contamination, and how aquifers recover after contaminant removal (resistance and resilience). S. Marozava (Anaerobic Degradation Group) aimed to understand the physiology of anaerobic degraders *in situ*. S. Qiu (Environmental Isotope Group) aimed at using compound-specific stable isotope analysis (CSIA) to quantify contaminant turnover *in situ*. The general aim of this PhD project (Molecular Ecology Group) was to unravel how and how fast an oligotrophic microbial community from an uncontaminated aquifer can develop aerobic and anaerobic degrader lineages upon contact with toluene, and to develop and apply molecular tools to monitor their succession.

Figure 1.3. View over the mesoscale indoor model aquifer.

The meso-scale dimension of the flume (5.0 m length x 0.8 m width x 0.7 m depth) allowed for a meaningful linking between micro-scale approaches (as in the lab) and the much larger dimension of contaminated aquifers. A wealth of sampling- and 'online' monitoring devices facilitated a high spatial and temporal resolution of analyses (Huenninger, 2011), often not realisable in the field. Also, only in such an initially pristine model aquifer the successional impact of a defined organic contamination (here, toluene), as well as the recovery of the system (resilience to the 'initial' state) after contaminant removal can be studied in realistic time frames. The sediment material consisted of natural sand (0.1-4 mm grain size) representative for the Munich area and was selected to be in geochemical equilibrium with the utilized groundwater. The sand was from a gravel pit in Bruckmühl, situated around 60 km south of the HMGU in Neuherberg. The groundwater derived from wells on site at HMGU. The natural groundwater was pumped from four wells on the campus and stems from a shallow, Quartenary aquifer. It is a relatively pristine, oligotrophic, oxic groundwater with low levels of nitrate, thus allowing the potential development of denitrifying contaminant degradation within an introduced plume. After installation, the indoor model aquifer system was allowed to stabilize for almost one year in its hydrological and geochemical properties and to develop a natural intrinsic microbial community.

The flow rate was approximately 1 m d^{-1} (0.5-1.5), with heterogeneities and preferred flow paths in the middle of the flume. A lens of coarse sand was installed after the inflow, in the center of the depth transect with the intent to focus the flow of groundwater, but especially of the plume (the position of the lens is shown in Figure 1.4). Due to an increased hydraulic conductivity in the sand lens, flow velocity is increased and also vertical and horizontal mixing. Therefore the introduced contaminant toluene can be diluted and dimensioned over the flow path. This focusing lead to a widening of the plume fringes downstream of the lens, and thus facilitated appropriate depth-resolved sampling of plume compartment. High resolution water sampling ports, in depth intervals as small as 4 cm in the central row (Fig. 1.4), were constructed with glass frits in order to prevent sediment alteration during pumping. Dissolved oxygen was measured using oxygen sensitive sensor spots with fluorescence depending on oxygen saturation (Lippitsch et al., 1988; Huenninger, 2011).

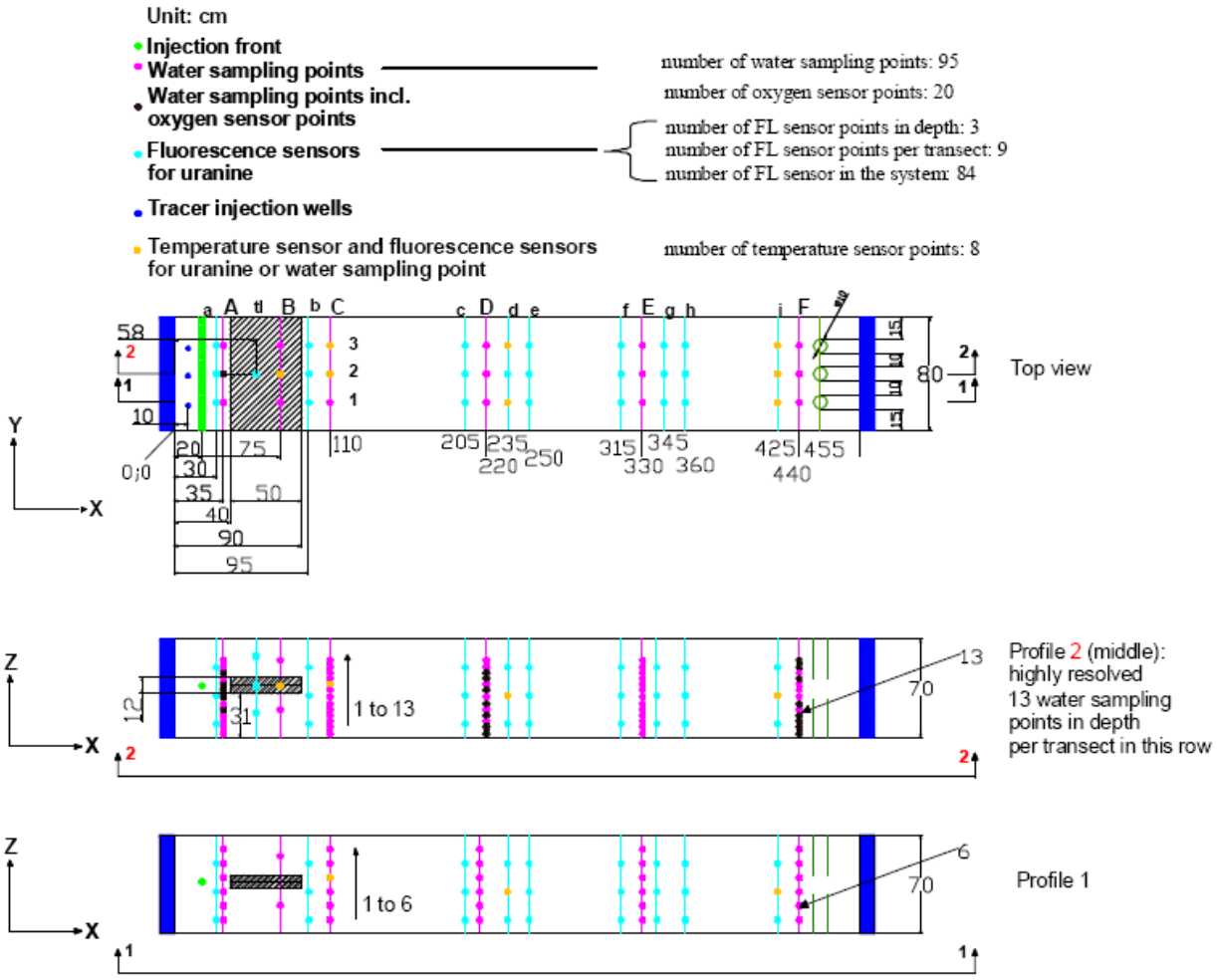

Figure 1.4. Schematic overview and cross-section of the indoor model aquifer, showing the technical installations and water ports for sampling and measuring (Huenninger, 2011).

The pristine system was successively contaminated with two different concentrations of toluene to investigate (in this thesis) (i) the emergence of toluene degraders (and general microbial community impacts) upon contamination, (ii) the spatial and temporal succession of degraders upon increasing contaminant, and (iii) distinctions in information obtained from the aquifer matrix (sediment) vs. free groundwater. To obtain a broad range of biogeochemical and microbiological parameters, several monitoring methods for microbial biomass, activity, diversity and functionality were applied for water and sediment samples by the team. Concentrations of microbially relevant anions and cations, such as nitrate, sulphate, and iron were measured by the PhD candidate with additional help from M. Stoeckl (technician) and P. Knappet (PostDoc). Oxygen concentrations, pH-value and temperature of the model aquifer were also monitored in regular time intervals by the PhD candidate, in exchange with M. Stoeckl or S. Kaschuba (technician). A comparison of steady-state bromide concentrations (a non-reactive tracer

which does not undergo sorption and degradation) in vertical depth profiles of the different transects was used as basis for the estimation of the toluene flow path and plume spreading. A measurement of steady-state bromide distribution (breakthrough of bromide), pre-toluene injection, conducted by S. Qiu (PhD student) and P. Knappett suggested the aquifer to be rather homogeneous initially. Also oxygen and nitrate concentrations resulted in lateral symmetry in the same experiment. In the field, the reproducibility and representativity of sediment samples is an issue often faced due to natural aquifer heterogeneity. In the indoor aquifer, reproducible sampling of sediment cores and free groundwater to study the temporal evolution of physical, chemical, and biological processes at high resolution (in space and time) now becomes possible for the first time.

1.6. Aims of the Ph.D. project

The main objective of the PhD thesis is to improve the understanding of the partitioning of aerobic and anaerobic biodegradation potentials at redox gradients and in heterogeneous hydrogeochemical settings. For this, integrated catabolic marker gene-based assays for the monitoring of distinct toluene degradation potentials in polluted aquifers are developed and applied. More specifically, catabolic gene-based monitoring of aerobic and anaerobic toluene degradation are applied to detect degrader diversities and to quantify degrader distribution. Thus, the uncovery of potential quantitative couplings between local aerobic and anaerobic degrader abundances and the importance of the respective degradation processes is pursued, especially across biogeochemical redox gradients, which are the hot spots of biodegradation *in situ*. Thus, catabolic marker gene assays are used to elaborate a more ecological perspective of contaminant plumes, a prerequisite for developing integrated monitoring strategies for natural attenuation. The following specific aims are addressed:

(i) **Development of an optimized set of combined quantification assays for aerobic and anaerobic toluene degradation genes in aquifers.** Focus is directed especially towards the adaptation of detection assays for aerobic toluene degradation genes (*tmoA*), while anaerobic marker assays (*bssA*) are applied as developed in the PhD projects of C. Winderl (Winderl et al., 2008).

(ii) Comparative identification and quantification of aerobic and anaerobic toluene degraders at an oxic/anoxic redox gradient at a contaminated field site. Quantitative couplings between local aerobic and anaerobic degrader abundances and the importance of the respective degradation processes are to be verified. Based on this, hot spots of distinct degradation processes in correlation to geochemical and contaminant data are to be identified.

(iii) Elucidation of the reactivity of a pristine aquifer towards toluene contamination. Degrader establishment and succession of biodegradation processes upon increasing contaminant concentration are unravelled for a unique indoor aquifer model system. Population information is provided in high resolution in time and space. Both overall communities as well as specific degrader lineages are traced (via pyrosequencing approaches and catabolic marker gene assays).

(iv) Application of gene-based monitoring in combination with 'classical' site monitoring as a contribution to a stakeholder bioremediation campaign. In the frame of an internship with Isodetect GmbH, a comprehensive monitoring of natural attenuation processes at a former Gasworks site in Augsburg is to be conducted. The aim is to demonstrate the added value of molecular monitoring tools in addition to standard geochemical and isotopic monitoring, to assist in strategic decision making in site management and remediation.

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2. Fine scale degrader community profiling over an aerobic/ anaerobic redox gradient in a toluene contaminated aquifer

Abstract

Hydrocarbon contaminants in groundwater can be degraded by microbes under different redox settings, forming hot spots of degradation especially at the fringes of contaminant plumes. At a tar-oil contaminated aquifer in Germany, it was previously shown that the distribution of anaerobic toluene degraders as traced via catabolic and ribosomal marker genes is highly correlated to zones of increased anaerobic degradation at the lower fringe of the plume. Here, the respective distribution of aerobic toluene degraders over a fine-scale depth transect of sediments taken at the upper fringe of the plume and below was traced, based on the analysis of 16S rRNA genes as well as catabolic markers in intervals of 3-10 cm. Well-defined small-scale distribution maxima of typical aerobic degrader lineages within the *Pseudomonadaceae, Comamonadaceae*, and *Burkholderiaceae* are revealed over the redox gradient. An unexpected maximal abundance of 9.2 x 10^6 toluene monooxygenase (*tmoA*) genes per g of sediment was detected in the strongly reduced plume core, and gene counts did not increase towards the more oxidised upper plume fringe. This may point towards unusual ecological controls of these yet unidentified aerobic degraders, and indicates that competitive niche partitioning between aerobic and anaerobic hydrocarbon degraders in the field is not yet fully understood. These findings demonstrate the potential of catabolic marker gene assays in elaborating the ecology of contaminant plumes, which is a prerequisite for developing integrated monitoring strategies for natural attenuation.

2.1. Introduction

Groundwater represents ~30% of global freshwater resources (Foster and Chilton, 2003). Microbial processes can play an important role in the protection against and remediation of groundwater contaminants, therefore a better integration of respective understanding in current management schemes is needed. Currently, compound-specific isotope analysis of contaminants to qualify and quantify degradation, as well as monitoring of metabolites and electron acceptor consumption represent widely used methods to assess natural attenuation and bioremediation (Meckenstock et al., 2004; Wilson et al., 2004). Due to limited oxygen availability, contaminant plumes within hydrocarbon-contaminated aquifers are predominantly anoxic, structured in a succession of distinct redox compartments. Different microorganisms are capable of utilizing hydrocarbon contaminants as a source of carbon and energy under aerobic and distinct anaerobic conditions (Andreoni and Gianfreda, 2007). However, especially in heterogeneous geochemical settings, monitoring of overall degradation and also of the net contribution of different redox processes is often not easy to determine.

Here, molecular detection systems for degraders can greatly advance our understanding of the importance of different degrader guilds in complex systems. Gene-based monitoring strategies based on the detection of catabolic markers in environmental samples are of increasing interest and may offer attractive advantages in monitoring of degradation processes at natural sites (Lovley, 2003; Andreoni and Gianfreda, 2007). A molecular method for monitoring specific pollutant degraders should target a functional gene strictly associated with the microbes involved in the respective process, to allow direct correlations between the number of gene copies and respective degradation potentials residing in a given zone (Beller et al., 2002; Colombo et al., 2004; Piskonen et al., 2008). It has been shown that local microbial community patterns and especially the distribution of specific degrader lineages can provide valuable insights on the localisation and control of on-going degradation processes (Winderl et al., 2008; Baldwin et al., 2009; Staats et al., 2011). In particular, contaminant plume fringes and redox gradients are considered as hot spots of biodegradation and other biogeochemical processes, evident also in residing specialized microbial degrader communities (Cavalca et al., 2004; Wilson et al., 2004; Winderl et al., 2008).

It was previously shown that the distribution of anaerobic toluene degraders as traced via functional marker genes is highly correlated to zones of increased anaerobic

degradation activities at the lower fringe of a hydrocarbon (mainly toluene) plume in Germany (Winderl et al., 2008). However, the distribution and ecology of different degrader guilds at the upper plume fringe, directly underlying the groundwater table at this site, is far from understood. The upper fringe can be hypothesised to harbour a spatial succession of both aerobic and anaerobic toluene degrader lineages which, if quantified, may be indicative even of the relative importance of respective catabolic pathways. However, certain aerobic toluene degraders are notoriously detected also in anaerobic compartments of contaminated aquifers (Hendrickx et al., 2005; Hendrickx et al., 2006b); yet, their quantitative spatial distribution has not been comprehensively investigated.

In this work, comparative detection and quantification assays for aerobic and anaerobic toluene degrader lineages based on both 16S rRNA genes as well as catabolic markers were applied to a unique fine-scale depth transect of sediments taken at the upper fringe of the hydrocarbon plume. My main research questions were: (i) which aerobic and anaerobic toluene degraders can be detected in which plume compartments, (ii) how are comparative catabolic gene abundances distributed over the redox gradient, and (iii) can gene-based monitoring help to better understand the ecology of the "plume system" and distinct intrinsic degrader populations as a whole?

2.2. Material and Methods

2.2.1. Site location, sampling, and geochemistry

Aquifer sediment samples from a well-studied tar-oil contaminated site in Düsseldorf-Flingern, Germany, sampled in February 2006 were analysed in this study. For a description of the site, sampling of sediments, measurements of the redox potential, nitrate, and toluene concentration see (Anneser et al., 2008; Anneser et al., 2010). To resolve especially the transect from the groundwater table into the strictly anoxic plume core, sediment samples at intervals between 3 and 10 cm between 6.45 and 6.90 m below ground surface were analysed.

2.2.2. DNA extraction and PCR

Total DNA was extracted using ~1 g of sediment (wet weight) in three independent extracts for every depth, following the procedure described by (Winderl et al., 2008). All PCRs were performed in 50 µl-reactions containing nuclease-free H_2O, 1x PCR buffer, 1.5

mM $MgCl_2$, 0.1 mM dNTP's, 1 U Taq polymerase (all Fermentas GmbH, St. Leon-Rot, Germany), 10 µg BSA (Roche Diagnostics GmbH, Basel, Switzerland), 0.5 µM of each primer (biomers.net GmbH, Ulm, Germany), and 1-2 µl of template DNA. 16S rRNA gene-PCR was performed under the following thermal cycling conditions: 3 min of initial denaturation at 94°C, 26-28 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 55°C, and 60 s extension at 72°C), and 5 min of final extension at 72°C. The amplification of toluene dioxygenase (*tod*) and monooxygenase (*tmoA*) genes followed the same thermocycling program but with different annealing temperature and 30 cycles (see Table 2.1). Primers used for oxygenase detection are given in Table 2.1.

Table 2.1. Primers and PCR conditions used for the detection of toluene dioxygenase (*tod*) and toluene-para(4)-monooxygenase (*tmo*) genes in this study.

Primer	Sequence (5' – 3')	Annealing [°C]	Reference
todA-f	CTTAGCGGTGAGCGCAGTGTG	55	
todA-r	CCACGATGTAGCGACTTGGGG	55	(Shinoda et al., 2004)
todC1-f	CAGTGCCGCCAYCGTGGYATG	55	
todC1-r	GCCACTTCCATGYCCRCCCCA	55	(Hendrickx et al., 2006b)
tmoA-f	CGAAACCGGCTTYACCAAYATG	60	
tmoA-r	ACCGGGATATTTYTCTTCSAGCCA	60	(Hendrickx et al., 2005)

2.2.3. Microbial community fingerprinting, cloning and sequencing

T-RFLP (terminal restriction fragment length polymorphism) analysis of bacterial 16S rRNA genes was performed with PCR-amplicons of primers Ba27f-FAM / 907r by *MspI* digestion, as previously described (Pilloni et al., 2011). 16S rRNA gene amplicons (obtained with unlabelled primers) and *tmoA* amplicons (primer pair tmoA-f and tmoA-r) were cloned according to (Winderl et al., 2007) via the pGEM-T cloning kit (Promega, Madison, WI) and sequenced on a ABI 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) using BigDye terminator v3.1 chemistry according to the manufacturer's protocol.

2.2.4. Quantitative PCR

All real-time quantitative PCR (qPCR) measurements were performed on a MX3000P qPCR cycler (Stratagene, La Jolla, CA). For 16S, standard PCR assays as described (Winderl et al., 2008) were used in the presence of 0.1x SYBR Green (FMC

BioProducts, Rockland, MA). qPCR of benzylsuccinate synthase (*bssA*) genes was performed with the TaqMan system previously developed by (Winderl et al., 2008), with the Universal PCR Master Mix, No AmpErase UNG kit (Applied Biosystems, Carlsbad, CA). The Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA) was applied for toluene-para-monooxygenase (*tmoA*)-qPCR, using the manufacturer's protocol with minor modifications. Primers tmoA-f and tmoA-r were used with two-step thermal cycling (45 cycles at 95°C for 20 s and 60°C for 20 s). qPCR standards were generated with a PicoGreen-quantified partial tmoA gene amplicon (clone DV7tmo13, GenBank accession no. JX307448) retrieved from the same aquifer sediment. qPCR gene counts were tested for statistically significant differences by One Way ANOVA in SigmaPlot (Systat, Erkrath, Germany).

To correct for potential extraction, amplification and/or detection biases in our workflow, defined biomass amendments were conducted for the Flingern sediments, as previously reported also for *bssA* quantification (Winderl et al., 2008). For this, defined amendments of Pseudomonas mendocina KR1 cells were spiked to sterilised Flingern sediment in concentrations between 1.13×10^5 and 1.36×10^7 cells g^{-1} wet of sediment as counted on a LSR II flow cytometer (Becton Dickinson, Heidelberg, Germany) according to (Anneser et al., 2010). DNA was extracted from spiked sediments, and the detected versus expected gene quantities were used to infer specific correction factors for the used *tmoA* vs. 16S rRNA gene qPCR assays assuming 4 rrn and 1 tmo operon per cell and genome (factors of 2.7 for *tmoA* and 5.5 for 16S genes were inferred, respectively). The detection limits of our qPCR assays were 2.3×10^4 copies per g of sediment for 16S rRNA genes, 9.2×10^3 for *bssA*, and 3.3×10^4 *tmoA* genes per g of sediment.

2.2.5. RNA extraction and rt-PCR

In addition to DNA work, RNA extraction was accomplished in two independent replicates, each with ~20 g of sediment, taken between 6.57-6.65 m bgs. Extraction of total RNA was performed following an adaptation of the method from (McIlroy et al., 2008; Lloyd et al., 2010), based on bead-beating in TCA (trichloroacetic acid) lysis buffer followed by phenol/chloroform cleanup. DNA digestion was accomplished with the RQ1 RNase-free *DNase* (Promega Corporation, Madison, WI) following the manufacturer protocol by using the same concentrations in adjusted amounts. Reverse transcription (rt-)PCR was performed with the AccessQuickTM RT-PCR System (Promega) in 50 µl-reactions containing 1x AccessQuickTM Master Mix, additionally 10 µg of BSA, 0.5 µM

of each primer (tmoA-f and tmoA-r), 0.1 U of AMV Reverse Transcriptase, and 2-5 µl of total RNA (undiluted and in different dilutions, to circumvent potential inhibition). Thermal cycling conditions were analogous to the PCR for *tmoA*-genes but with a preceding rt-step of 30-60 min at 45°C. Additional rt-PCR reactions were set up with a lower annealing temperature of 58°C and/or an increase to 40 cycles.

2.2.6. Data analysis

T-RFLP electropherograms were analysed using the GeneMapper 4.0 software (Applied Biosystems) and then evaluated as in (Winderl et al., 2008). Sequence reads from clones were assembled with SeqMan II software (DNASTAR Inc., Madison, WI), screened against all publically available sequences by BLAST (McGinnis and Madden, 2004), and checked for possible chimeric formations trough the Bellerophon server (used window size of 200-400 bp as breakpoint of the sequences, 5 putative chimera were found (Huber et al., 2004)). Clone affiliation was classified using the RDP classifier (Wang et al., 2007) at a default confidence threshold of 80%. If possible, the T-RF's were predicted in ARB for sequenced clones and correlated to the respective experimentally observed T-RF's with clear relation in their abundance and length (bp). Possible differences between predicted and measured T-RFs were accounted for in accordance to our previous verification of in silico vs. in vitro T-RFs of cloned amplicons from the same site (Winderl et al., 2008). Publically available and sequenced *tmoA* gene data was integrated into an alignment using the ARB software (Ludwig et al., 2004). Phylogenetic dendrograms of partial *tmoA* sequences were constructed from distance matrices using Fitch analysis and further verified via neighbour-joining and maximum likelihood methods (all in ARB), as previously performed also for *bssA* phylogeny (Winderl et al., 2007).

2.2.7. Nucleotide sequence accession numbers

All sequenced 16S rRNA gene clones reported in this study were deposited in GenBank under accession numbers JX307455-JX307590, the *tmoA* clones under accession numbers JX307412-JX307454.

2.3. Results

2.3.1. Bacterial 16S rRNA gene distribution over depth

Already directly at the groundwater table, redox conditions were reducing (~0 mV) and further decreased with depth (Figure 2.1A), alluding possible oxygen supply from the unsaturated zone above the groundwater table only for the uppermost cm of saturated sediment. For a first screening of overall bacterial community stratification over this oxic/anoxic redox gradient and into the plume, fine-scale depth-resolved bacterial community fingerprinting was performed at sampling intervals of 3-10 cm. Over the entire depth transect of 35 cm, this revealed defined specific abundance maxima of different bacterial terminal restriction fragments (T-RFs, Figure 2.1B). For example, the 154, 477, and 492 bp T-RFs were found to be especially abundant at the upper plume fringe. T-RFs of 486, 499 and 502 bp were found most abundant in the centre of the plume (the core), whereas e.g. the 130, 159 and 509 bp T-RFs increased towards the lower fringe.

To identify the bacterial lineages represented by these T-RFs characteristically distributed across depth, and especially to screen for bacteria related to potential aerobic toluene degraders, two clone libraries at 6.5 m (upper plume fringe) and at 6.65 m depth (plume core) were sequenced and revealed pronounced distinctions in depth-resolved bacterial communities (Table 2.2). The majority of the clones belonged to the *Beta*- and *Deltaproteobacteria*. While *Betaproteobacteria* were similarly abundant in both libraries, the *Deltaproteobacteria* were clearly more abundant in the plume core.

Important T-RFs characteristic for the upper fringe were thus identified to represent clones affiliated to the *Rhizobiales* (154 bp T-RF), *Thiobacillus* spp. (477 bp) and *Pseudomonas* spp. (492 bp). In the plume core, the characteristic 486 bp fragment was affiliated to clones within the *Commamonadaceae*, while the 499 and 502 bp T-RFs represented unclassified *Rhodocyclaceae*. Towards the lower fringe, the characteristic 130 and 159 bp T-RFs have already been described to represent *Geobacter* spp. and *Desulfobulbaceae* populations (Winderl et al., 2008), the latter (159 bp fragment) representing the dominating anaerobic toluene degraders on site (Pilloni et al., 2011).

Table 2.2. Composition of bacterial 16S rRNA gene libraries and identified T-RFs associated to defined lineages in the contaminated Flingern-aquifer.

Phylogenetic affiliation[c]	No. of clones[a] Library name, depth		T-RF length (bp)[b]	
	DV02Ba, 6.50 m	DV07Ba, 6.65 m	predicted	observed
Alphaproteobacteria				
unclassified *Alphaproteobacteria*	1	1	n.a.	
Bradyrhizobium spp.		2	152	154
Rhizobium spp.	1	1	n.a.	
Pseudolabrys spp.	1		152	154
Rhodospirillaceae	2		n.a.	
Betaproteobacteria				
unclassified *Betaproteobacteria*	2		n.a.	
Burkholderiaceae		2	329, 473	471
unclassified *Comamonadaceae*	3	5	298, 305	n.a.
Simplicispira spp.		2	488	486
Comamonas spp.		1	n.a.	
Acidovorax spp.		1	488	486
unclassified *Hydrogenophilaceae*	1		n.a.	
*Sulfuricell*a spp.	1		n.a.	
Thiobacillus spp.	11		477	477
unclassified *Rhodocyclaceae*		7	500	499, 502
Dechloromonas spp.		1	n.a.	
Gammaproteobacteria				
unclassified *Gammaproteobacteria*	2		n.a.	
Pseudomonas spp.	3	1	490	492
Rhodanobacter spp.	3	1	156, 497	495
Methylobacter spp.		2	447	n.a.
Deltaproteobacteria				
Bacteriovorax spp.		1	n.a.	
Geobacter spp.	1	3	132	130
Desulfobulbaceae	3	9	162	159
Epsilonproteobacteria				
Sulfurimonas spp.		1	n.a.	
Sulfuricurvum spp.		1	n.a.	
Sulfurospirillum spp.	2		61, 466	60
unclassified *Proteobacteria*	1	1		
Firmicutes				
Desulfosporosinus spp.		2	229	228
Clostridiales	2		167	n.a.
Sedimentibacter spp.		2	280	279
Chloroflexi				
Anaerolineaceae	4	4	454, 518, 519	518
Sphaerobacter spp.	2		469	n.a.
Bacteroidetes				
unclassified *Bacteroidetes*	2	2	n.a.	
Flavobacterium spp.	1		n.a.	
Acidobacteria				
Gp6	1		n.a.	
Geothrix spp.		1	174, 276	172
Actinobacteria	4	1	139, 141, 146	135, 137
OD1	5		75	n.a.
Spirochaetes				

Treponema spp.	1		n.a.	
Armatimonadetes	1		333	n.a.
Planctomycetes	1		n.a.	
unclassified *Bacteria*	8	11	n.a.	

[a] The libraries at 6.50 (upper fringe) and 6.65 m (plume core) contained 70 and 66 clones, respectively.

[b] Characteristic T-RF lengths (bp) predicted from the sequence data for all clones of a given lineage are indicated together with the corresponding T-RF lengths observed in T-RFLP analysis. Values separated by comma indicate more than one characteristic T-RF for that lineage. n.a., not assigned (no conserved T-RF within a given taxonomic group).

[c] The affiliation of clones was done using the RDP classifier (Wang et al., 2007).

Several putative degrader populations related to known aerobic toluene and aromatics degraders were identified within these taxa, and showed distinct abundance distribution patterns. Sequences related to genus *Pseudomonas*, harbouring the known toluene-degrading *P. putida* F1 (Zylstra and Gibson, 1989), 94 - 97% sequence similarity to our detected sequences) or *P. mendocina* (Yen et al., 1991), 95 - 98% similarity) were retrieved exclusively at the upper fringe. The distribution of the connected 492 bp T-RF also supported this pattern. In contrast, clones within the *Comamonadaceae* (T-RF 486 bp), related to *Acidovorax* sp. NA3 (Singleton et al., 2009) and *Comamonas testosteroni* (Teramoto et al., 1999), both known degraders of aromatics, although not of toluene, were found mostly in the plume core where strictly anoxic conditions prevail. A small number of clones from the plume core also belonged to the *Burkholderiaceae,* for which known toluene degraders have also been described (Johnson and Olsen, 1995; Shields et al., 1995; Ayoubi and Harker, 1998; Perez-Pantoja et al., 2008). Moreover, certain sequences within the *Rhodocyclaceae* were remarkably dominant in the centre of the plume, especially on T-RF level, but were not clearly assigned to a specific genus (they grouped closest to the genera *Sulfuritalea* and *Azovibrio* in ARB, data not shown).

2.3.2. Genes for aerobic toluene catabolism

Next, depth-resolved samples were screened for different aerobic toluene degradation markers. Although aerobic toluene degradation is possible via a number of different pathways, the focus was on genes encoding the well-known toluene di- and monooxygenases, which differ in their mechanisms of primary substrate attack (Jindrova et al., 2002). Faint bands were observed after PCR (qualitatively) targeting toluene dioxygenase genes at the upper fringe. PCR for *todA* and *todC* showed weak signals only

in these upper sediment depths (data not shown). In contrast, surprisingly, toluene monooxygenase genes were most readily amplified from the actual plume core. Cloned *tmoA* genes from the upper plume fringe (6.5 m), the plume core (6.65 m), and in the lower fringe (7.1 m) were mostly not assigned to known *tmoA* sequences. A few were related (~90 % nucleotide similarity) to *Burkholderia*- and *Ralstonia-tmoA* (Figure 2.2, clones 'DV02 tmoA 02'; 'DD05 tmoA 06' and '08'). However, most sequences from the actual plume core were related to environmental *tmoA* clones from a BTEX contaminated oil-refinery site in the Czech Republic (Hendrickx et al., 2006a), up to 98% sequence similarity), and to clones from benzene-amended soils in Japan (Iwai et al., 2009), up to 85% similarity). Furthermore, these sequences were loosely affiliated to the *tmoA* genes of *Methylibium petroleiphilum* PM1 (Nakatsu et al., 2006), 80% similarity) and *Pseudomonas stutzeri OX1* (Bertoni et al., 1998), 78% similarity). Despite the fact that these unassigned *tmoA* sequences formed two distinct clusters, no clear distinction in *tmoA*-lineage distribution was found over depth, i.e. between the different plume compartments. This was also further confirmed via preliminary *tmoA* DGGE fingerprinting (Hendrickx et al., 2006a) over depth (data not shown).

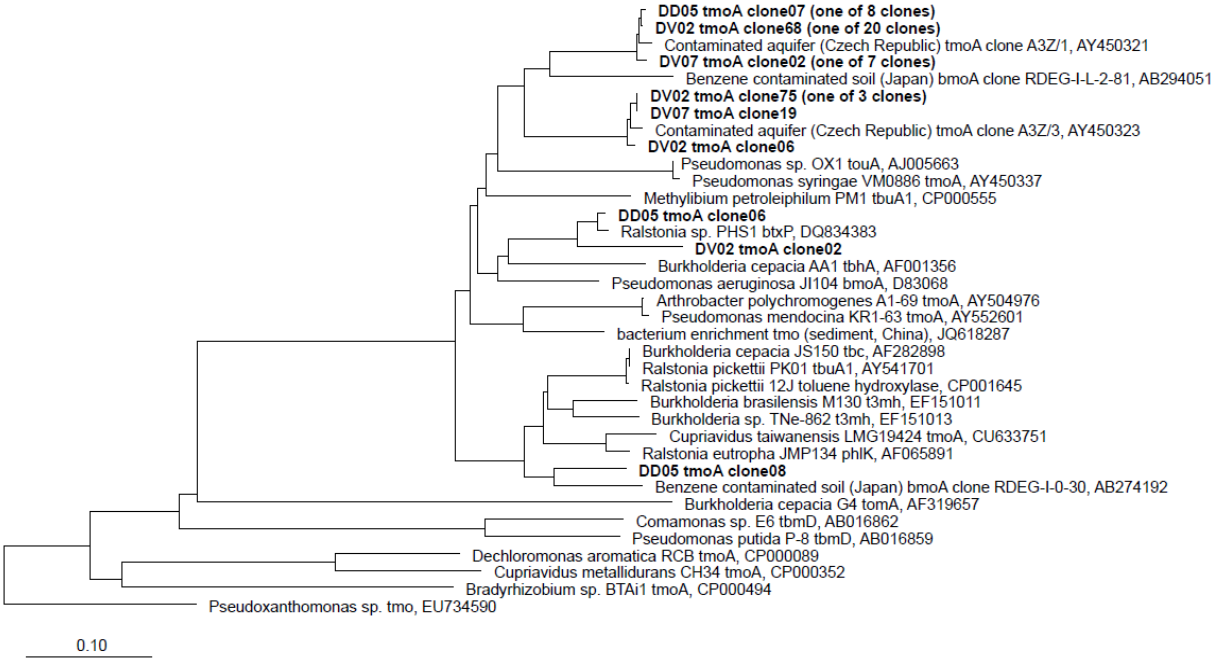

Figure 2.2. Condensed phylogenetic tree of representative *tmoA* (toluene-para-monooxygenase α-subunit) genes cloned from the upper plume fringe (6.50 m, DV02 clones), the plume core (6.65 m, DV07 clones), and the lower fringe (7.10 m, DD05 clones) of the Flingern plume. Related reference sequences of *tmoA* genes (or similar oxygenase genes encoding BTEX-catabolic enzymes) from cultivated representatives or environmental DNA are given.

2.3.3. Marker gene quantification

qPCR quantification of ribosomal, as well as aerobic and anaerobic toluene degradation genes over the depth of the contaminated aquifer was performed (Figure 2.1C). In contrast to previous findings from the same site (Winderl et al., 2008), possibly connected to the even higher resolution employed in our study, both the minimum and the maximum of total bacterial rRNA gene abundance was found in the centre of the toluene plume. While this may illustrate small-scale heterogeneities in sedimentary microbial distribution, maximal bacterial gene counts (8.7 x 10^8 bacterial rRNA gene copies g^{-1} at 6.65 m depth) were well comparable to previous measurements at the site (Winderl et al., 2008). At this depth, quantities of toluene degradation genes were comparably low, showing *bssA* and *tmoA* to 16S gene abundance ratios of only 0.013 and 0.002, respectively. The highest abundance of *bssA* genes involved in anaerobic toluene degradation (9.1 x 10^7 gene copies g^{-1}, 16S to *bssA* abundance ratio of 0.2) occurred towards the lower fringe and was again comparable to gene counts from 2005 sediments (Winderl et al., 2008). In contrast, the maximal abundance of tmoA genes was found not at the upper plume fringe, but in the plume core, ~7.5 cm above maximal bacterial 16S rRNA gene abundances. However, even at the maximal quantity of 9.2 x 10^6 genes g^{-1} of sediment, *tmoA* counts were slightly below *bssA* gene copy numbers in the same depth (1.9 x 107 g-1). A slight but statistically insignificant (P = 0.468) decrease of *tmoA* gene quantities was observed towards the upper fringe of the plume (~4 x 106 genes g^{-1}), connected to a much higher variability of counts there. Thus, while *tmoA* gene counts did not increase towards the upper fringe, a clear decrease was observed towards the lower fringe.

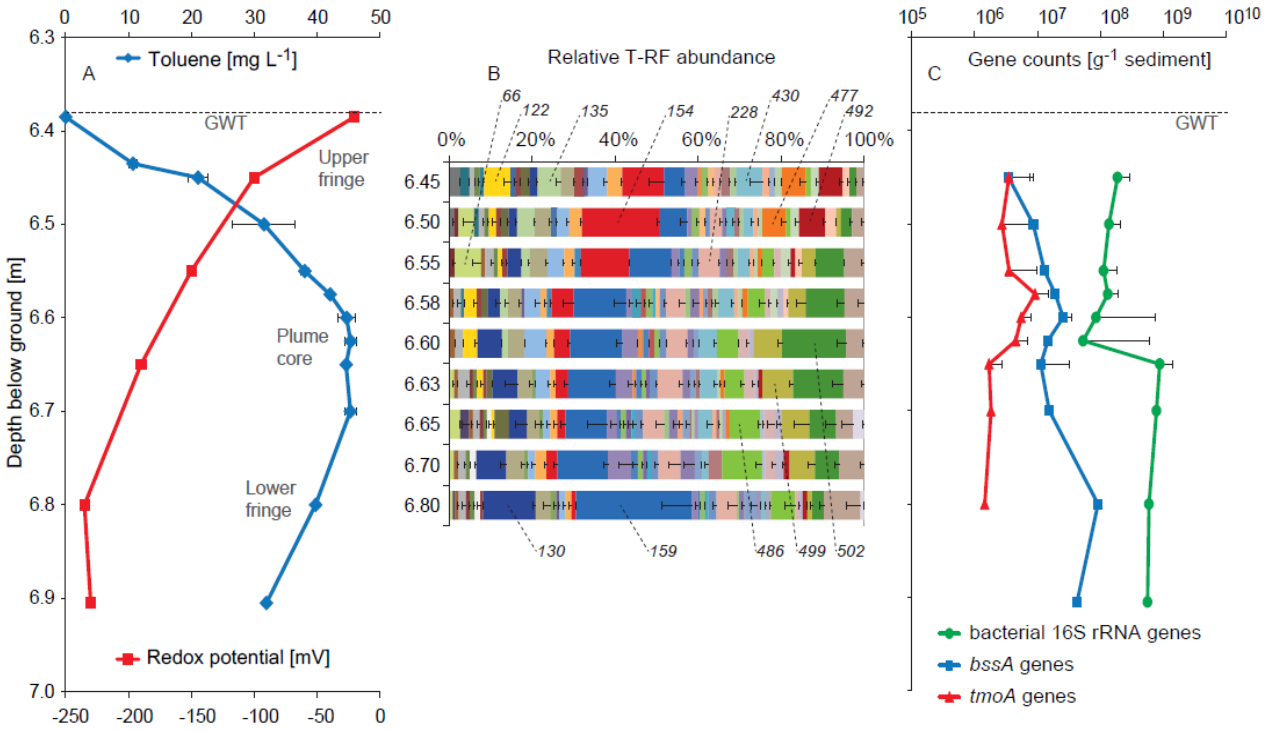

Figure 2.1. Depth profile of the tar oil contaminated Düsseldorf-Flingern site showing (A) toluene concentration, redox potential and plume zonation over depth (modified from (Anneser et al., 2010). Toluene concentrations and redox were measured by B. Anneser ans A. Bayer (HMGU, Microbial Ecology Group). (B) Averaged bacterial T-RF abundances from triplicated DNA extracts over the high-resolution depth transect. Selected T-RFs with pronounced abundance maxima in distinct plume compartments are highlighted. (C) Quantitative depth profile of bacterial 16S rRNA, *bssA* and *tmoA* genes as measured via qPCR. Error bars of standard deviations are shown for qPCR measurements from triplicate DNA extracts for each depth. GWT, groundwater table.

2.3.4. Search for *tmoA* transcripts

Since maximal *tmoA* gene abundances were detected in a plume zone not expected to harbour aerobic toluene degraders, attempts to detect actively transcribed respective mRNA were made. rRNA was indeed extractable from the sediments, as indicated by visual inspection of 16S rRNA RT-PCR amplicons from our extracts. However, *tmoA*-mRNA was undetectable with the used extraction/purification protocol and also diverse other extractions tested in comparison. Only unspecific PCR products and not the expected 505 bp *tmoA* gene-fragment were obtained with the used tmoA-f/r primer pair. Thus, an expression of *tmoA* genes was not detectable, suggesting either a lack of expression, or general problems in mRNA extraction from these contaminated subsurface sediments (we

also failed to exemplarily detect *dsrB* transcripts in our extracts, which should principally be expressed, since sulphate reduction is an important process at the site).

2.4. Discussion

Here, first spatially highly-resolved insights into distinct and partially unexpected quantitative distribution patterns of aerobic and anaerobic toluene degraders over the redox gradients and compartments of a real-world BTEX plume were provided. It was hypothesized that potential aerobic degrader lineages and degradation genes would be established typically at the upper plume fringe, where a constant replenishment of oxygen would warrant their sustained activity. However, this was only partially confirmed by our results. As hypothesized, putative degraders belonging to genus Pseudomonas were present mostly at the upper plume fringe, potentially connected to the sparse detectability of toluene dioxygenases (*tod*) in this compartment.

In contrast, bacterial lineages related to potential aerobic toluene or aromatics degraders within the *Burkholderiaceae* and *Comamonadaceae* were found mostly, or exclusively, in the reduced plume core. Within the *Burkholderiaceae*, especially the genera *Burkholderia* (e.g. *B. cepacia* (Shields et al., 1995); *B. sp.* JS150 (Johnson and Olsen, 1995)) and *Ralstonia* (*R. eutropha* (Ayoubi and Harker, 1998)), but also *Cupriavidus* (*C. necator* (Perez-Pantoja et al., 2008)), harbour known toluene degraders with described monooxygenases (Cavalca et al., 2004; Rhee et al., 2004; Piskonen et al., 2008). In Flingern, rRNA and *tmoA* sequence types related to *Ralstonia* -or *Burkholderia* spp.-reference sequences were found only in the plume core, albeit not abundant. In contrast, rRNA sequences related to potential aerobic aromatics degraders within the *Comamonadaceae* (related to *Acidovorax* spp., a known PAH degrader (Singleton et al., 2009) and *Comamonas testosteronii*, a phenol degrader (Teramoto et al., 1999)), were abundant in the plume core on both clone and T-RF level. The *Comamonadaceae* also are known to harbour aerobic toluene degraders within the genus *Methylibium* (Nakatsu et al., 2006).

Last but not least, rRNA gene sequences within the *Rhodocyclaceae* were also found abundant in the center of the plume at the Düsseldorf site. Although these clones were not clearly affiliated to a specific genus, this family includes many mostly denitrifying hydrocarbon degraders within the genera *Azoarcu*s and *Thauera*. Interestingly,

Thaurea sp. DNT-1 in this family has been described capable of both aerobic and anaerobic toluene degradation by carrying both *bss*- and *tod* catabolic genes (Shinoda et al., 2004). Also other close relatives are capable of both aerobic and anaerobic aromatics degradation, e.g. *Aromatoleum aromaticum* EbN1 (Trautwein et al., 2008), albeit not for toluene.

We are aware that such 16S rRNA marker gene studies allow only for a tentative detection of potential toluene or aromatics degraders based on a close affiliation with known isolates. Clearly, as even within well-known degrader lineages such as *Pseudomonas* or *Ralstonia* spp. there are many non-aromatics degrading strains and species, such data must be interpreted with caution. An impeccable inference of the respective function and actual activity of detected lineages on site is not possible based merely on rRNA data. This is why it was chosen to screen our sediments also for catabolic genes, which allows much more direct insights on in situ bacterial populations potentially involved in toluene oxidation. Since toluene is the dominating contaminant and electron donor in the Flingern plume (Anneser et al., 2010), it was considered at least plausible that respective degraders should be detectable via both catabolic and ribosomal markers. Ideally, phylogenetic congruencies between both approaches should allow for a placing of detected monooxygenases within on site total bacterial communities.

In Flingern, mostly unassigned *tmoA* genes related to clones from a BTEX contaminated refinery in the Czech Republic (Hendrickx et al., 2006a) and benzene-amended soil from Japan (Iwai et al., 2009) occurred in the highly contaminated plume core. This points towards a potentially widespread occurrence of an as-yet unidentified group of aerobic aromatics degraders in contaminated aquifers and similar environments. These *tmoA* sequences were placed within the wider phylogenetic radiation of marker genes from *Methylibium* spp. (*Comamonadaceae*), but also of *Pseudomonas* spp. Thus at least on the family-level, there was some congruency between detected abundant 16S rRNA and *tmoA* genes. On the other hand, the *tmoA* gene is not a very strong phylogenetic marker, and an intermixed phylogeny between different members of the *Beta*- and *Gammaproteobacteria* (Figure 2) suggests occasions of lateral gene transfer (Hendrickx et al., 2006b). Several genes connected to aerobic toluene degradation are located on mobile genetic elements (Sentchilo et al., 2000), therefore potential links have to be interpreted with due caution. To clearly identify these degrader populations and to link detected *tmoA* and 16S rRNA genes, dedicated labeling studies, i.e. via DNA-based stable isotope probing (SIP) are currently planned.

In contrast to the already documented highly correlated depth-distribution of anaerobic toluene degraders to anaerobic degradation processes (Winderl et al., 2008), a distinct stratification of toluene oxygenase genes was observed with respect to oxygen availability. As shown previously (Winderl et al., 2008; Anneser et al., 2010), highest *bssA* quantity correlated with maximal sulphide concentrations as well as overlapping toluene and sulphate gradients at the lower plume fringe. In clear contrast, monooxygenase (*tmoA*) genes were found (and most abundant) in plume zones considered as highly reduced, i.e. the actual plume core, and did not increase in abundance towards the upper fringe. This provides interesting insights on the depth stratification of on-site 'aerobic' toluene degraders.

It seems warranted that populations with different oxygen demands for toluene degradation develop and become selected for in different zones of a contaminant plume (Hendrickx et al., 2006b). Thus *tod*-dependent degraders (e.g. *Pseudomonas putida*) seem to localise close to the groundwater table, dependent on a higher oxygen availability. Under more limited oxygen availability, degraders with monooxygenases can be expected to be more readily detectable than those with dioxygenase genes (Leahy and Olsen, 1997). Additionally, also nitrate could sustain aerobic BTEX degradation under hypoxic conditions (very low concentrations of dissolved oxygen), i.e. in oxygen-requiring but nitrate-respiring biodegradation of BTEX (Wilson and Bouwer, 1997) as documented for *Ralstonia pickettii* PKO1 and *Burkholderia cepacia* G4 (Leahy and Olsen, 1997), *Acidovorax facilis* and *Pseudomonas veronii* (Nestler et al., 2007), or also for microcosm experiments (Leahy and Olsen, 1997; Nestler et al., 2007; Kim and Jaffe, 2008). Sequences affiliated to *Comamonadaceae* have been found abundant in an aromatic hydrocarbon (mainly BTEX) contaminated hypoxic groundwater from Hungary, both on functional (catechol-2,3-dioxygenase) and ribosomal (16S rRNA) level (Tancsics et al., 2010). At an oil refinery site in the Czech Republic, *Pseudomonas* spp. (esp. *P. veronii*) were always the dominant colonizers and putative key degraders in mesocosms incubated in different zones of an hypoxic, highly BTEX-contaminated aquifer. Typical anaerobic degraders (denitrifiers or sulphate reducers) did not become dominant, even if nitrate and sulphate were present in sufficient amounts (Hendrickx et al., 2005). Together with the spatial distribution of potential toluene degraders for the Flingern site reported here, this suggests that competitive niche partitioning between aerobic and anaerobic hydrocarbon degraders in the field may not yet be fully understood.

The catabolic gene detection assays utilized here were performed with published primer systems for *tmoA* (Hendrickx et al., 2005) and for *bssA* (Winderl et al., 2007). It cannot be excluded, that at least some potentially important catabolic gene lineages for toluene degradation at the site were missed. In fact, the *tmoA*-detection assay utilized for (q)PCR represents only one of several different possible pathways for aerobic toluene degradation (Jindrova et al., 2002). Thus, the presence of other aerobic catabolic potentials at the upper plume fringe and also in the plume core can be expected. Therefore, the development of further optimized primer sets or platforms (Vilchez-Vargas et al., 2012) for a more comprehensive detection and quantification of aerobic BTEX degraders in polluted groundwater is still an important task.

Nevertheless, this maximal localisation of presumably aerobic degraders inside the actual plume core allows developing some interesting working hypotheses to follow up on. Either, this localisation can be interpreted as a memory effect, from a previously lower localisation of the groundwater table and thus the upper plume fringe. Before the sediments were taken, the groundwater table at the site was relatively constant over three months (data not shown), while it was ~5 cm higher in the winter season previous to sampling. The time scales over which contaminant plumes as a microbial ecosystem are dynamic are still poorly understood at the moment, and clearly merit future scientific attention. On the other hand, it can be also speculated on potential aerobic toluene degradation activities under anaerobic conditions, via mechanisms similar to oxygenic, *pmoA* dependent methane oxidation under nitrite dismutation (Ettwig et al., 2010). Similarly, also *Dechloromonas aromatica* is able to oxidize benzene via oxygenase reactions in the absence of external oxygen while reducing chlorate or nitrate and generating catalytic amounts of oxygen for the enzymatic attack (Coates et al., 2001). In fact, nitrate required for potential oxygenic nitrite dismutation has been detected in concentrations of up to 2 mg L^{-1} in the core of the Flingern plume and below, while it was around detection limits (0.1 mg L^{-1}) at the upper fringe (Anneser et al., 2008).

At any rate, this scenario remains speculative, as long as no direct evidence for specific toluene oxidising activity is available. A detection of *tmoA* transcripts and a direct determination of respective in situ toluene oxidation rates would certainly be the next step. In fact, all attempts to detect *tmoA* mRNA (data not shown) were unsuccessful, suggesting either a lack of expression, or general problems in mRNA extraction from these subsurface sediments. Nevertheless, the simultaneous monitoring of catabolic genes for aerobic and anaerobic toluene degradation linked to fine-scale bacterial community distribution

reported here provides novel insights into the partitioning of distinct catabolic potentials across plume compartments, and may help to identify unexpected new hot spots of biodegradation in situ.

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3. Fast microbial community response and development of aerobic and anaerobic degrader populations upon toluene contamination of a pristine indoor model aquifer

Abstract

An indoor aquifer mesocosm system was used to assess, for the first time, the initial impact of toluene contamination on natural microbial communities of a pristine aquifer system, and degrader succession during the onset of natural attenuation processes. For reasons of legacy contaminations, these processes are mostly unobservable in the field. It is hypothesised that distinct levels of contamination should be directly reflected in the development and spatial succession of distinct aerobic and anaerobic degrader populations. After introduction of a moderate toluene contamination, sediment and groundwater of the indoor aquifer were analysed and compared in two dimensions and over time. A rapid development of abundant aerobic toluene degraders as shown by toluene oxygenase (*tmoA*) genes (up to 2.1 x 10^6 genes mL^{-1} groundwater and 1.5 x 10^6 genes g^{-1} sediment) was found only 16 days after contamination over the entire plume as active zone. Highly significant differences in the microbial communities between the sediment and the groundwater ($R = 0.73$, $p = 0.0001$) were revealed via T-RFLP (terminal fragment length polymorphism). Pyrotag sequencing revealed an immediate response towards the toluene plume via a selection of *Pseudomonas* spp. populations as putative primary aerobic degraders, with successional dominances also of *Zoogloea* spp., *Dechloromonas* spp., unclassified *Comamonadaceae*, but also *Bdellovibrio* spp., hinting even at a toluene-based intra-microbial food web evolving in the aquifer. Under secondary high toluene loading, a further clear change in overall microbiota and degraders was observed. Denitrifying hydrocarbon degraders related to *Azoarcus* spp. appeared in the highly-contaminated sediment, while degraders within the *Pseudomonadaceae* became more frequent towards the plume fringes. These findings provide unique insights into the reactivity of a pristine aquifer microbiota to contamination, and the spatial and temporal development of degrader populations adapted to local contaminant and redox scenarios.

1. Introduction

Microorganisms are important driving forces for the degradation of hydrocarbon contaminants in aquifers under different redox settings. However, the initial reactivity of subsurface ecosystems to contamination and the temporal dynamics of the development of contaminant-degrading microbial communities are still poorly understood. Normally, monitoring of natural attenuation in the field starts only a considerable time span after the actual contamination event, since contaminations are mostly historic. Therefore, insights on early microbiome adaptations and short-term successions are mostly still at lack. Yet, they can be hypothesised as crucial for understanding the driving forces of ecosystem adaptation to contamination, and for long-term predictions on ecosystem recovery.

As deliberate contamination of pristine aquifers in nature is not an acceptable experimental practice, experimentally designed aquifer systems can be used for such research purposes. There are indeed numerous studies working with different small-scale sediment columns or similar microcosm systems focussing on groundwater biodegradation (e.g. Amirbahman et al., 2003; Bauer et al., 2008; Burland and Edwards, 1999). Frequently, respective microbiological investigations have been conducted in rather small laboratory column systems while larger flume systems have been used for hydro-chemical experiments. However, results from both scales are not easily linked or extrapolated to the field scale. Here, a three-dimensional indoor model aquifer was used as a powerful tool to address these gap, a system very close to a natural subsurface ecosystem. This mesoscale indoor model aquifer was planned and developed in 2009 at the Institute of Groundwater Ecology at the Helmholtz Zentrum München (Huenninger, 2011). The 3-D model aquifer was intended to allow for contamination experiments under well-controlled hydrology, geochemistry, and defined mass fluxes/balances. The system simulated a natural aquifer consisting of natural sand-gravel sediment typically found in Southern Bavaria and was fed with natural oxic groundwater from the Helmholtz campus, at a realistic flow velocity of ~1 m d^{-1}. The geochemistry was dominated by high amounts of carbonate, calcium (in a range of 40 - 90 mg L^{-1}), magnesium (~20 mg L^{-1}), and chloride (~50 mg L^{-1}), with molecular oxygen being the dominant electron acceptor (initially 7.5 - 8.5 mg L^{-1} dissolved O_2), but also nitrate (7 - 9 mg L^{-1}) and sulphate (~10 mg L^{-1}) being naturally present. Also, low concentrations of DOC (< 0.1-0.5 mg L^{-1}) were characteristic for the groundwater. As contaminant, an artificial toluene plume was introduced to the system, which can be

considered a general model substance for aromatic hydrocarbon degradation by microbes (Piskonen et al., 2008).

There are many bacterial lineages and species known to degrade toluene and other aromatic hydrocarbons under aerobic and anaerobic conditions (Cavalca et al., 2004). In 'young' contaminant plumes, small scale gradients in hydrogeochemistry and also community distribution can be expected to co-evolve, as important characteristics of degradation hot spots, but also limitations of biodegradation (Anneser et al., 2008; Winderl et al., 2008). The simultaneous local availability of electron donors and acceptors as controlled by mixing is considered as a main limiting factors for contaminant decomposition in aquifers (Bauer et al., 2008). In aged contaminant plumes, the contribution of aerobic respiration to overall pollutant breakdown is currently considered to be negligible. If at all, aerobic degraders are considered to be restricted to the upper contaminated zones directly underneath the capillary fringe. Even there, most of the oxygen replenished via the vadose zone is immediately consumed by the abiotic and biotic re-oxidation of e.g. sulphide and ferrous iron, which are reduction products of anaerobic pollutant degradation deeper within the plume.

However, in 'young' (or fresh) contaminant plumes, a simultaneous (or overlapping) occurrence of aerobic and anaerobic degradation activities can be expected (Massol-Deya et al., 1997). In fact, this could even be considered a primary adaptation mechanism of aquifer microbes to counteract contaminant impacts with maximum efficiency. Unfortunately, the early development of degrader communities as well as the distinct spatial and temporal succession of catabolic potentials upon contamination has never been observed to date. Respective insight are central to understand the impacts of contamination and the dynamics of natural attenuation processes in natural groundwater systems. Therefore, the aim of this work was to follow early hydrocarbon degrader community development upon toluene contamination of a pristine aquifer model system. The main focus of this project was on the emergence and of key bacterial toluene degraders and their spatial and temporal succession. It is hypothesised that distinct levels of contamination should be reflected in the temporal and spatial succession of distinct aerobic and anaerobic degrader populations. Potential distinctions in degrader development and general microbial community impacts visible in the aquifer matrix (sediment) vs. the groundwater were also addressed. Finally, the overall consistency between parallel biogeochemical and microbiological lines of evidence on active processes and was to be tested.

A multidisciplinary approach was chosen to integrate multiple parameters (molecular- and microbiological, hydrogeological, chemical, and stable isotope data) on the contamination event and the onset of biodegradation in the aquifer mesocosm. The team working on the indoor model aquifer consisted of the PhD students funded by the GOODWATER ITN, all with distinct own project objectives and aims, as well as most of the technical assistants of the IGÖ helping in system maintenance, routine measurements, and technical support. The distinct objectives included the use of compound-specific stable isotope analysis (CSIA) for process monitoring (S. Qiu, Environmental Isotope Chemistry Group); the reaction and recovery of general aquifer microbial parameters upon and after contamination (A. Herzyk (Microbial Ecology Research Group); as well as the physiology of degraders *in situ* (S. Marozava, Anaerobic Degradation Group). The general aim of the PhD project presented here was to unravel how and how fast an oligotrophic microbial community from an uncontaminated aquifer adapts to toluene contamination, as well as how the temporal and spatial succession of processes and populations are correlated throughout the developing plume. For this, the development and succession of aerobic and anaerobic degrader lineages and the quantitative distribution of aerobic vs. anaerobic degradation genes (degradation capacities) were traced.

3.2. Material and Methods

3.2.1. Model aquifer description, sampling, and physico-chemical measurements

The mesoscale indoor model aquifer measured 5.0 x 0.8 x 0.7 m (length x width x depth). It was filled with natural sand and fine gravel sediment from a South Bavarian gravel pit (0.1 - 5 mm grain size) and saturated with natural groundwater from the campus site in Neuherberg (Germany) at a flow of ~1 m d^{-1}. The indoor model aquifer was constructed with capillaries for sampling groundwater in 3D, with a depth resolution of up to 4 cm, over six vertical transects located at 0.35, 1.10, 2.20, 3.30, and 4.40 m flow distance (named as transect A, C, D, E, F, respectively). At the beginning of the mesocosm (at 0.2 m length, 0.27 m depth) a horizontal well for contaminant injection was integrated. A high-conductivity lens of quarz-sand was situated immediately downstream of the injection well, to allow a mixing of the incoming groundwater with injected toluene, but also for a uniform distribution of the contaminant plume across the flume width. Additionally, each sampling transect contained fluorescence sensors for online

measurement of dissolved oxygen (FIBOX3, PreSens GmbH, Regensburg, Germany). The temperature was monitored online via temperature sensors and showed an increase from the inlet to the outflow of ~12 to ~19°C, with notable seasonal fluctuations. Initially, uncontaminated groundwater was allowed to flow through the mesocosm for almost one year, in order to stabilize the system and allow for the development of an undisturbed natural background community of intrinsic microbes.

For the contamination with toluene, the spreading and expected concentrations of toluene were pre-modelled by Marko Hünniger (Huenninger, 2011). An experiment to assess the steady-state spatial distribution of bromide as a conservative tracer over vertical profiles and depth transects was conducted. For practical calculation, the migration of toluene was assumed to be conservative, controlled only by advection and dispersion processes. For the actual contamination experiment, water fully saturated with toluene (500 ± 30 mg L^{-1}) and amended with 250 mg L^{-1} bromide (as tracer) was injected into the aquifer over a period of 133 days with a volumetric flow rate of 0.78 L day^{-1}. This corresponds to a contaminant mass of 390 ± 23.4 mg day^{-1}. In a second phase of the project, following the moderate continuous injection, a massive 6h pulse of 200 mL pure toluene was injected into the aquifer. Toluene was injected into the horizontally placed and perforated mixing tube near to the inflow of the system via 16 stainless steel capillaries, allowing distribution of the contaminant across the entire width of the model aquifer to generate a uniform contaminant plume. As predicted from the preceding tracer experiment, vertical spreading of toluene was restricted to a narrow zone (approx. 6 cm wide) at transect A (0.35 m flow). After passing the high-conductivity lens, the plume had a vertical thickness of approximately 10 cm and 20 cm at transect C and F, respectively.

Toluene injection started on November 22, 2010 (day 0), aimed at relatively low concentrations of maximally ~20 mg L^{-1} in the resulting plume. Two subsequent sampling campaigns were conducted: the 1st sampling of groundwater and sediment took place after 16 days of toluene injection, and the 2nd after 63 days. Then, on April 3th/4th, 2011 (day 129/130), 200 ml pure toluene resulting in a plume concentration of >100 mg L^{-1} was injected over ~6 h. The next sampling was performed on April 11th, 2011, 137 days after the first introduction of toluene contamination. For a more detailed description of the indoor model aquifer-setup, functioning, and experimental strategies, see figures 1.4. and 3.1 this thesis, as well as Huenninger (2011) and Herzyk (2013).

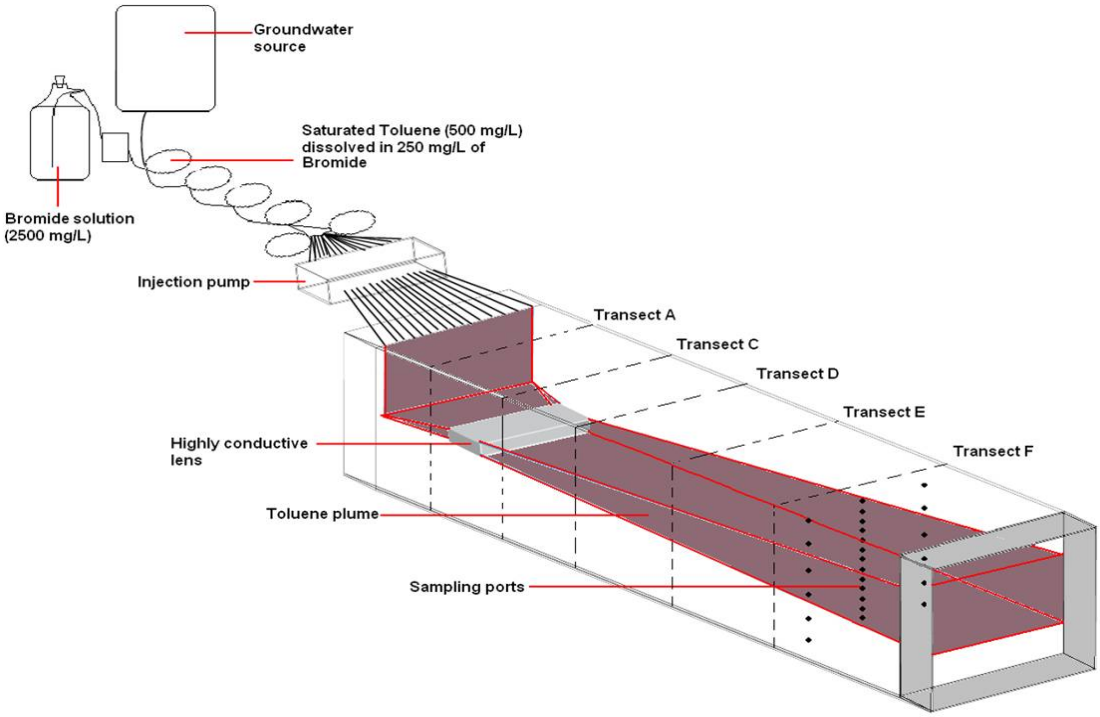

Figure 3.1. Schematic setup of the indoor model aquifer used in this study. Shown are the toluene injection devices, the location of the highly conductive quarz-sand lens, and the different transects for groundwater sampling (mentioned explanatory only for one transect). For more details on the experimental setup see also figure 1.4. and Huenninger (2011).

Sampling campaigns were planned and carried out by the PhD candidate together with A. Herzyk, S. Marozava, S. Qiu, M. Granitsiotis (the GOODWATER PhD students), and also practical help of the technical assistants K. Hörmann, G. Barthel, S. Kaschuba, M. Höche, and G. Hinreiner (all from IGÖ). Groundwater was sampled in autoclaved 1L Schott-bottles cooled on ice, with a pumping rate of ~0.5 mL min^{-1}. In order not to alter the hydrology of the system, pumping was done simultaneously for all sampled depth-ports of one transect. Sampled groundwater was filtered as soon as possible (within 8-14 hours after conclusion of sampling) via sterile 0.22 µm pore size filters (Durapore, Millipore, Bedford, MA). Only half of the filter (corresponding to 500 mL groundwater) was used for DNA extraction and further analyses. Sediment samples were taken by using a hand-operated 'Multisampler' for sediment coring (Eijkelkamp Agrisearch Equipment BV, Griesbeek, Netherlands). Obtained sediment cores were divided into intervals of 5-10 cm depending on the desired depth-resolution. The remaining holes in the aquifer matrix collapsed only partially, and were re-filled with fresh uncontaminated sediment from a

smaller model aquifer system consisting of the same sediment and flooded also with the same groundwater. Anions and cations (including nitrate and nitrite) in groundwater were prepared for quantifications and measured via ion chromatography (Dionex DX500, Thermo Fisher Scientific Inc., Waltham, MA) as previously described (Anneser et al., 2008) by the PhD candidate, partly with help from Michael Stöckl and Peter Knappet. Toluene concentrations were measured on a Trace DSQ GC-MS (Thermo Electro, Germany) as described previously (Anneser et al., 2008) by Shiran Qiu (Environmental Isotope Chemistry group, IGOE).

3.2.2. DNA extraction, PCR, and microbial community fingerprinting

Nucleic acid extractions were shared between the PhD candidate and A. Herzyk, with occasional support from IGÖ technicians. Microbial community fingerprinting and gene quantification were done by the PhD candidate. Total DNA was extracted using ~1 g of sediment (wet weight) in three parallel extracts for every depth, or from half a filter from1 L of filtered groundwater (corresponding to 0.5 L), following the procedure described by Winderl et al. (2008). For sediment, DNA was extracted in replicates. For groundwater biological replication was unfortunately not possible, due to the large amount of water needed for DNA extraction (0.5 L). Considering the water flow of approximately 8-9 L h^{-1} through the whole aquifer, the amount sampled via each port was high and required ~24 h sampling effort in order not to alter the overall hydrology. Therefore, only technical replication was done for water analyses. All PCRs were performed in 50 µl-reactions containing nuclease-free H_2O, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP's, 1 U *Taq* polymerase (all Fermentas GmbH, St. Leon-Rot, Germany), 10 µg BSA (Roche Diagnostics GmbH, Basel, Switzerland), 0.5 µM of each primer (biomers.net GmbH, Ulm, Germany), and 1-2 µl of template DNA. 16S rRNA gene-PCR was performed under the following thermal cycling conditions: 3 min of initial denaturation at 94°C, 26-28 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 55°C, and 60 s extension at 72°C), and 5 min of final extension at 72°C. The amplification of toluene monooxygenase (*tmoA*) genes followed the same thermocycling program but with higher annealing temperature of 60°C and 30 cycles (Larentis et al., 2013). T-RFLP (Terminal-Restriction Fragment Length Polymorphism) analysis of bacterial 16S rRNA genes was performed with PCR-amplicons of primers Ba27f-FAM (5'-6-carboxyfluorescein labelled 5'-AGA GTT TGA TCM TGG CTC AG-3') and 907r (5'-CCG TCA ATT CMT TTR AGT TT-3') by *Msp*I digestion, as previously described (Pilloni et al., 2011).

3.2.3. qPCR

All quantitative PCR (qPCR) measurements were performed on a MX3000P qPCR cycler (Stratagene, La Jolla, CA). For bacterial 16S rRNA gene quantification, standard PCR assays as described (Winderl et al., 2008) were used in the presence of 0.1x SYBR Green (FMC BioProducts, Rockland, MA). qPCR of benzylsuccinate synthase (*bssA)* genes was performed with the TaqMan system previously developed by Winderl et al. (Winderl et al., 2008), with the Universal PCR Master Mix 'No AmpErase UNG kit' (Applied Biosystems, Carlsbad, CA). This TaqMan assay was used because sulphate was present in the aquifer and a development of sulphate reducing toluene degraders could potentially be expected. The Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA) was applied for toluene-para-monooxygenase (*tmoA)*-qPCR, using the manufacturer's protocol with minor modifications. Primers tmoA-f and tmoA-r were used with two-step thermal cycling (45 cycles at 95°C for 20 s and 60°C for 20 s). qPCR standards were generated with a PicoGreen-quantified partial *tmoA* gene amplicon (clone DV7tmo13, GenBank accession no. JX307448) retrieved from the Düsseldorf-Flingern aquifer sediment. A qPCR-correction factor to overcome potential comparative gene extraction, amplification, and/or detection biases in the workflow was applied as also in our previous study (chapter 1 in this thesis, Larentis et al., 2013).

3.2.4. Pyrotag-sequencing

For bacterial 16S rRNA gene amplicon pyrosequencing, PCRs were performed with primers Ba27f and Ba519r (5'-TAT TAC CGC GGC KGC TG-3') with respective multiplex identifiers (MID barcodes) and conditions described previously (Pilloni et al., 2011; Pilloni et al., 2012; and as for T-RF - PCR described in chapter 2 of this thesis). The amplicons were then checked for integrity on standard agarose gel and purified by Agencourt AMPure XP - PCR Purification Kit (BeckmanCoulter, Brea, USA) following the manufacturers' manual. After PicoGreen dsDNA quantitation (Invitrogen, Carlsbad, USA) amplicons were diluted to $1x10^9$ molecules μl^{-1} and pooled for the generation pyrosequencing libraries. The libraries were again diluted to $1x\ 10^7$ molecules μl^{-1} prior to emulsion PCR (emPCR) and 454 sequencing. The latter were done by K. Hörmann (IGÖ technician) as well as M. Engel and B. Hai of the HMGU core facility for 454 sequencing on a GS FLX system with Titanium chemistry (Roche, Basel, Switzerland) following supplier protocols.

3.2.4. Data analysis

T-RFLP (terminal restriction fragment-length polymorphism) electropherograms were analysed using GeneMapper 4.0 software (Applied Biosystems, Carlsbad, CA) and then evaluated with the T-REX online-software (Culman et al., 2009). Variations in bacterial community structure, based on T-RFLP analyses, were evaluated by principal component analysis (PCA) based on Bray-Curtis dissimilarities with ANOSIM (analysis of similarities) procedure (Clarke, 1993).

Pyrosequencing reads were first quality-trimmed through the TRIM function of GREENGENES (DeSantis et al., 2006) with the standard settings of good-quality score 20, window size 40 bp, and window threshold 90%. Then, sequence-reads were length-filtered (sequences <250 bp were removed), forward and reverse reads were separated with BIOEDIT (Hall, 1999), and classified using the RDP classifier (Wang et al., 2007) at a default confidence threshold of 80%. In addition, reads were assembled into contigs for dominating amplicons (assembly threshold of 98% sequence similarity in 50 bp-window) with SEQMAN II (DNAStar, Madison, USA). These contigs were then classified using the RDP classifier (Wang et al., 2007) at a default confidence threshold of 80%.

3.3. Results

3.3.1. Hydrogeochemistry of the indoor aquifer

The 3-D model aquifer was intended to allow for contamination experiments under well-controlled hydrology, geochemistry, and mass fluxes and balances (Huenninger, 2011). The system simulated a natural aquifer consisting of natural sand-gravel sediment infiltrated with natural oxic groundwater at a flow velocity of ~1 m d^{-1}. Daily hydro-chemical monitoring was performed by G. Hinreiner (IGÖ technician) and the PhD candidate (in turns), oxygen measurements by the PhD candidate or S. Kaschuba (IGÖ technician). Geochemical measurements including nitrate and nitrite quantification were done by the PhD candidate, partly with help from M. Stoeckl (IGÖ technician) and P. Knappet (GOODWATER PostDoc). Toluene in groundwater samples was measured by S. Qiu (GOODWATER PhD student).

The redox of the indoor aquifer was clearly oxic before contamination, with dissolved O_2 between 7.0 and 9.4 mg L^{-1} over the whole flow length and over all depth transects. Also nitrate was constant, in all dimensions, between 5 and 6 mg L^{-1} (Fig. 3.2), as well as sulphate (~7.5 mg L^{-1}). 16 days after the beginning of initial toluene injection (moderate concentrations of maximally ~25 mg L^{-1} in the plume), toluene was actually only detected in one port of the foremost depth of transect A (~2 mg L^{-1} at 27 cm of depth). This indicated that in comparison to a hypothetical conservative contaminant spreading, almost 95% of the pollutant was already degraded along this short flow path. This interpretation is based on toluene spreading predicted by the conservative transport model, generated by S. Qiu and P. Knappett (GOODWATER project colleagues), building on the original tracer-modelling by M. Huenninger (Huenninger, 2011). According to the model, the added contaminant mass was originally dimensioned to produce a plume of maximally 25 mg L^{-1}.

As a result, oxygen and also nitrate were locally depleted in the corresponding depths of transects A, and oxygen decreased even more in transect C (down to 3.7 mg L^{-1} at 0.23 m depth). Nitrate was not significantly depleted in any depth of the latter transects. It must be stated that nitrate concentrations were elevated to 8 – 10 mg L^{-1} on day 16, possibly reflecting natural fluctuations in the pumped groundwater.

After 63 days of constant (low) contaminant injection, toluene was detectable over the whole length of the aquifer at decreasing maximal concentrations of ~20, ~1.4, and ~0.2 mg L^{-1} at transects A, C, and F, respectively. The corresponding overall degradation was ~30, ~96, and ~99 % in successive transects. A resulting strong depletion of oxygen was observable at A, C, and F, while nitrate depletion was only apparent in transects C and F (Fig. 3.2). Here, nitrite actually became detectable in the centre of the toluene plume, albeit only at minor concentrations of maximally 0.3 mg L^{-1}.

After the subsequent short-term injection of high toluene loads, dimensioned to result in a conservative plume concentration of >100 mg L^{-1}, toluene concentrations maxed at ~92 mg L^{-1} in transect A, and were still maximally ~22 mg L^{-1} in transect F. This corresponded to a total attenuation of only ~20% over the flume length, connected to a noticeable upward shift and vertical spreading of the plume with distance. After 137 days, oxygen concentrations decreased towards zero in the centre of the plume over the entire flume length, i.e. the aquifer turned anoxic in the highly contaminated plume core (Fig. 3.2). Also nitrate concentrations were clearly depleted (below 1 mg L^{-1}) after this event, and nitrite was measurable in amounts of up to almost 1 mg L^{-1}.

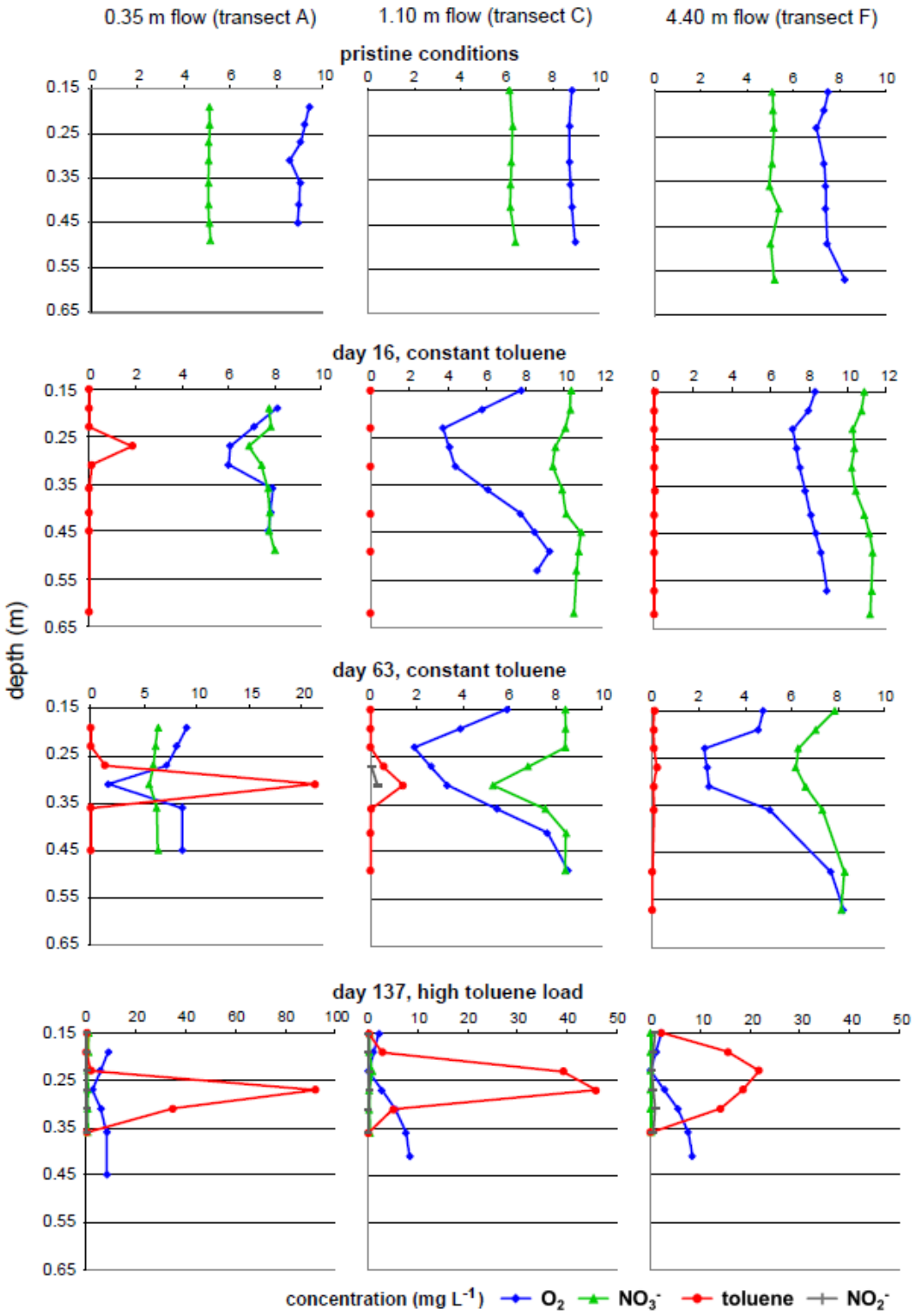

Figure 3.2. Depth transects of dissolved toluene and electron acceptors along the flow of the indoor model aquifer. Distinct time points and contaminant loads are resolved. Oxygen was measured by the PhD candidate or S. Kaschuba (IGÖ technician), nitrite and nitrite by the PhD candidate and partly by M. Stoeckl (IGÖ technician) and P. Knappet (GOODWATER PostDoc). Toluene in groundwater samples was quantified by S. Qiu (GOODWATER PhD student). Shown are means of duplicate measurements (technical replicates), which showed only minimal variation (SD not shown).

In sum, toluene contamination resulted in a surprisingly rapid initiation of biodegradation coupled to oxygen consumption. Also a slight decrease in nitrate concentrations could be measured already under oxic conditions, at moderate toluene loading. Under high toluene impact, the aquifer readily turned anoxic over large parts, and most of the nitrate was consumed by denitrification (traces of nitrite could be measured) in the plume centre.

3.3.2. Marker gene quantification with changing contaminant/redox conditions

Next, depth-resolved water and sediment samples were quantified for marker genes over time and flume transects. Total bacterial rRNA gene counts were put into comparison with aerobic (*tmoA*) and anaerobic (*bssA*) degradation marker genes. Hence, the relative contribution of the respective catabolic potentials to the reduction of contaminant mass, successions and shifts, as well as biodegradation hot spots were identified. Additionally, information from sediment vs. groundwater samples was differentiated at a high spatial and temporal resolution, to provide important molecular information on the development of the plume and redox-zonations. This molecular work was the major contribution of the PhD candidate to the joint GOODWATER indoor aquifer experiment. It aimed to provide molecular evidence for degrader community development and on-going key degradation processes.

In groundwater of the uncontaminated aquifer, qPCR of bacterial 16S rRNA genes revealed a low bacterial abundance (10^4 - 10^5 genes mL^{-1}). 16 days after the beginning of moderate toluene injection, 16S rRNA gene abundance clearly increased in zones affected by the toluene plume. Even more pronounced, the same was observable for toluene monooxygenase (*tmoA*) abundances (Fig. 3.3). In fact, *tmoA* was initially not detected in uncontaminated groundwater, but appeared readily upon contamination and increased drastically to up to 2 x 10^6 gene copies mL^{-1} in the plume core. Intriguingly, *tmoA* abundance was almost as high as total 16S rRNA genes (~3 x 10^6 16S genes mL^{-1}) in these samples. It must be noted here however, that unlike as for sediments, no qPCR extraction-and-detection correction factors were applied for groundwater DNA. This was because initially, PCR bias and inhibition was assumed to be much less affected by humic acids and similar PCR inhibitors known to be present in the sediment, but not in groundwater,. Generally, both 16S rRNA and *tmoA* gene quantities in groundwater clearly reacted to toluene contamination with increasing abundances (Fig. 3.3). After 63 days of toluene

exposure, maximal 16S rRNA and *tmoA* gene numbers were 2.1 x 10^6 and 8.3 x 10^5 mL^{-1}, respectively, both at 0.35 m flow distance (transect A) in 0.31 m depth.

In groundwater of all transects and all time points (except the initial uncontaminated situation), anaerobic toluene degradation genes were indeed detectable in depths with maximal toluene concentrations, albeit only in negligible amounts, just above the detection limit. Also, a notable increase after high-contamination was not observed. However, as the utilised *bssA* assay was not specific for denitrifying degraders, but designed for sulphate reducing *Deltaproteobacteria*, these result do not exclude an even higher abundance of denitrifying toluene-degrading bacteria that was expected to develop in the respective plume zones. In contrast, *tmoA* gene abundances showed generally elevated quantities after high contamination, but decreased with depth. The highest abundance (1.7 x 10^6 *tmoA* genes mL^{-1}) was detected in 0.19 m depth of transect F.

In DNA extracted from sediments, 16S rRNA- and *tmoA* gene quantities showed a similar pattern as for groundwater, reacting to the toluene plume with an increase in abundance. For sediment qPCR, the comparative extraction-and-detection correction factors applied also for Düsseldorf-Flingern sediments were used (Larentis et al., 2013). Both systems had a very similar sediment composition. Surprisingly, *tmoA* was not detected in transect A after 16 days of toluene contamination, while it was clearly detectable in the corresponding groundwater (Figs. 3.3 and 3.4). In contrast, high amounts of *tmoA* genes (10^4 to 10^6 genes g^{-1}) were detectable in sediments of transects C and F. A maximum of 1.5 x 10^6 *tmoA* genes g^{-1} sediment was reached at 0.33 m depth at transect C, This sediment sample was situated just underneath the actual plume core and corresponded to the zone of maximal oxygen depletion at this time point (Fig. 3.2). 63 days after the start of toluene injection, *tmoA*-detectability almost perfectly matched the localisation of the actual plume, and widened with flow length. In one specific depth (0.28 m) of transect C, deltaproteobacterial *bssA* was also detectable at a relatively high abundance (1.2 x 10^3 g^{-1}). However, after the introduction of the high toluene load (day 137) and the depletion of oxygen in the actual plume, anaerobic catabolic potentials for sulphate reducing bacteria were more apparent in transects C and F. Simultaneously, *tmoA* gene detectability shifted more upwards, towards the upper plume fringe.

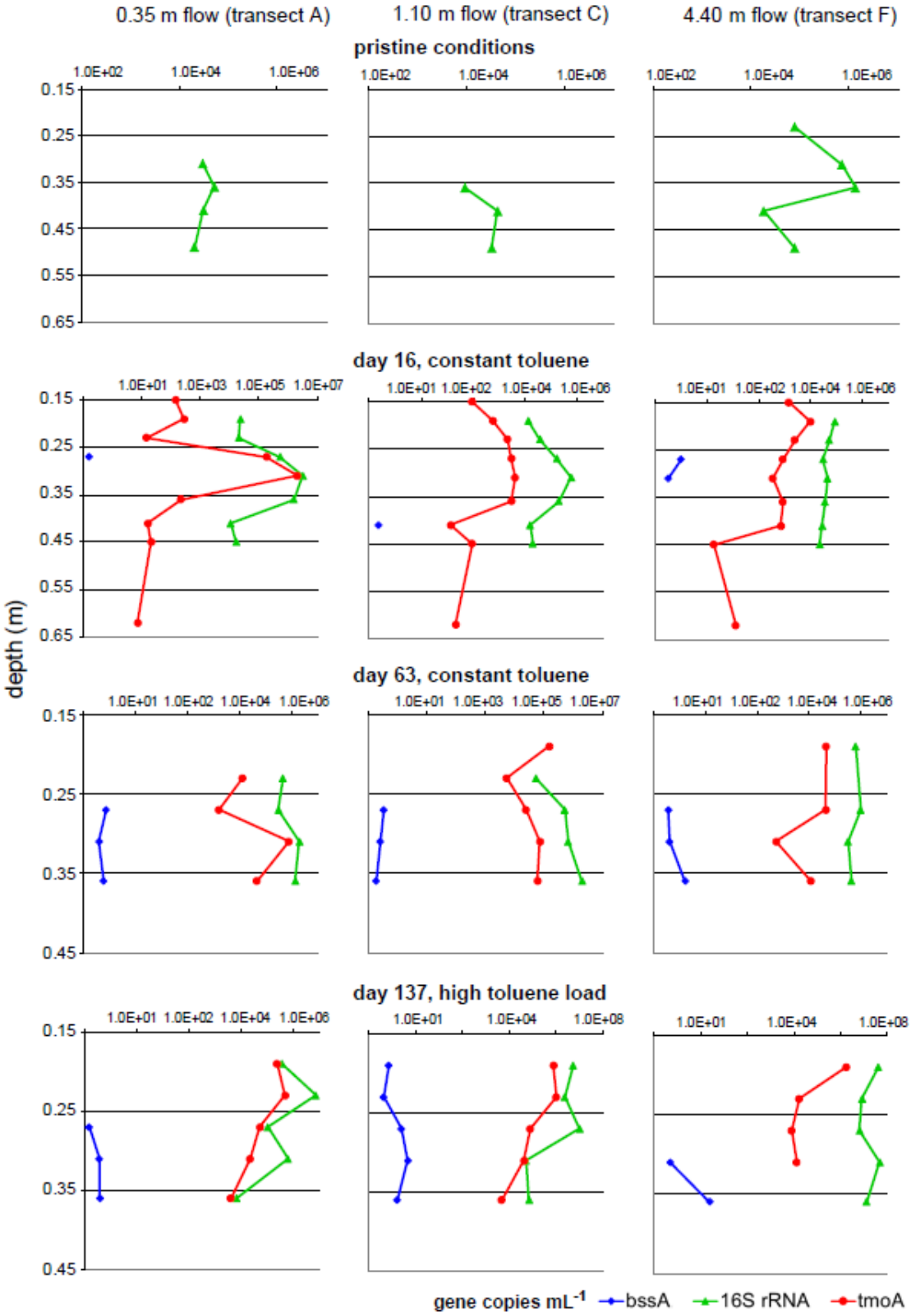

Figure 3.3. Depth transects of bacterial 16S rRNA, *tmoA*-, and *bssA*-gene quantification in groundwater along the flow path of the indoor aquifer. Different time points and toluene loadings are resolved. Shown are means of triplicate measurements (technical replicates), which showed only minimal variation (SDs not shown).

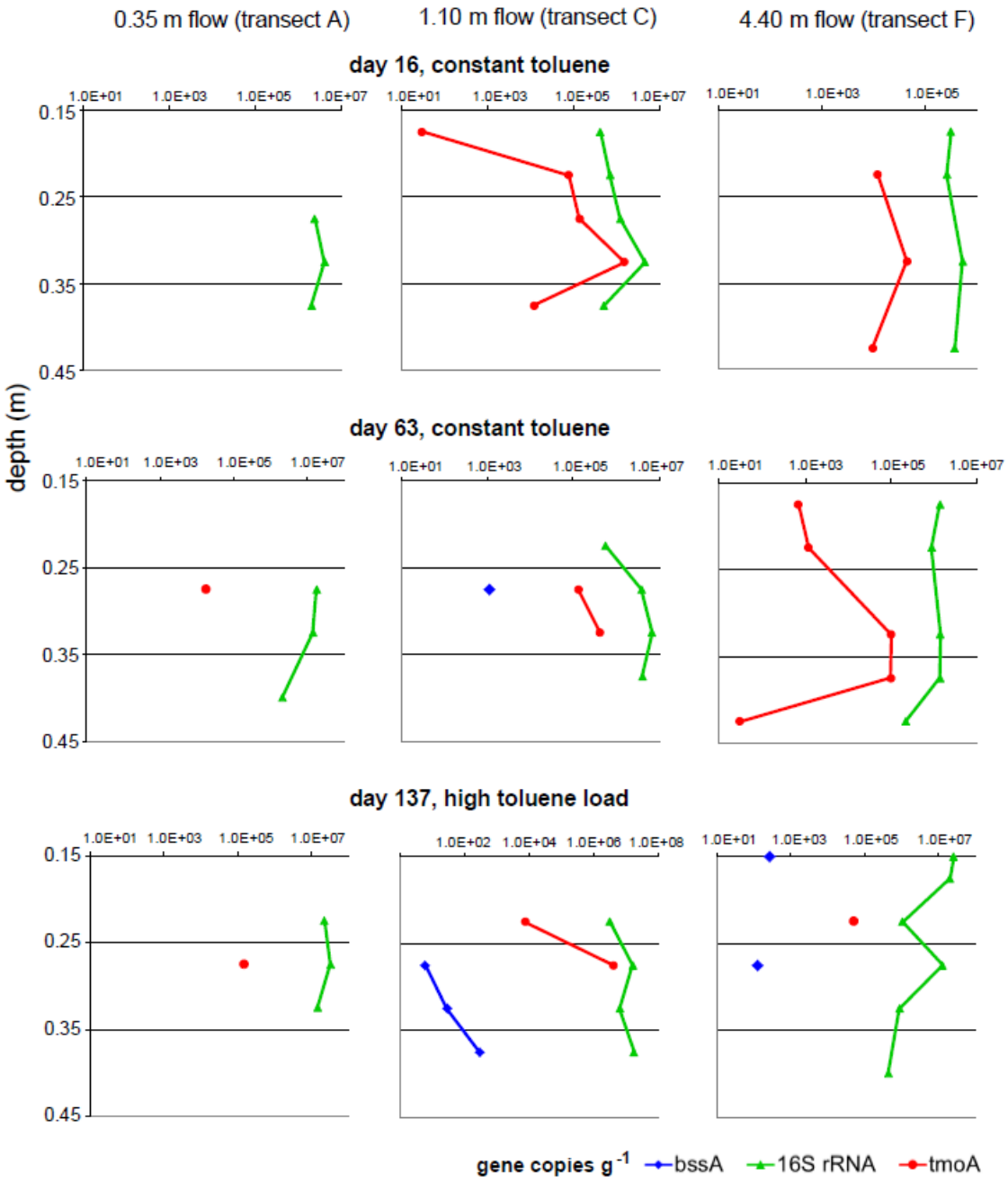

Figure 3.4. Depth transects of bacterial 16S rRNA, *tmoA*-, and *bssA*-gene quantification in sediments along the flow path of the indoor aquifer. Different time points and toluene loadings are resolved. Shown are means of triplicate measurements (biological replicates), which showed only minimal variation (SDs not shown).

In summary, aerobic degradation genes evolved immediately after toluene contamination and showed high abundance in and around the plume, also under massive secondary contaminant loading. Total bacterial rRNA gene (≈ cell) counts also increased with the toluene contamination, while only small amounts of anaerobic degradation genes of sulphate-reducing degraders were measurable.

3.3.3. Bacterial community response to contamination and development of degrader populations

As the next step, total bacterial community composition was studied in detail, with the special objective of following putative degrader populations over the experiment, and to differentiate between sediment and water phase. At rather small scales, sediment populations can be hypothesised to evolve differently from communities in water, and to be functionally redundant to sediment microbiota but active in different niches. This work was done in cooperation with A. Herzyk (GOODWATER PhD student), who aimed to understand the reaction and recovery of general aquifer microbial parameters (such as community diversity) upon and after contamination. The main objective of the PhD candidate was here to track the development and succession of local aerobic and anaerobic degrader lineages, in coupling to the quantitative distributions of degradation capacities.

Microbial community composition analysed via T-RFLP fingerprinting revealed highly significant differences ($R = 0.73$ and $p = 0.0001$) between bacterial populations in groundwater and sediment of the indoor model aquifer (Fig. 3.5). Moreover, community distinctions were apparent with flow distance, as well as over depth and especially in contaminated vs. uncontaminated (or less impacted) aquifer zones. More details on this data, especially for sediments, as well as an extensive statistical evaluation of fingerprinting T-RFs correlating to different toluene concentrations and time points of sampling is documented in the partnering thesis (Herzyk, 2013). In this thesis at hand, only a very general overall overview of community variability between water and sediment samples is to be provided, which was a specific additional objective and own contribution to the collaborative project.

For much more extensive community details, 454-pyrotaq sequencing was applied. The simultaneous sequencing of very high numbers of gene fragments allowed to identify members of flume populations at high saturation. Pyrosequencing revealed, on phylum level, a dominance of *Actinobacteria, Firmicutes, Acidobacteria,* and *Alphaproteobacteria* in the sediments of all transects (Fig. 3.6A). However, especially the uncontaminated groundwater showed high abundances of unclassified reads, much higher than in other recent respective work of our group (Pilloni et al., 2011; Pilloni et al., 2012).

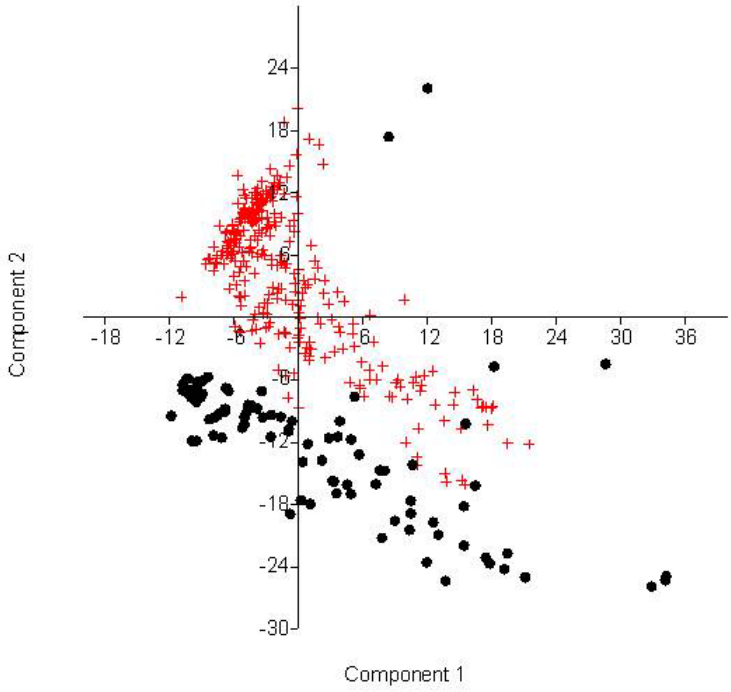

Figure 3.5. Principal component (ANOSIM) ordination of the overall similarity of T-RFLP fingerprinting data sets of bacterial communities in groundwater (black circles) versus sediments (red) of the indoor aquifer. Different flow distances and depth transects were included also with different toluene contaminant concentrations.

After toluene contamination, both the attached as well as the suspended bacterial communities shifted towards a dominance of reads affiliated to the *Gamma-, Delta-*, and/or *Betaproteobacteria*, as well as to the *Bacteroidetes* (Fig. 3.6A and 3.6B). At more detailed taxonomic resolution (Fig. 3.7), the uncontaminated and early toluene-impacted sediments harboured more reads affiliated to the *Acidobacteria*, *Actinomycetales, Burkholderiales* (esp. *Oxalobacteraceae),* as well as the genera *Massilia* and *Herbaspirillum*. In sediment depths corresponding to the actual toluene plume, reads of *Pseudomanas* spp. became a dominant population on days 16 and 63, together with *Zoogloea* and *Bdellovibrio* apparently enriched especially in transect A. Also in the groundwater, *Pseudomonas*-related pyrotags appeared to represent a dominating degrader population under low toluene loads, together with reads related to *Dechloromonas,* unclassified *Comamonadaceae,* and *Rhodocyclaceae* (Fig. 3.7B). This list of the development of putative toluene degrader lineages in the flume is based on known isolates and findings of other researches. Nevertheless, many known toluene degraders are available in these lineages, and many other close relatives are known to degrade other BTEX comounds.

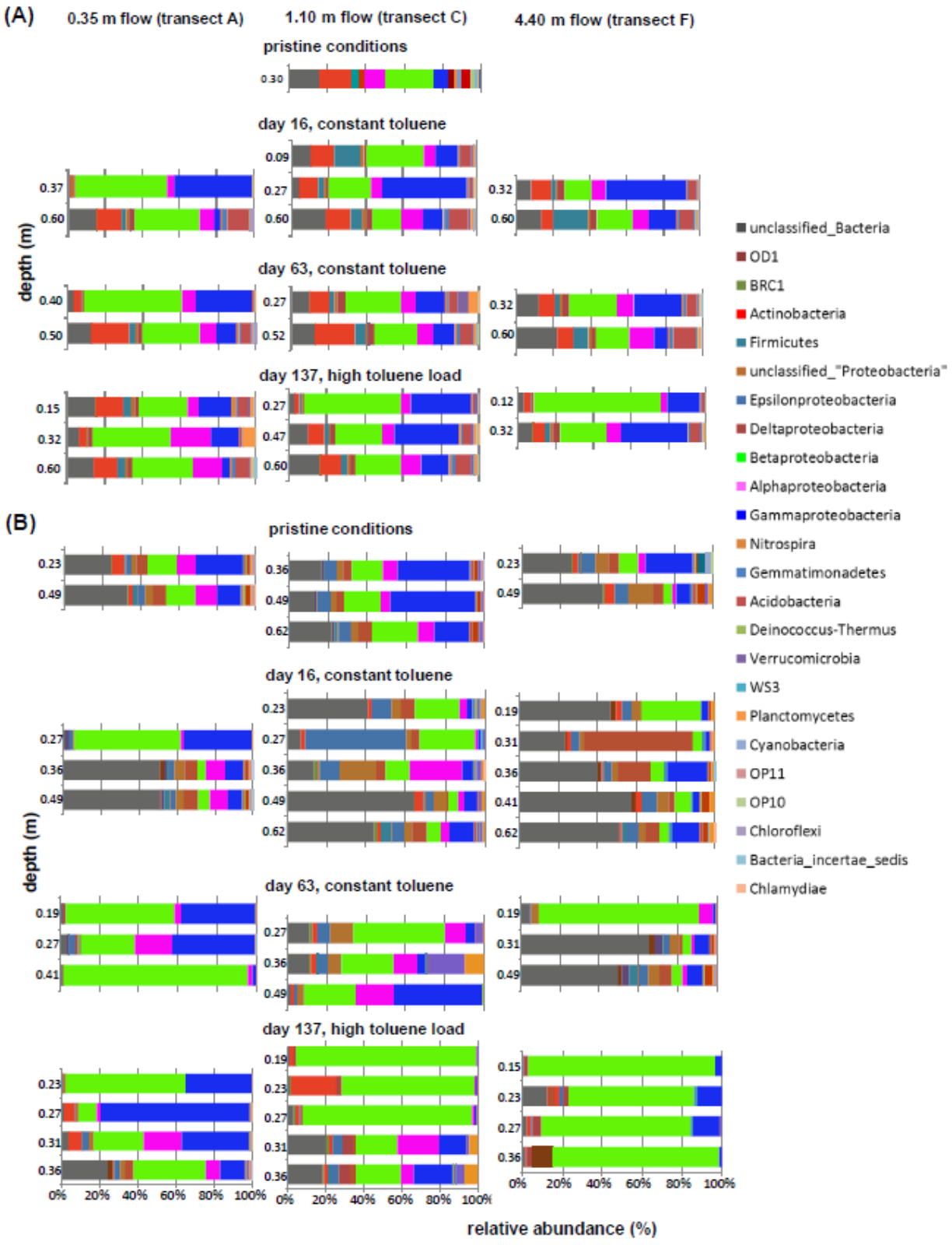

Figure 3.6. Depth transects of phylum- and class-level bacterial community composition along the flow path of the indoor aquifer. Different time points, toluene loadings as well as sediment (A) vs. groundwater (B) samples are resolved.

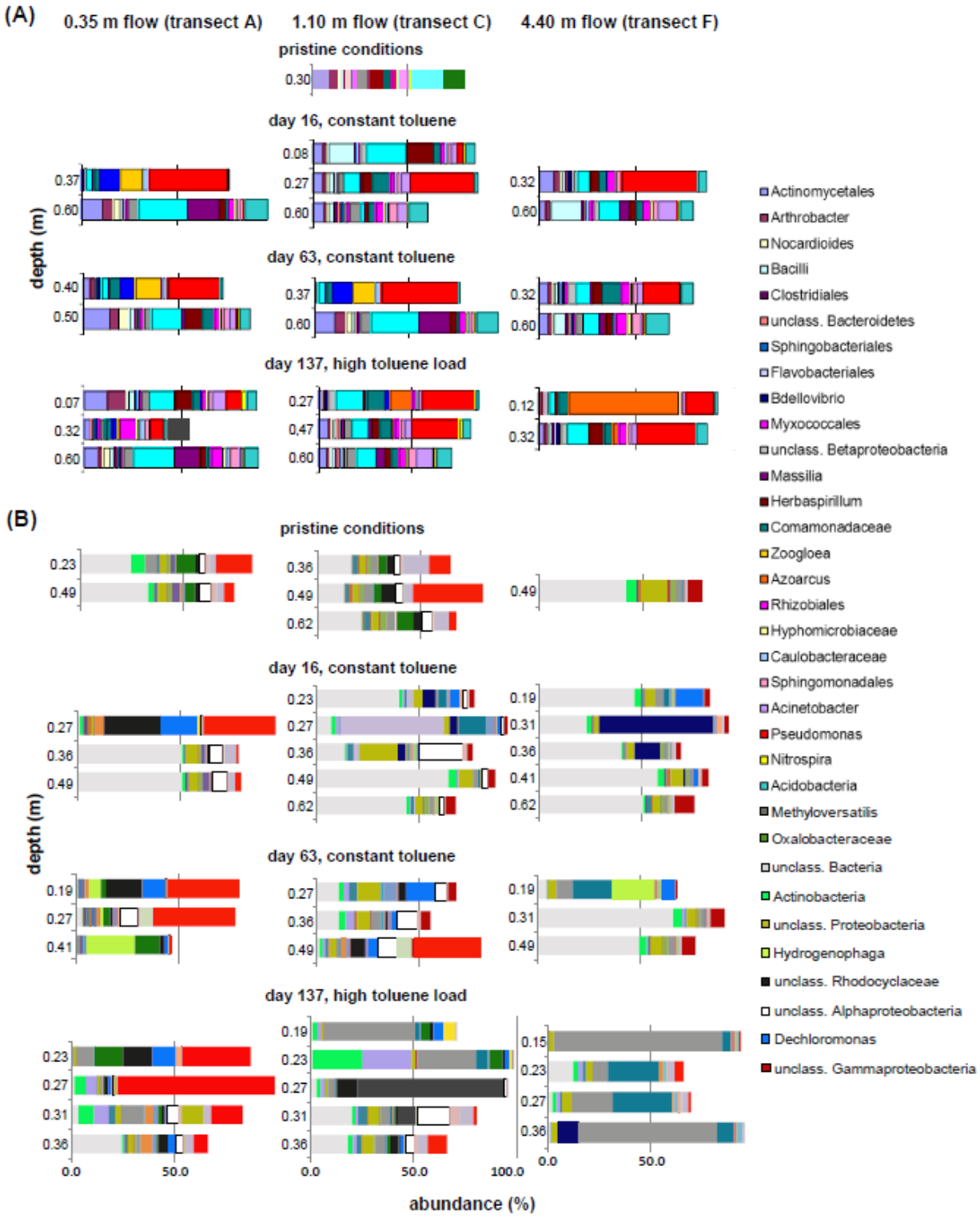

Figure 3.7. Depth transects of the distribution of selected dominating sub-class-level bacterial taxa along the flow path of the indoor aquifer. Different time points, toluene loadings as well as sediment (A) vs. groundwater (B) samples are resolved.

After high toluene contamination (day 137), a pronounced change in overall bacterial community composition as well as dominating presumed key players in toluene degradation were observed. While *Pseudomonas* spp. populations were still important in groundwater samples from transect A, a dominance of *Betaproteobacteria*, esp. *Methyloversatilis* spp., unclassified *Comamonadaceae,* and other *Betaproteobacteria* emerged in the plume core towards the distal end of the aquifer. In the sediments, a marked dominance (56 %) of *Azoarcus* spp. reads was observed in the upper zones of transect F. *Pseudomonas* spp. related reads were still abundant in transects C and F, and thus in clear contrast to results from the groundwater. At transect A, *Methyloversatilis* was also apparent at high abundance (12 %) in the centre of the plume.

3.4. Discussion

In this study, detailed and elaborate insights into the initial impacts of a contamination event on a pristine aquifer system are presented. The joint research on a mesocosm model aquifer was conducted to comprehensively elucidate central aspects relevant for natural attenuation and it's monitoring in nature. The collaborative GOODWATER experiment allowed for an ease of sampling access for biotic and abiotic parameters at high resolution in space and time. The multidisciplinary approach included microbiological, hydrogeological, chemical, and stable isotope expertise. The PhD candidate's specific contribution to the collaborative experiment was, with integrated molecular biology methods, to elucidate the impacts of contamination on the spatial and temporal succession of overall bacterial communities, and on the establishment of key contaminant-degrading populations.

The immediate response of the system towards initially low toluene contamination was apparent in drastically increased toluene monooxygenase (*tmoA*) gene abundance, already in the primary transect A of the flume, connected to an almost complete degradation of the amended toluene in the first ~1 m flow path of the aquifer system. Such a surprisingly rapid response was never documented before for a pristine, almost natural groundwater ecosystem. To date, an even more rapid initiation of toluene degradation (within hours) has been observed only for a two-dimensional model aquifer 'tank'; however this system was inoculated with pre-grown biomass of *P. putida*, a known aerobic toluene degrader (Bauer et al., 2008).

In groundwater of the indoor aquifer, *tmoA* genes were detected in high numbers over all transects and most depths upon contamination, suggesting the establishment of an abundant toluene oxidising microbiota in the high-conductivity lens installed for mixing purposes, and its respective detachment into the mobile phase. In contrast, local maxima and minima in *tmoA* gene abundances remained detectable in sediments. Interestingly, 16 days after the onset of contamination, *tmoA* genes were not detectable in sediments of transect A, either because of an inadequate depth resolution (i.e. the narrow plume of only 5 to 6 cm width was missed in sediment sampling), or of an insufficient time frame for establishing detectable attached catabolic potentials.

The marker gene for anaerobic toluene degradation (*bssA*) was also detected in groundwater already 16 days after toluene contamination, albeit at very low quantities, which also remained unchanged in anoxic compartments after high toluene loading (at day 137). Likely, this may have been due to the fact that an assay not fully appropriate for denitrifier *bssA* was employed, because the appropiate assay was not available. Sulphate reducing degraders with the targeted *bssA* would probably have needed more time and more reduced conditions to establish. But still, the results do not exclude the development of more abundant toluene degrading denitrifiers in the aquifer upon high toluene loading.

A drastic increase of *bssA* gene abundances and denitrifying BTEX degradation has been previously observed in sediment microcosms already after four days (Beller et al., 2002). In our study, 16S rRNA gene counts indicated a considerable increase in total bacterial cell numbers triggered only by toluene availability. The same applies for earlier column experiments, where a biomass increase coupled to high degradation rates was detected close to the toluene inlet, showing a correlation of bacterial cells to the toluene concentration (Kim and Jaffe, 2007). A rapid development of specialized degrader populations upon contaminant coupled to an increase of total biomass was also observed for natural field sites (Da Silva and Alvarez, 2007). This substantiates that toluene is a readily utilisable carbon source for microbes even in pristine aquifer environments with no previous history of hydrocarbon contamination.

In addition to qPCR and T-RFLP community fingerprinting, pyrosequencing was applied in order to differentiate and classify the bacterial lineages in correlation to catabolic gene counts and redox conditions. 454 pyrotag sequencing is indeed known to be well reproducible and can be interpreted in a semi-quantitatively robust manner (Pilloni et al., 2012). In this thesis, for the first time, this technology was applied to resolve community distinctions between groundwater and sediment at high resolution. In general,

overall communities and the abundance of degrader lineages in water were more variable and fluctuating over time, in contrast to sediment data where degrader populations evolved rather constantly, but with much more pronounced spatial variation. As a clear difference, the water phase harboured more (and more abundant) putative aerobic degrader lineages, potentially because of constant oxygen supply with the inflow, most readily consumed during flume passage. On the other hand, abundant denitrifying toluene degraders were detectable and became dominant under respective contaminant loading and redox conditions only for the sediment matrix.

Dynamics of intrinsic microbiota in the indoor model aquifer upon toluene impact suggested the development of a specialized bacterial community adapted to toluene degradation under locally available electron acceptors and biogeochemical settings. Populations of *Pseudomonas* spp. appeared clearly selected as dominant aerobic degraders (up to 40 % of pyrotag abundance) in the entire plume and especially in the plume core, at least during initially low contaminant concentrations. *Pseudomonas* species have been detected as important toluene degraders in a contaminated aquifer even under fluctuating aerobic/anaerobic conditions (Hendrickx et al., 2005). Also in our study, *Pseudomonas* spp. remained detectable even in zones subject to anaerobiosis after strong toluene contamination, albeit at reduced abundance.

Microbial communities in sediment above and below the toluene plume were relatively stable, at least during the initial phase of low-level contamination. A change in the community was evident mostly in the plume over space and time, showing, together with *Pseudomonas* spp., an appearance of *Zoogloea, Bdellovibrio*, and unclassified *Comamonadaceae* (all known to harbour degrader representatives in sediment-attached bacteria). In the groundwater, also *Dechloromonas*-related reads became abundant as putative toluene degraders (Coates et al., 2001; Chakraborty et al., 2005; Salinero et al., 2009), but also *Bdellovibrio*, a bacterial micropredator, and unclassified *Rhodocyclaceae* emerged.

This provides evidence for the development of a complex bacterial food web based on toluene, not apparent in overall communities without the high carbon loads of contamination. *Bdellovibrio* (*B. bacteriovorus*) can tolerate microaerophilic as well as (short) anoxic conditions; and resistance to organic solvents and a high motility towards prey and/or oxygen has been reported (Rendulic et al., 2004; Chauhan and Williams, 2006). In fact, *Bdellovibrio* species were shown to preferentially predate on gram-negative bacteria, e.g. *Pseudomonadaceae*, and to have a short generation time in the presence of

high numbers of host cells (Stolp and Starr, 1963). This reasoning is also supported by the observation of increased protozoan grazing activity in the same indoor aquifer model, described in the partnering thesis (Herzyk, 2013).

The unique data set on bacterial population shifts after toluene contamination presented here shows pronounced temporal and spatial succession. Upon high toluene contamination, sediment microbiota showed a shift of *Pseudomonas* spp. detectability towards the plume fringes, while they remained detectable in groundwater only near the toluene inlet, and 'disappeared' with increasing flow path. To date, pronounced microbial successions and fluctuations have been observed only in a real tar-oil contaminated groundwater system, albeit over a 16-year record of plume monitoring and contaminant attenuation (Yagi et al., 2009). Thus the data presented here provides a totally new perspective of the time frames over which contaminant plumes are dynamic as a microbial habitat. Downstream, in the upward-moving plume core, reads affiliated to the genus *Azoarcus* became strikingly abundant in the sediment (56 % read abundance) under high toluene loading. Even then, rapidly decreased toluene concentrations provided clear evidence for efficient overall biodegradation. Interestingly, denitrifying toluene degraders could only be enriched under initially low toluene concentrations (5 ppm) in another study, resulting in isolates related to the genus *Azoarcus* and capable of degrading toluene under both anaerobic and also aerobic conditions (Fries et al., 1994).

Intriguingly, the majority of the bacterial groups detected in the water phase after high toluene impact in this study seemed to be unclassified on a higher taxonomic rank. Possibly, methodological bias during the preparation (changing of emPCR kit chemistry by Roche) of libraries for FLX sequencing could have been responsible for this. Alternatively, developing degrader lineages not yet present in public databases could have been responsible for this.

In this study, multiple biogeochemical and molecular lines of evidence are provided for a rapid initiation of aerobic (under low toluene loading) and anaerobic degradation (upon high toluene concentrations) after relatively short periods of time (10 days). Toluene consumption was immediately coupled to oxygen and nitrate reduction. A variety of denitrifying toluene degraders are known, and can become dominant in different environments (Fries et al., 1994; Wilson and Bouwer, 1997). It is interesting to mention here that both *Azoarcus* spp., possessing the *bss* gene (Achong et al., 2001) and also *Dechloromonas* spp. (Coates et al., 2001), possessing aerobic BTEX-catabolic pathways, were found upon heavy contamination in our model aquifer. Denitrification in conjunction

with toluene removal has been observed at different initial oxygen levels, up to saturation (Durant et al., 1999; Nestler et al., 2007; Kim and Jaffe, 2008). Also, the biodegradation of monoaromatics continued without lag-phase upon shifting from microaerophilic to anaerobic conditions with nitrate as terminal electron acceptor in microcosm experiments developed with aquifer material from a contaminated site (Hutchins, 1991). Together with our results this indicates that a simultaneous degradation of toluene with various electron acceptors can indeed occur in such systems, or that processes are spatially separated only on a micro-scale.

In sum, several synchronous lines of evidence for the rapid development of an adapted, specialised degrader community upon toluene contamination are provided. Thus, bacterial 16S rRNA gene information nicely matched the dominating biogeochemical processes going on in a given compartment over time. While clear distinctions were found comparing groundwater and sediment data; the water-phase appeared more dynamic and expressed relatively strong temporal fluctuations. Monitoring results from the sediments were more stable and more coupled to dominating processes over time. These findings provid unique insights into the initial reaction of a pristine aquifer microbiota to contamination, something that has not been investigated in the field to date. It was shown how ongoing reactive processes and involved degrader populations evolve simultaneously, adapting spatially to local contaminant and redox scenarios but staying highly variable in groundwater.

Acknowledgments

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4. Molecular monitoring strategies for the assessment of natural attenuation based on catabolic gene abundances in correlation to geochemical and isotopic field data

Abstract

An accurate and integrated monitoring of natural attenuation processes is generally important, especially at heterogeneous contaminated aquifers, where the net contribution of different redox processes to overall contaminant removal may be hard to delineate. Here, the value of degradation gene-based monitoring tools for contributing to the assessment of aromatic hydrocarbon degradation at a tar-oil polluted aquifer in Augsburg, Germany, was verified. Genetic monitoring strategies based on the fingerprinting (T-RFLP) and quantification of ribosomal and catabolic marker genes were applied. In addition to the lines of evidence for aerobic aromatic degradation provided by 'conventional' isotopic monitoring strategies applied at the site and in the lab, a clear spatial resolution of overall aerobic vs. anaerobic catabolic pathways was shown via molecular monitoring. Also an underestimated, punctually dominating anaerobic degradation potential was demonstrated in certain deeper sediment depths. Also in areas strongly exposed to contaminants, anaerobic degradation potentials dominated in marker gene screening. Potential hot-spots of anaerobic degradation were thus identified especially in the Tertiary aquifer. This provides a more detailed understanding of overall contaminant removal in a natural, heterogeneous aquifer. The generation of congruent lines of evidence between different monitoring methods such as stable isotopes or molecular approaches will contribute to the design and implementation of enhanced site-specific remediation strategies.

4.1. Introduction

There are potentially more than 200,000 contaminated sites in Germany, mainly with BTEX and PAH hydrocarbons, representing a risk of affecting the groundwater in surrounding areas (Prokop et al., 2000). Microorganisms are capable to degrade hydrocarbons and to utilize them as carbon and energy source under both aerobic and anaerobic conditions (Andreoni and Gianfreda, 2007) so that biodegradation is one of the most important processes for natural attenuation (NA) and groundwater contaminant remediation. Therefore, an accurate and comprehensive monitoring system for NA is very important, especially at heterogeneous contaminated aquifers, where overall processes may be hard to delineate.

Here, compound-specific isotope analysis (CSIA) can be applied to qualitatively and quantitatively assess biodegradation in groundwater. Natural stable isotopes appear in certain ratios in nature, such as $^{13}C/^{12}C$, $^{15}N/^{14}N$, or $^{18}O/^{16}O$. If a contamination with hydrocarbons occurs, contaminants within lighter isotopes are preferentially metabolized, causing an enrichment of the heavier isotope in the residual substrate. This shift is called isotope fractionation (Morasch et al., 2001; Richnow et al., 2003; Steinbach et al., 2004). Therefore, measuring shifts in contaminant stable isotopes, usually $^{13}C/^{12}C$-ratios, allows an assessment of microbiological degradation in the field (Richnow et al., 2003; Meckenstock et al., 2004). Another established method is the exposure of microcosms (BACTRAPs®) loaded with isotopically labelled contaminants corresponding to the local scenario in order to demonstrate incorporation of label into the biomass of active bacterial degraders on site (Geyer et al., 2005). A labelling of bacterial cell components with heavy isotopes shows that specific contaminant-degrading bacteria are active within the site.

However, especially in hydrologically and geochemically heterogeneous settings, CSIA monitoring results are often not easy to interpret. Isotope fractionation effects may be masked by mixing of different water flow paths or by passage of contaminant fluxes through different redox strata. At the same time, there is an increasing interest in methods capable to monitor and quantify hydrocarbon biodegradation in the field also under heterogeneous settings. It has been shown that local microbial community patterns and especially the distribution of specific degraders can provide valuable insights on the importance of ongoing degradation processes. Microorganisms have developed specific degradation pathways for contaminants adapted to distinct biogeochemical conditions (Gulensoy and Alvarez, 1999; Junca and Pieper, 2004). Genetic monitoring strategies

based on the detection of respective catabolic genes in environmental samples are becoming of increasing interest and may be very attractive for application in monitoring of aerobic and anaerobic degradation processes at heterogeneous sites. Here, molecular analysis can provide quantitative data on the presence of a certain degradation gene and *in situ* bioremediation capacity.

In practice, qPCR analyses seem most easily implemented in routine site monitoring. A qPCR method for monitoring pollutant degradation should target a functional gene associated with the contaminant, such as to produce a positive correlation between the number of gene copies in a given sample and degrading activity (Beller et al., 2002). For anaerobic degradation of BTEX, benzyl succinate synthase (*bss*) is a key enzyme that catalyzes the first activation step of degradation, and environmental *bssA* gene counts are well correlated to ongoing contaminant degradation processes (Beller et al., 2002; Beller et al., 2008; Winderl et al., 2008).

On the other hand, the initial step of aerobic BTEX degradation is encoded via a variety of oxygenases (Gulensoy and Alvarez, 1999), including mono- and dioxygenases for aromatic ring cleavage. Monooxygenases are more efficient under limiting or fluctuating oxygen conditions (Leahy and Olsen, 1997) and display significant sequence homologies (Hendrickx et al., 2006a; Andreoni and Gianfreda, 2007) that allow quantitative molecular assay development for detecting degradation potentials for different hydrocarbons. The great advantage of i.e. qPCR assays for anaerobic and aerobic marker genes is the direct applicability to aquifer sediment or groundwater samples without the need for labelling, incubation, or other laboratory experimentation. Via assays for BTEX catabolic genes, *in situ* degradation capacities can be quantified and spatially allocated, thus providing valuable information on the most relevant degradation processes, and identifying potential biodegradation-hot spots.

Here, in the frame of an SME internship within the GOODWATER ITN, the usefulness of the application of molecular markers for degrader populations (diversity-screening and quantification of aerobic vs. anaerobic toluene degradation genes) together with hydro-geochemical (done by mplan eG) and isotopic monitoring (examined by Isodetect GmbH) of biodegradation at a heterogeneous contaminated aquifer has to be demonstrated. This stakeholder internship was, as requested by the ITN project scheme, carried out with an SME enterprise, in my case with the specialized isotopic monitoring

company Isodetect GmbH (Neuherberg). Field work was done at a tar-oil contaminated site in Augsburg (Germany) currently remediated by the engineering company mplan eG (Munich). The Ph.D. candidate conducted sediment sampling at the site and all respective molecular analyses, as well as comprehensive data evaluation and interpretation. On site hydrogeochemical analyses were performed from certified labs engaged by mplan eG, laboratory biodegradation assays were done by Isodetect GmbH in collaboration with UFZ (Leipzig). The results are shown in this thesis as background information relevant for the interpretation of the Ph.D candidate's molecular field data.

In summary, the aim this specific part of the thesis was to (i) provide supportive evidence for ongoing natural degradation of aromatic hydrocarbons on-site by the use of molecular monitoring assays, (ii) inform stakeholders on which redox processes (aerobic/anaerobic) key natural attenuation processes may depend in distinct zones of the contaminated aquifer, and (iii) unravel possible factors driving or limiting microbial contaminant transformations *in situ*.

4.2. Materials and Methods

4.2.1. Site description, sampling, and chemical analyses

The investigated area was a former gasworks site in Augsburg-Oberhausen, Germany. The aquifer was composed of two distinct and clearly separated layers, a highly permeable sand and gravel groundwater layer (Quaternary) underlined by a fine matrixed silt and clay layer with low permeability (Tertiary). The tertiary aquifer layer derived from an earlier geological period and its origin is non-glacial but probably tectonic/weathering. In contrast, the upper aquifer layer is composed of 'younger' sediment from the quaternary period, formed through de-/glaciation processes (deposition). From 1915 until 2001 the gaswork plant was used for production (till 1969 of coal, including coke stocking) and storage of natural gas on an area of ~8 ha. Production processes but especially the storage and disposal of all intermediate products contaminated the aquifer over a long period with different hydrocarbons, especially polycyclic (but also monocyclic) aromatics.

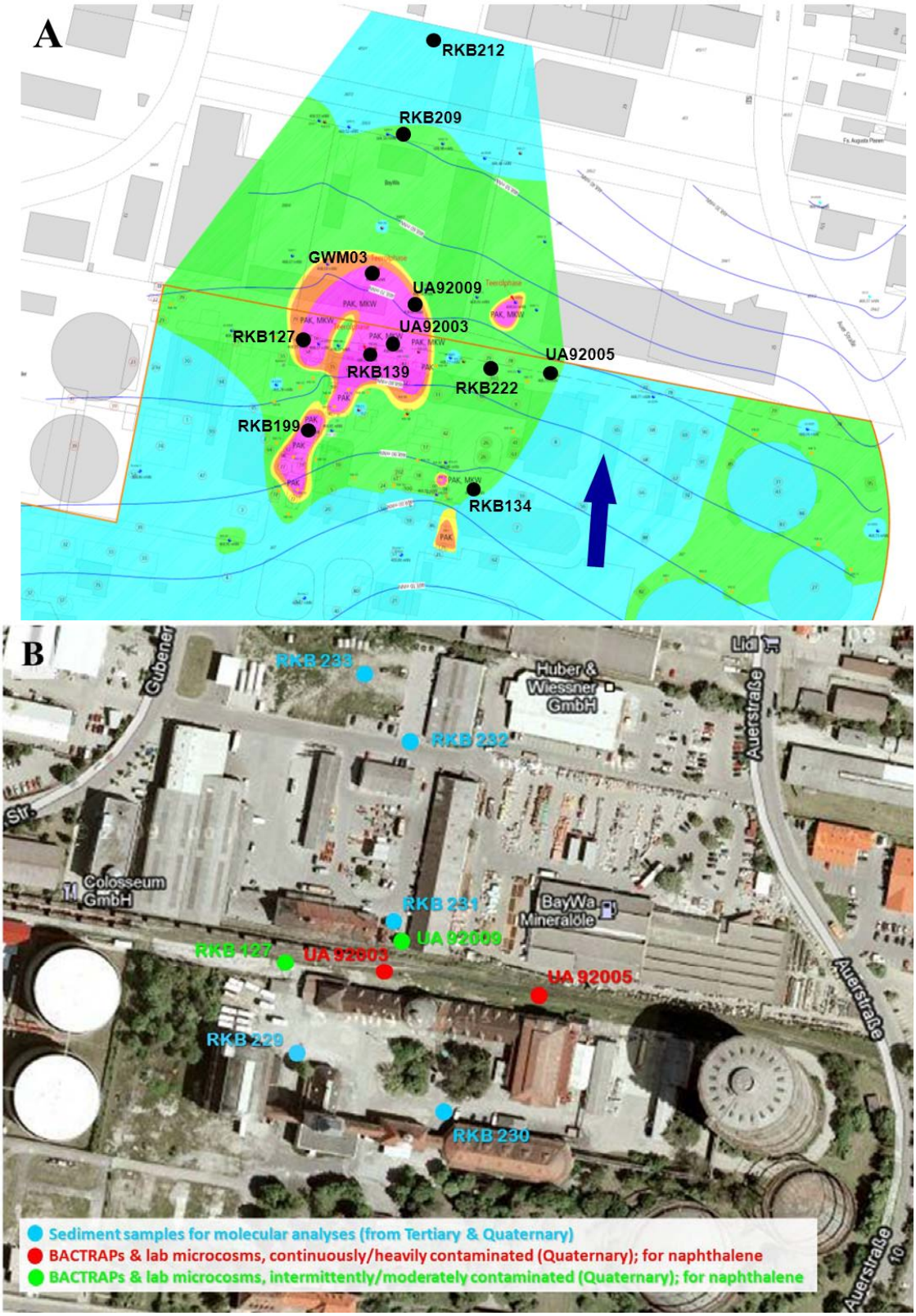

Figure 4.1. (A) Area of the former gasworks site in Augsburg Oberhausen showing contaminant concentrations of polycyclic aromatic hydrocarbons (PAH) and mineral-oil hydrocarbons (MKW): pink colour indicates high-, green moderate-, and blue low hydrocarbon concentrations (measured by certified labs appointed by mplan eG). The arrow indicates groundwater flow. Black circles show the locations of monitoring wells used for chemical and contaminant measurements in this study. (B) Localisation of groundwater wells where microcosms (BACTRAPs) were incubated by Isodetect GmbH and location of sediment samples taken by me for molecular analyses during the drilling of new monitoring wells.

Different monitoring wells, distributed across the whole area of the former gaswork, were used to measure the chemical parameters and the contaminant concentration in the groundwater. In June 2010, mplan eG (the Engineering Bureau hired by the Augsburg municipality) planned the drilling of several new boreholes through the aquifer for a more detailed hydrogeological inspection of the aquifer and the implementing of additional monitoring wells. From this drilling campaign, sediment samples were taken by me from five different locations and depth intervals (between 3 and 28 m below surface, based on contaminant measurements of November 2007): samples RKB 229 and RKB 231 from heavily/continuously contaminated areas, RKB 230 and RKB 232 from moderately contaminated locations (also fluctuations), and RKB 233 from outside the plume area with very dilute contamination (Fig. 4.1). Approximately 50 g of sediment were taken in sterile Falcon tubes and immediately frozen on dry ice until further analyses in the molecular ecology laboratory at IGOE, Helmholtz Zentrum Munich.

All chemical analyses, measurements of the different contaminant concentrations, and geological examinations were carried out by certified labs engaged by mplan eG. Contaminant concentrations (PAH and BTEX), electron acceptors, oxygen content, pH, and DOC in groundwater were measured by a routine analytical laboratory. Modelling and mapping were performed by the engineering bureau, while final data evaluation as well as preparation of the results/reports was performed by mplan eG and Isodetect GmbH, with support of the Ph.D. candidate in the frame of an SME internship.

4.2.2. Laboratory microcosms and BACTRAPs

Isodetect GmbH (Neuherberg) and its subsidiary at the Helmholtz Zentrum für Umweltforschung (UFZ) Leipzig performed *in situ* exposure of isotopically-labelled microcosms (BACTRAPs) at the contaminated site, for detection of on-site biodegradation. In the laboratory, naphthalene labelled with heavy isotopes (^{13}C, >99%) was adsorbed to sediment particles, as described previously (Geyer et al., 2005). Then these 'biotraps' were exposed to the contaminant plume in selected monitoring wells of the field site for a certain time. Labelling of attaching microbial cells provides direct evidence that contaminant-degrading bacteria are present and active at the site (Stelzer et al., 2006). Specifically, BACTRAPs for naphthalene degradation (100 d incubation) were exposed into the monitoring wells UA92005 and UA92003 with continuous heavy contamination, UA92009 and RKB127 with moderate and intermittent pollution. The depth of all installed microcosms ranged at ~6 m below well top level.

As an additional tool to define the PAH-metabolization rates, BACTRAPs inoculated for 62 d in the same monitoring wells were used for lab microcosm measurements. They served as an inoculum incubated with groundwater from the corresponding wells and under aerobic conditions at 14°C in the dark, for measurements (in time series) of complete degradation. Microbial conversion of ^{13}C-labelled PAH-contaminants to CO_2 can be quantified through carbon isotope signatures, i.e. $^{13}CO_2$ quantification, as documented and validated previously (Richnow et al., 1998; Morasch et al., 2007). Each set-up comprised two active replicates and one sterile control, to discriminate against abiotic non-biodegradative transformations. These laboratory microcosms were also conducted by Isodetect GmbH, the results are shown in this thesis as relevant background information for the molecular field data of the Ph.D. candidate.

4.2.3. DNA extraction and microbial community fingerprinting

Total DNA was extracted, using ~1 g of sediment (wet weight) in three independent extracts for every depth, following the procedure described by Winderl et al. (Winderl et al., 2008). All PCRs were performed in 50 µl-reactions containing nuclease-free H_2O, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP's, 1 U Taq polymerase (all Fermentas GmbH, St. Leon-Rot, Germany), 10 µg BSA (Roche diagnostics GmbH, Basel, Switzerland), 0.5 µM of each primer (biomers.net GmbH, Ulm, Germany), and 1-2 µl of template DNA. 16S rRNA gene-PCR was performed under the following thermal cycling conditions: 3 min of initial denaturation at 94°C, 26-28 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 55°C, and 60 s extension at 72°C), and 5 min of final extension at 72°C. T-RFLP (Terminal-Restriction Fragment Length Polymorphism) analysis of bacterial 16S rRNA genes was performed with PCR-amplicons of primers Ba27f-FAM (5'-6-carboxyfluorescein labelled 5'-AGA GTT TGA TCM TGG CTC AG-3') and 907r (5'-CCG TCA ATT CMT TTR AGT TT-3') by *MspI* digestion, as previously described (Pilloni et al., 2011).

4.2.4. qPCR measurements

All real-time quantitative PCR (qPCR) measurements were performed on a MX3000P qPCR cycler (Stratagene, La Jolla, CA). qPCR of benzylsuccinate synthase (*bssA*)-genes was performed with the TaqMan system previously developed by Winderl et al. (Winderl et al., 2008), with the Universal PCR Master Mix, No AmpErase UNG kit (Applied Biosystems, Carlsbad, CA). The Brilliant III Ultra-Fast SYBR Green qPCR

Master Mix (Agilent Technologies, Santa Clara, CA) was applied for toluene-para-monooxygenase (*tmoA*)-qPCR, using the manufacturer's protocol with minor modifications. Primers tmoA-f and tmoA-r were used with two-step thermal cycling (45 cycles at 95°C for 20 s and 60°C for 20 s). qPCR standards were designed via PicoGreen-quantifications from partial *tmoA* gene amplicon (clone DV7tmo13, GenBank accession no. JX307448). To correct for potential extraction and/or amplification biases, defined cells amendments of *Pseudomonas mendocina* KR1 cells were spiked to sterilised sediment in concentrations between 1.13 x 10^5 and 1.36 x 10^7 cells g^{-1} wet of sediment (as counted on a LSR II flow cytometer, Becton Dickinson, Heidelberg, Germany, according to (Anneser et al., 2010)). DNA was extracted from spiked sediments, and the detected versus expected gene quantities were used to infer a specific correction factor for the used *tmoA* gene qPCR (the resulting correction factor was 2.7). The detection limit for all used qPCR assays was ~10^4 genes per g of sediment.

4.3. Results

4.3.1. Hydrogeochemical properties

Most of the aquifer at the former gasworks site in Augsburg appeared oxic (Table 4.1) by mere groundwater sampling from wells. Only depth zones with high amounts of pollution and/or impermeable layers showed evidence for anoxic conditions, such as wells GWM3, RKB209, and RKB212. Anion concentrations indicated the presence of nitrate and sulphate as alternative electron acceptors and the DOC content (excluding hydrocarbon contaminants) was generally low. In contrast, considerable amounts of total PAHs together with naphthalene were found as major contaminants over the whole area of the former gasworks site, but also BTEX polluted zones were present (see Fig. 4.1 for a general view; Table 4.1 shows relevant wells). In the area of the sediment sampling cores RKB231, RKB229 and RKB232, high amounts of PAHs and naphthalene, together with substantial BTEX concentrations, were detected in the Tertiary aquifer, suggesting prevailing strictly anaerobic conditions there, despite low oxygen concentrations still measured in the well water. Here, nitrate concentrations also decreased clearly, while sulphate remained almost constant over the entire field site.

Table 4.1. Hydrochemical parameters and contaminant concentrations in groundwater of the monitoring wells used for microcosm incubations, and groundwater wells located near the sediment sampling points used in this thesis.

Well name	O_2 (mg L^{-1})	NO_3^- (mg L^{-1})	NH_4^+ (mg L^{-1})	SO_4^{2-} (mg L^{-1})	DOC (mg L^{-1})	tot. PAH (µg L^{-1})	naphthalene (µg L^{-1})	BTEX (µg L^{-1})
UA92003	5.7	26	n.d.	27	1	8	16	n.d.
UA92005	7	21	n.d.	24	0.6	3.2	8.8	n.d.
UA92009	5.6	27	n.d.	27	0.7	n.d.	0.1	0.7
RKB127	5.8	23	n.d.	27	0.6	0.4	3.4	n.d.
RKB134	7	29	n.d.	26	0.7	0.5	0.1	50
RKB139	6.3	29	n.d.	26	0.5	0.3	0.2	n.d.
RKB199	6.1	29	n.d.	27	n.d.	2.4	2.8	n.d.
RKB209	0.1	16	1.7	29	1	190	120	30
RKB212	0.3	17	1.2	27	0.7	0.1	n.d.	26
RKB222	4.5	22	n.d.	24	1	2.6	0.4	n.d.
GWM3	4.3	28	n.d.	27	0.5	1,550	650	16

n.d., not detectable

All chemical analyses and hydrogeological measurements were carried out at certified labs engaged by mplan eG upon groundwater sampling in November 2007. The results are shown in this thesis as background information relevant for the interpretation of my own molecular field data.

4.3.2. BACTRAPs - *in situ* degradation

To measure contaminant stable isotope shifts and herewith microbiological degradation directly in the aquifer, BACTRAP microcosms loaded with isotopically labelled contaminants corresponding to the local scenario were exposed in the groundwater wells. This was performed by Isodetect GmbH and was fundamental for comparative monitoring of natural attenuation, including molecular and isotopic approaches. The concentration of total amino acids (AA) in naphthalene-spiked microcosm was measured as a proxy for microbial biomass formation during down-well *in situ* exposure. The highest biomass growth was measured for wells UA92009 (4403 µg AA $BACTRAP^{-1}$) and RKB127 (1196 µg AA $BACTRAP^{-1}$), both exhibiting very low concentrations of naphthalene. In comparison, wells continuously polluted (UA92005 and UA92003, with max. 16 µg L^{-1} naphthalene) showed only $1/5^{th}$ of biomass formation (807 and 800 µg AA $BACTRAP^{-1}$, respectively). Hence, a higher colonization of naphthalene-spiked BACTRAPs occurred in less polluted groundwater. Backed up by the above electron

acceptor measurements (Table 4.1), this biodegradation of naphthalene in BACTRAPs exposed in monitoring wells *in situ* was assumed to occur primarily under aerobic conditions.

The enrichment of ^{13}C in AA, determined through analysis of $^{13}C/^{12}C$-carbon isotope enrichment, results from the metabolization of ^{13}C-substrate. In the BACTRAP biomass, it directly proves biological naphthalene degradation (Pelz et al., 1998; Scott et al., 2006). In contrast to total biomass accumulation, BACTRAPS exposed in continuously polluted groundwater wells showed higher carbon isotope enrichment of AAs compared to the less polluted wells (Fig. 4.2). Carbon isotope signatures were generally high, showing a strong biodegradation of naphthalene in groundwater on-site. However, after calculation of the total ^{13}C-content in the amino acids for all BACTRAPS, total ^{13}C-incorporation was similarly in three wells (UA92005, UA92003, and RKB127) with ~90 µg ^{13}C per BACTRAP. In the less contaminated well UA92009, total ^{13}C-incorporation was even higher (219 µg ^{13}C per BACTRAP), indicating an even more active biodegradation in these exposed microcosm.

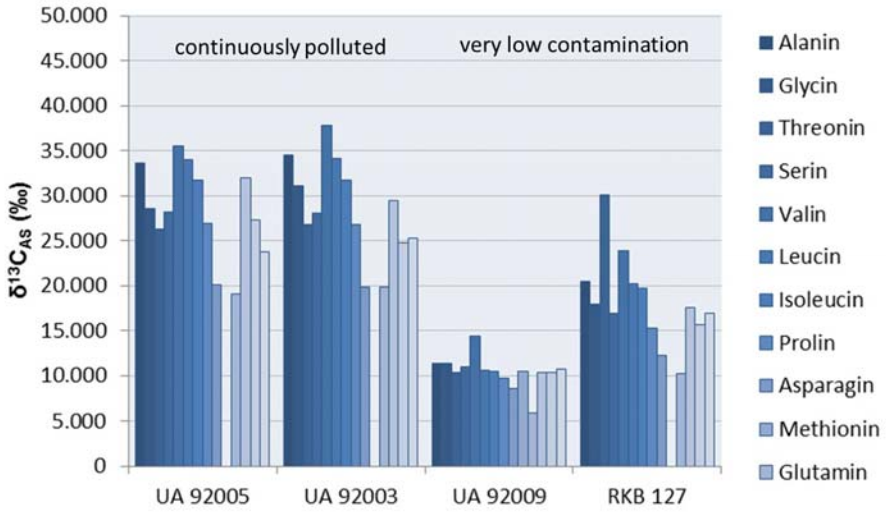

Figure 4.2. ^{13}-C isotope enrichment of amino acids (AA, named on the right) extracted from naphthalene-spiked BACTRAPs after 100 d of incubation in groundwater monitoring wells at the Augsburg site. Isotopic site monitoring was carried out and analysed by Isodetect GmbH (Neuherberg) and its subsidiary at the Helmholtz Zentrum für Umweltforschung (UFZ) in Leipzig. These results are shown in this thesis as background information relevant for the interpretation of my own molecular field data.

4.3.3. Laboratory microcosm incubations to assess degradation rates

As an additional tool to define the biodegradation rates (albeit *ex situ*), BACTRAPs inoculated for 62 d in the same monitoring wells were transferred to lab microcosms. This was also performed by Isodetect GmbH in collaboration with the UFZ Leipzig. Generally, a high potential for (aerobic) biodegradation of naphthalene and also other PAHs (data not shown) was confirmed for the investigated BACTRAP materials also via laboratory microcosm approaches (Table 4.2). No significant difference in the biodegradation of naphthalene was observed for BACTRAPs originating from distinct contamination zones. The overall degradation of ^{13}C-naphthalene to $^{13}CO_2$ in 62 d was 3.0-4.6 mg L-1, corresponding to a mineralization rate of 24-37 % (~1/3) independently from the contamination of the original groundwater well (Tab. 4.2). In summary, a clear evidence for active aerobic naphthalene biodegradation was determined for BACTRAPS exposed *in situ*, which can be interpreted as an indicator of general aerobic (polycyclic) aromatic hydrocarbon degradation potentials at the site.

Table 4.2. $^{13}CO_2$ formation, degradation and respective overall mineralisation of naphthalene after 62 d of incubation of *in situ*-exposed BACTRAP materials from the different monitoring wells in laboratory microcosms.

contaminant	Origin of BACTRAP[a]	Well contamination	^{13}C-CO_2 (mg L^{-1})	Degrad. (mg L^{-1})	Mineralis.. (%)
Naphthalene	UA92005 (a)	continuously	6.8	3.4	27
	(b)		7.0	3.5	28
	UA92003 (a)	continuously	9.3	4.6	37
	(b)		6.4	3.2	25
	UA92009 (a)	intermittent/low	7.4	3.7	29
	(b)		6.0	3.0	24
	RKB127 (a)	intermittent/low	7.5	3.7	30
	(b)		7.2	3.6	29

[a] name of the groundwater monitoring well where the BACTRAP was exposed.

- The laboratory microcosm incubations were carried out and analysed by Isodetect GmbH (Neuherberg) and its subsidiary at the Helmholtz Zentrum für Umweltforschung (UFZ) in Leipzig. The results are shown in this thesis as background information relevant for the interpretation of my own molecular field data.

4.3.4. Sediment bacterial community monitoring in the field

The specific contribution of the Ph.D. candidate to the site monitoring project was the molecular approach to detecting degradation potentials at high spatial resolution. This is the central pillar for this chapter of the thesis, and provides important additional information about degrader communities and degrading capacities on site, otherwise not available for site management and remediation. For this purpose, the Ph.D. candidate was responsible for choosing relevant locations and depths for sediment analyses during a general drilling campaign.

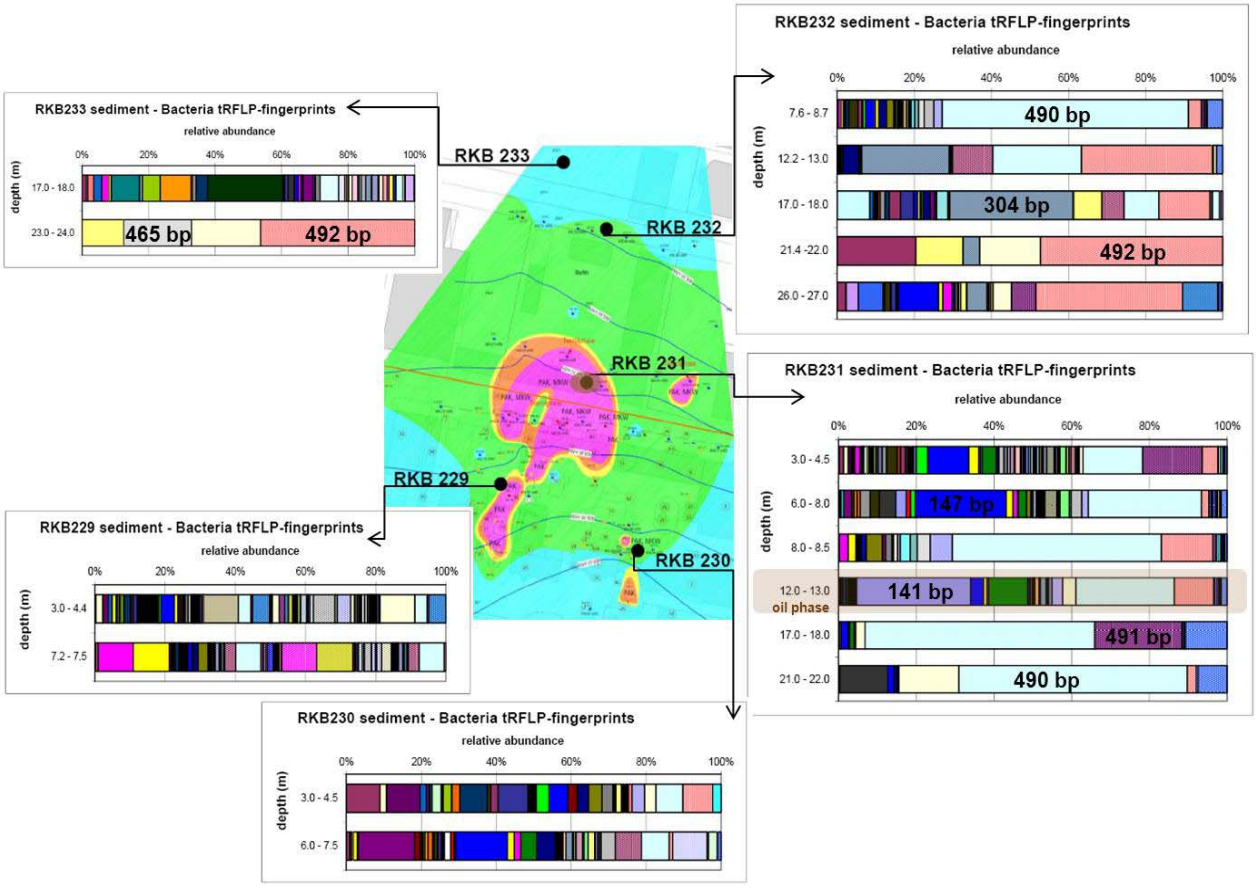

Figure 4.3. Bacterial 16S rRNA gene T-RFLP fingerprinting profiles over depth of distinct sediment sampling locations at the Augsburg gasworks site. Shown are averaged bacterial T-RF abundances (in %) from triplicated DNA extracts. Important dominant T-RFs are specified with their length [in bp].

For the fresh sediment samples taken *in situ*, T-RFLP fingerprints of the microbial communities were first generated. Spatial and depth resolution was applied, reflecting the distinct aquifer geology (visual inspection) and contaminated zones. Samples RKB229 and RKB230 were obtained from rather heavily and less polluted zones, respectively. For both locations, sediment was sampled only from the upper (tertiary) aquifer layer. Sediment transect RKB233 was divided into two different geological layers over depth, Tertiary and Quaternary, from which only the tertiary layer was exposed to a weak contamination. The depth transect of core RKB232 also reflected the different aquifers; again only the tertiary sediment was polluted here, albeit quite heavily. Hydrocarbon concentrations at location RKB231 were continuously high. Here, the sediment cores retrieved six depth samples, four from the tertiary (more contaminated) aquifer and two from the deeper quaternary layer.

The upper tertiary aquifer sediments (until a depth of ~8 m below surface) were mostly characterized by a higher bacterial diversity than the deeper quaternary. This may suggest a higher potential of the aquifer community to 'switch' between different redox processes and metabolic pathways under changing environmental and pollutant conditions. However, the most heavily contaminated depth layers (i.e. 8 - 8.5 m and 17 - 22 m at RKB231; 7.5 - 13 m and 21.4 - 22 m at RKB232) showed distinct community structures with distinct lineages becoming rather dominant. Probably, a less permeable geological formation was present in between these depths. Anyhow, this is consistent with the concept of more specialized degrader populations establishing in zones of highest biodegradation activity (Winderl et al., 2008). Especially the T-RF of 490 bp length became dominant (Fig. 4.3) in sediment zones considered as highly contaminated with PAHs and also BTEX. In sediment transect RKB231, from the most highly contaminated zone, the abundance of the 490 bp T-RF increased drastically with depth, especially above 8.5 m and below 17 m. The only exception was observed for the in-between sediment layers of 12 - 13 m, which showed a distinct community co-dominated by a T-RF of 141 bp. It is important to note that a residual oil phase was still detectable in these layers (Fig. 4.3).

At the downstream location RKB232, in a depth of ~13 m, the 490 bp T-RF, together with the very similar 492 bp T-RF, represented more than 50 % of the bacterial community. Maximal relative abundance of the T-RF 490 bp was at 7.6-8.7 m depth (64 %). Visibly, with increasing depth at sediment core RKB232 the bacterial community was changing and harboured distinct dominances of the 492 vs. 490 bp T-RFs.

4.3.5. Degradation gene quantification

The next step for the molecular monitoring was the quantification of the degradation marker genes, representing the key processes going on in the aquifer. qPCR quantification of aerobic (*tmoA*) and anaerobic (*bssA*) aromatics (toluene) degradation genes revealed an interesting stratification and also co-occurrence of the distinct oxic vs. anoxic contaminant degradation potentials in the field, along contaminant gradients between different locations and over depth (Fig. 4.4). In sediments from the more contaminated cores (RKB229, RKB231 and RKB232), high numbers of *bssA* gene copies were detected. Especially in depth zones heavily contaminated with PAHs and also BTEX, the quantity of *bssA* genes per g of sediment increased to up to ~2.6 x 10^5 (e.g. at 7.2-7.5 m depth in sediment RKB229). In fact, *bssA* genes, and therefore the potential for anaerobic aromatics degradation, were found in almost all sediments and depths.

tmoA quantities and the deduced aerobic degradation potential were much less abundant in heavily contaminated aquifer sediments (<4 x 10^3 *tmoA* gene copies per g sediment). At the distal RKB233 location, aerobic and anaerobic catabolic genes were detected only at a depth of 18 m. An almost 'classical' depth-pattern of redox succession was shown at RKB229: *tmoA* or *bssA* degradation genes were not detected in the unsaturated soil, aerobic degradation genes were most abundant below (at 6.6 m depth), while anaerobic catabolic gene quantity increased frther below (7.35 m, Fig. 4.4). Similarly, *tmoA* gene quantities decreased with increasing depth at RKB232, while *bssA* numbers reached a maximum of 2.2 x 10^4 gene copies at 17-18 m depth. Unexpectedly, only *bssA* genes were detected in the uppermost sediments of core RKB230, while *tmoA* became quantitatively more abundant below, at 6.75 m. Last but not least, the highly contaminated RKB231 cores demonstrated high aerobic and anaerobic degradation potential between 6 m and 13 m depth, with a generally higher abundance of *bssA* genes. However, below the oil phase at ~13 m, aerobic degradation genes disappeared.

It must be noted that especially in the tertiary aquifer, *bssA* abundance was always higher than *tmoA* abundance by approximately one order of magnitude. Thus, in addition to what was shown from BACTRAP and lab microcosm approaches, clear evidence for an anaerobic aromatics degradation potential in heavily contaminated aquifer zones and depths was substantiated by these molecular approaches. But even if anaerobic degradation genes dominated in the heavily contaminated, apparently reduced sediments, also aerobic aromatics monooxygenase potential was principally present.

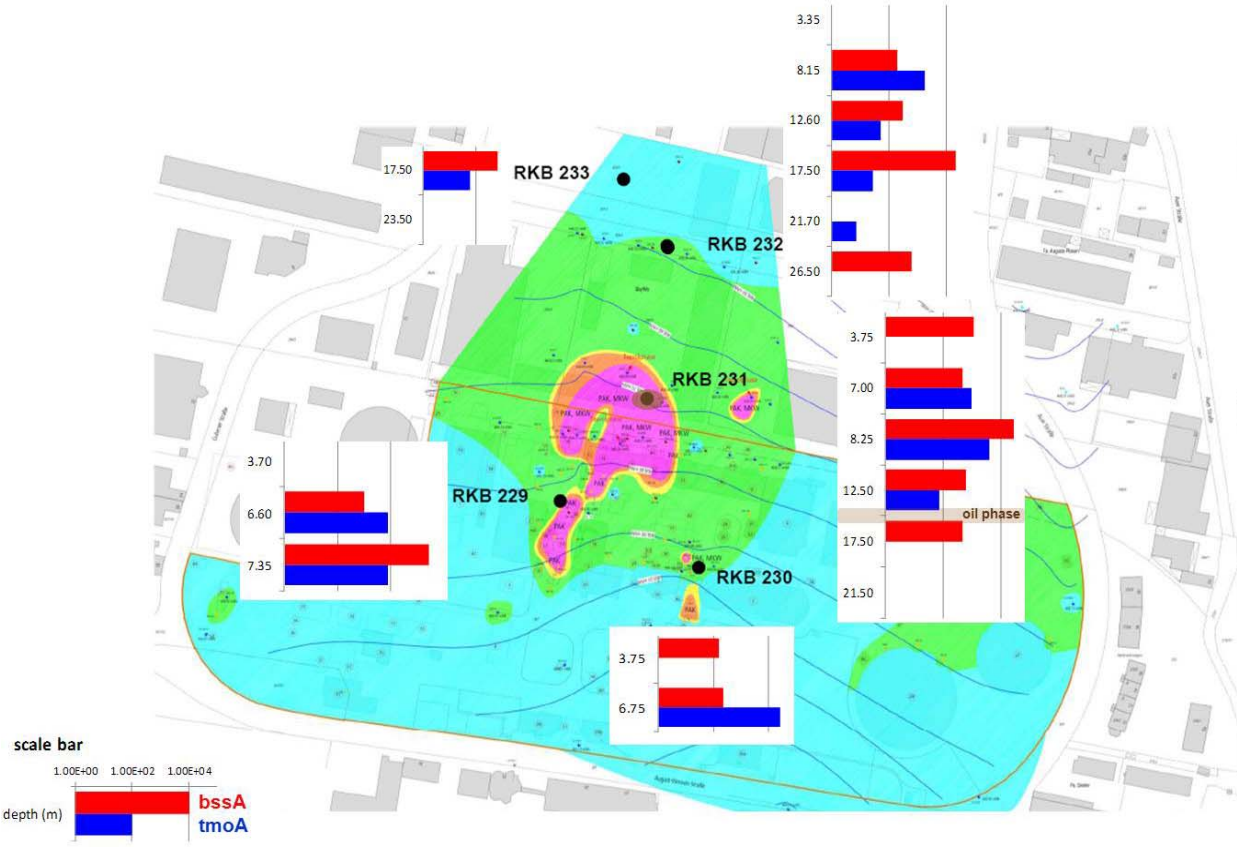

Figure 4.4. Quantitative depth distribution of *bssA* and *tmoA* genes at the site as measured via triplicate qPCR in sediments sampled in distinct aquifer sediment sampling locations. On the bottom right the scale bar for representation of absolute gene-values.

4.4. Discussion

Here, the additional value of the application of molecular markers for degrader populations and the quantification of key catabolic genes together with hydro-geochemical and isotopic monitoring of biodegradation at a contaminated aquifer was demonstrated. To test how molecular monitoring tools can provide supportive evidence for ongoing natural degradation of aromatic hydrocarbons on-site, previously developed marker gene assays (chapter 2 of this thesis, resp. Larentis et al., 2013) were applied in a routine monitoring campaign. New information was provided especially concerning on which redox processes (aerobic/anaerobic) key natural attenuation processes may actually depend in distinct zones of the contaminated Augsburg aquifer.

The geochemical data obtained by the hosts of my SME internship in this part of the thesis suggests important biodegradation processes in the investigated aquifer to occurr

especially (or only) under oxic conditions. This accelerates the degradation of persistent contaminants like PAHs (Bamforth and Singleton, 2005) versus monocyclic aromatic hydrocarbons (BTEX), compared to anoxic conditions. Therefore this information is vital to come to sound predictions on overall degradation rates and the time spans needed to accomplish clean-up objectives in site management. Amongst the PAHs, naphthalene can be metabolized relatively rapidly due to its relatively good water-solubility, thus bioavailability factors play an important role (Bamforth and Singleton, 2005; Johnsen and Karlson, 2007). This is, first, dependent on the sediment material itself (its geology) and secondly, on the chemical properties of the contaminant substance (Johnsen and Karlson, 2007). Another important factor here is the age of the contaminants (pollution history of the site), as older contaminations are usually more strongly sorbed to the sediment (Bamforth and Singleton, 2005). Generally, moderately polluted zones can be expected to favour increased biodegradation, due to less severe contaminant concentrations, and potential redox oscillations (increased mixing of electron donors and acceptors) and also electron acceptor fluctuations can enhance biodegradation (Anneser et al., 2010).

In this work, PAH degradation activities (naphthalene-Bactraps) were indirectly compared with BTEX degradation potentials (*tmoA* and *bssA* assays). Although not fully consistent, this approach was based on the fact that hydrocarbon contaminated sites including the Augsburg location are usually contaminate by mono- and poly-aromatic hydrocarbons, and that both catabolic potentials are usually to be found within very similar bacterial degrader lineages. More important, however, was the assumption that a dominant aerobic (or anaerobic) catabolic potential for BTEX would surely also be reflected in a dominating respective potential for PAH.

The maximal concentrations of total PAHs in a range of 1,550 μg L^{-1}, as measured in GWM3 in 2008 should impose toxic effects on microorganisms (Loibner et al., 2004) and hence inhibit general microbial activity, therefore also biodegradation. Also at coring RKB231 in ~12-13 m depth, where an oil phase was located (visible even by eye by the black sediment colour and a strong smell), toxic effects can be expected. These local distinctions in pollutant scenarios were clearly reflected on 16S rRNA gene level, with local change in overall bacterial community structure. The bacterial community screening via 16S – T-RFLP gave a first picture about the presence of potential pollutant-degrading microbiota, as well as their distribution at the site. Bacterial communities were generally less diverse and less even in highly contaminated zones. This is consistent with the concept of more specialized degrader populations establishing in zones of highest biodegradation

activity (Winderl et al., 2008). Although the information on the actual taxonomic affiliation of the most abundant T-RFs for this site is still missing, especially the 490 bp T-RF is known to frequently represent relatives of *Pseudomonas* spp., a genus including many imminent aerobic hydrocarbon degraders (Larentis et al., 2012).

In today's routine site monitoring, *in situ* and also laboratory microcosms are practical tools and can allow for a clear interpretation and also prediction of the on-going biodegradation processes (Richnow et al., 1998; Geyer et al., 2005; Stelzer et al., 2006; Morasch et al., 2007). Ultimately, degradation rates can even be inferred, based on intrinsic microbial communities that have developed on the BACTRAP carrier material amended with isotopically labelled substrate amendments. However, rate estimates from Bactraps may still be hard to transfer to processes in sediments over depth. BACTRAPs in the groundwater monitoring wells are exposed to a mixed groundwater phase, including potential oxygen exposure and also diluted levels of contamination. Interpretations of on-site degradation processes via hydrogeochemical parameters alone are even more difficult. Via the sampling of (mixed) well water, local hydro-geochemical parameters, pollutant concentrations and also important redox gradients established in the sediment on very small scales (Larentis et al., 2013) are likely to be missed. Also, patterns of microorganisms suspended in groundwater do mostly not neccesarily reflect the much more abundant sediment microbiota (Lehman et al., 2001; Anneser et al., 2010).

In this field investigation, the laboratory microcosms showed generally high aerobic PAH mineralization rates, in all replicates. The successful use of natural occurring groundwater and physical conditions for these tests, as well as a lack of biodegradation lag-phases in microcosms suggests a high density of intrinsic aerobic PAH-degrading microorganisms had been enriched on the BACTRAP material after ~3 months of down-well incubation. Unfortunately, this BACTRAP material was no longer available for a molecular characterisation of attached degrader populations by the time I entered the project for my internship, therefore a direct comparison of BACTRAP-enriched vs. natural sedimentary bacterial populations at the site was not possible.

Nevertheless, the catabolic markers traced at the site can provide important supporting information on the occurrence of distinct degrader populations and degradation processes in the field. However, it was not possible to directly apply catabolic marker genes for aerobic and anaerobic PAH degradation in my work, as these are not established yet for anaerobic PAH catabolism. Instead, aerobic and anaerobic mono-aromatics degradation genes were used as a proxy. Although both sets of data on the occurrence of

aromatics degradation potential may not be fully congruent, this proxy allows at least for some general statements on the distribution of aerobic vs. anaerobic catabolic potentials *in situ*.

At this specific site, due to the long-term pollution history with a mix of easily degradable and more recalcitrant hydrocarbon contaminants, BTEX concentrations appeared already reduced in groundwater and sediments compared to PAHs. Nevertheless, anaerobic degradation potentials clearly dominated in marker gene screening in deeper layers of the Augsburg aquifer as well as in areas strongly exposed to contaminants. Potential hot-spots of anaerobic degradation were found, via qPCR, especially in deeper aquifer compartments (with high contamination) of the tertiary groundwater layer. Generally, locally increased catabolic gene abundances indicates an importance of the respective catabolic processes in these zones (Winderl et al., 2008). Along this line, less contaminated zones of the Augsburg aquifer tended to provide more gene-based evidence for oxic degradation, whereas more heavily contaminated sediments tended to indicate an increased importance of anaerobic degradation processes.

In summary, in addition to the lines of evidence for aerobic aromatic degradation provided by conventional hydrogeochemical and isotopic monitoring strategies, I could substantiate a clear spatial succession of aerobic vs. anaerobic catabolic potentials via molecular monitoring. Potentially, even an un-seen dominating anaerobic catabolic capacity was demonstrated in specific sediment depths. For the stakeholders and monitoring bureaus involved at the site, this was substantial novel information. These results have the potential to influence site management and decision making in remediation in the future. Potentially, routine monitoring approaches will be extended to include catabolic gene qPCR analyses in the near future, or at least a higher resolution in sediment and groundwater sampling by the stakeholders. Although interpretation of such molecular field data may still be complex, this clearly highlights the strength of this integrated approach. Degrader community screening may offer a rapid and general means to inform on occurring or potential degradation processes at a site. Also, a principal congruency of lines of evidence resulting from different monitoring methods – stable isotopes to molecular approaches – can ideally complement one another. In the future, molecular and isotopic analyses should be better synchronized to obtain a comprehensive, integrated understanding of such heterogeneous sites, and will allow for next-generation monitoring concepts for natural attenuation.

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5. General Discussion and Outlook

5.1. The applicability of molecular tools to determine catabolic potentials and activities in contaminated groundwater

The use of molecular tools has many advantages (i.a. from Goldscheider et al., 2006; Andreoni and Gianfreda, 2007) compared to classical microbiological monitoring of contaminated groundwater. First of all, no culturing is needed; methodologies are often faster and capable to address also oligotrophic and also putatively novel microbial physiologies. Molecular biology methods are rapid, reproducible, and large international databases exist on respective markers. Different functional groups of microbes as well as entire communities can be targeted and quantified with similar workflows. A major challenge for all molecular approaches is the amount of intact and clean nucleic acids which can be extracted from environmental samples. It is often tricky to produce a sufficient amount and quality of DNA, and especially RNA, from aquifer samples. For (m)RNA, problems concern the generally short life time and lack of stability. Despite these given constraints, the use of catabolic marker gene assays provides direct evidence on degradation potentials *in situ*. Distinct redox zones and oxic/anoxic redox gradients can be accessed at fine spatial resolution, often not possible for other biogeochemical analytics *in situ*. Hot spots of biodegradation can be identified, often not straightforward with based on other monitoring results. An integration of molecular methods including the quantification of degradation markers as well as the identification of dominating populations can contribute to optimal monitoring strategies for natural attenuation. This is very important, considering the central role of microbial processes in the protection and remediation of groundwater resources.

To address this, in the first major building block of my thesis (chapter 2), a quantitative (q)PCR method to quantify the distribution of aerobic toluene degradation genes in sediments of the toluene contaminated Flingern aquifer was optimized, tested and applied. A primer system for the detection of *tmoA* genes involved in aerobic toluene degradation (Hendrickx et al., 2005) was synchronously applied with an assay for anaerobic catabolic marker (Winderl et al., 2007). To date, the overlap of aerobic and anaerobic catabolic potentials at redox gradients in aquifers has never been specifically addressed. Spatially highly resolved insights into the upper plume fringe with unexpected

quantitative distribution patterns of distinct degrader populations were provided. Two congruent lines of evidence based on aerobic catabolic marker (*tmoA*) genes (Figure 2.1) and bacterial 16S lineages harbouring potential aerobic aromatics degraders (Table 2.2) within the *Burkholderiaceae* (e.g. genera *Ralstonia, Cupriavidus*, and many species within the *Burkholderia* genus) and *Comamonadaceae* (e.g. genera *Methylibium*, *Comamonas,* and *Acidovorax*) (Cavalca et al., 2004; Rhee et al., 2004; Nakatsu et al., 2006; Piskonen et al., 2008) substantiate, that well-known potential aerobic degraders were abundant on-site in the reduced plume core. These known aerobic degrader lineages are frequently detected at contaminated sites (Cavalca et al., 2004). Some of them can even use several BTEX compounds, and degrade also chlorinated hydrocarbons.

As mentioned, it was intriguing to detect these typically aerobic degrader lineages in plume compartments considered as strictly reduced. Therefore, the next important steps would now be to link the detected genotypes to actual catabolic activity *in situ*. For this purpose, two well-established approaches of the molecular ecology toolbox can be employed, the detection of mRNA transcripts by means of reverse transcription (RT-)PCR and the use of stable isotope probing (SIP). In the first approach, RNA is extracted directly from the sediments, and specific mRNA transcripts can be detected and quantified by quantitative reverse transcription (RT-qPCR) with distinct BTEX catabolic gene primers. The use of RT-qPCR is an accurate and sensitive method to quantify actively transcribed BTEX catabolic genes directly in the examined environmental samples. The power of transcript analysis is based on the short half-life time of mRNA, sometimes staying intact only for some minutes (especially in surface waters) after gene expression (Alifano et al., 1994). As regulation at the transcription level affects the rate of protein synthesis almost instantaneously, the detection of mRNA expression can be used for investigation of on-going microbial activities in real-time (Alfreider et al., 2003).

In the tar-oil contaminated Flingern aquifer, attempts to detect respective *tmoA* mRNA were made in this thesis. As described in the results of chapter 2, 16S rRNA was indeed extractable from aquifer sediments of different redox zonations and amplifiable via RT-PCR. However, *tmoA*-mRNA was undetectable with diverse tested RNA extraction and purification protocols. This suggests either a lack of *in situ* gene expression, already degraded mRNA by repeated freezing and thawing of sediments, or general problems in effective mRNA extraction from these contaminated subsurface sediments. The latter two scenarios are indeed likely, as we also failed to exemplarily detect transcripts of the dissimilatory sulphite reductase (*dsrB*) in our extracts, which should principally be

detectable since sulphate reduction is undisputedly an actively on-going process at the site. Nevertheless, all attempts to detect *tmoA* mRNA were unsuccessful, thus leaving the question of tentative activity of aerobic degraders in reduced plume zones unanswered for now.

Only in a small number of studies, mRNA of specific (and abundant) marker genes has been detected in aquifer samples to date. However, none of these successful RT-PCR detections has targeted aerobic degradation genes involved in the initial oxidation of monoaromatic hydrocarbons. An important example is the mRNA of particulate methane monooxygenase (*pmoA*), which could be detected qualitatively from contaminated groundwater wells (Cheng et al., 1999). In another study, mRNA of naphthalene dioxygenase was amplified and sequenced after filtration of 5 L groundwater samples (Wilson et al., 1999). As a possibly successful further strategy for the oxic-anoxic Düsseldorf aquifer, fresh, unfrozen sediments should be sampled and immediately examined for mRNA, thus substantiating respective degradation activity. Anyhow, this thesis showed how molecular methods can unravel degrader potentials *in situ* at high spatial resolution, hence enhancing our present understanding of the stratification of biodegradation processes going on in aquifers. A detailed picture of the distribution of active catabolic potentials as well as their temporal development in contaminated groundwater systems significantly increase our understanding of occurring processes even further. This information is vital for designing and optimizing monitored natural attenuation or bioremediation strategies, especially if combined with classical biogeochemical site monitoring, as discussed further down.

5.2. Linking ribosomal and catabolic markers for degraders *in situ*

The detection of catabolic gene populations in the field comes with a high probability of detecting unidentified or at least distantly related marker gene lineages (Ravatn et al., 1998; Teske et al., 2003; Winderl et al., 2008). Therefore, the next crucial step in characterising *in situ* degrader populations is to link catabolic potentials to taxonomically defined lineages, and thus to unambiguously identify the dominating populations. This, however, is not trivial and several strategies can be chosen. As classical microbiological approaches, enrichment, isolation, and cultivation can deliver clear links between taxonomy and functionality. However, despite considerable efforts to mimic exact

in situ growth conditions, it is often not possible to successfully retrieve the most important community members involved actively in degradation *in situ*, which may even be playing an important role but not be very abundant *in situ*. Here, a approach based on DNA stable isotope probing (Pilloni et al., 2011) could be chosen under aerobic and anaerobic conditions, in order to identify actively involved degraders. Then, these identified community members can be further analysed in their genetic and physiologal characteristics. Novel metagenomic sequencing strategies would also be an innovative alternative to link phylogenetic and functional genes, but would require quite an effort in terms of time and money to optimize and establish it specifically for aquifer sediments and groundwater.

At the Flingern site, this thesis also aimed to phylogenetically affiliate the detected dominating toluene monooxygenase genes. Since toluene is the dominating contaminant and electron donor at the site (Anneser et al., 2010), it was expected that respective degraders would be detectable via both catabolic and ribosomal marker genes. Ideally, phylogenetic congruencies between both approaches should allow for an affiliation of detected monooxygenases within total bacterial communities. Consistently, rRNA and *tmoA* sequence types related to *Ralstonia* or *Burkholderia* spp. were found in the plume core, although not very abundant. Here, it was highly probable although not directly proven, that both represented the same components of the *in situ* degrader community. On the other hand, rRNA sequences related to potential aerobic aromatics degraders within the *Comamonadaceae* were abundant in the plume core on both clone and T-RF level, but not clearly classifiable at the *tmoA* level. The *Comamonadaceae* are indeed known to harbour aerobic toluene degraders (Nakatsu et al., 2006) with toluene monooxygenase (*tbm*) genes. These toluene monooxygenase genes are only distantly affiliated with the *tmoA* gene family, but were potentially also detected and quantified with the used primer set. Hence, theoretically, these dominating *tmoA* OTU *in situ* could indeed be linked to the phylogenetic group of *Comamonadaceae*.

One should be aware that 16S rRNA marker gene studies allow only for a tentative detection of potential toluene or aromatics degraders based on the close affiliation of detected lineages with known degrader isolates. Furthermore, the tentative phylogenetic affiliation of catabolic markers have to be interpreted with caution because several genes connected to aerobic BTEX degradation are located on mobile genetic elements (Sentchilo et al., 2000). To clearly link detected *tmoA* and 16S rRNA genes, dedicated labelling studies, i.e. via DNA-based stable isotope probing (SIP) would be necessary. It is also

important to keep in mind that the divergence of hydrocarbon-degrading microorganisms probably occurred quite early in evolution (Junca and Pieper, 2010). This might explain their widespread occurrence in different phylogenetic lineages and inversely, due to stimulated genetic exchange mechanisms under contamination (van der Meer 2006), the appearance of dissimilar catabolic genes in closely related species.

Also, it cannot be excluded that further potentially important catabolic gene lineages for toluene degradation in Flingern were missed by the detection assays employed in this thesis. Therefore, the development of more extensive screening platforms (Vilchez-Vargas et al., 2012) for a more comprehensive detection and quantification of aerobic BTEX degraders in polluted groundwater is still an important task (for example by metatranscriptomic approaches).

5.3. Correlating degrader populations to geochemical and isotopic groundwater monitoring

In the second part of this thesis (chapter 3), an indoor aquifer mesocosm system was investigated as an integrated approach to unravel the response of a pristine aquifer ecosystem towards toluene contamination under 'close-to-natural' but controlled experimental conditions. Controlled lab experiments allow to follow effects of a contamination through various phases, but, in most cases they are not conducted at relevant scales. Many studies focussing on biodegradation in groundwater have actually been working with different small-scale column setups or similar (batch) microcosm systems in the lab. Other experiments were performed with 2D-tank systems, but predominantly addressing hydrological and geochemical aspects of bioremediation (e.g. Bauer et al., 2008; Heiderscheidt et al., 2008). Most frequently, micro- and molecular biology approaches were applied in microscale (column) systems, while larger setups were employed for hydrogeochemical experiments.

Small scale experiments are only partially relevant for processes *in situ*. Frequent limitations of such 'classical' lab experiments are the lack of groundwater flow, the use of artificial groundwater and sediments, the absence of a pristine background microbial community, and no-adequate temporal and spatial resolution and succession. Exactly these points were the main advantages of the mesoscale model aquifer investigated in this thesis, and therefore it was planned and within the GOODWATER project, and operated in a joint

synergistic research project. The team working with the indoor model aquifer consisted of several PhD-researchers, each with specific own project objectives, including a comprehensive linking of all possible process indicators (geochemistry, isotopes, biomass, general microbiology, and molecular data) to test how the different lines of evidence for ongoing natural attenuation conform.

The up-scaling of findings on processes and community dynamics from model systems (micro- as well as mesoscale) to the field scale is difficult and not straightforward. In the used indoor model aquifer system, longitudinal and depth transects of bacterial population shifts upon toluene contamination were elaborated and showed a pronounced temporal and spatial succession. Most prominently, it was clearly shown that toluene is a readily utilisable carbon source for microbes even in pristine aquifer environments with no previous history of contamination. The 16S rRNA gene abundance even indicated a considerable increase in total bacterial cell numbers upon toluene contamination. The same was observed for column experiments, where a biomass increase coupled to high degradation rates showed a correlation of bacterial cells to the toluene degradation (Kim and Jaffe, 2007). Such a fast development of specialized degraders coupled to an increase of total biomass, and specially of degradation genes has never been observed at a natural field site to date. If at all, this would be possible only for a 'young' BTEX plume migrating downstream with groundwater flow.

Finally, in an actually on-going site remediation campaign (chapter 4), the catabolic gene-based monitoring concept established in this thesis was combined with routine biogeochemical site monitoring by stakeholders. The objective was here to demonstrate the added benefit of molecular approaches, gained in surplus to classical geochemical – and also isotopic monitoring, and to help in developing enhanced natural attenuation and bioremediation strategies. Catabolic marker gene counts provided important additional information on the occurrence of distinct aerobic and anaerobic hydrocarbon degradation zones *in situ* (Figure 4.4). This was relevant novel information, as the stakeholders engaged at the site had to date not been aware of extant anaerobic degradation processes. A clear spatial resolution of overall catabolic potentials was shown and potential hot-spots of anaerobic degradation were found especially in compartments strongly exposed to contamination. This compartmentalisation was not depicted by any of the other applied conventional monitoring strategies, thus demonstrating the merit of molecular tools to assist decisions on appropriate bioremediation strategies.

All available geochemical data from the Augsburg site suggested biodegradation processes in the aquifer to occur under oxic conditions. Oxic degradation accelerates the degradation of persistent contaminants like PAHs (Bamforth and Singleton, 2005) compared to monocyclic aromatic hydrocarbons (BTEX). The local distinctions in pollutant scenarios of the explored field site were clearly visible on 16S rRNA gene level, with local changes in overall bacterial community structure and lineage-specific abundance patterns. Although detailed taxonomic information is still missing for that site, a first image of the occurrence and distribution of microbial populations potentially involved in ongoing attenuation processes is thus now available. In contrast, interpretations of on-site processes via hydrogeochemical characterisations alone are reliable only on a much more general level or scale. Sampling of groundwater in wells mixed over depth and the resulting lack of spatial resolution is a major caveat here.

Thus, catabolic markers can provide important additional information on the occurrence of distinct degrader populations and degradation processes in a contaminated aquifer. Although catabolic marker genes were for aerobic and anaerobic PAH degradation were not applied in this field study, mainly because a functional marker assay for anaerobic PAH degradation is not available yet, markers for mono-aromatic hydrocarbon degradation allowed at least some generic statements on *in situ* aerobic vs. anaerobic catabolic potentials. In addition to the lines of evidence for aerobic aromatic degradation provided by the geochemical and isotopic monitoring strategies applied in parallel (by the Isodetect project partners), a clear spatial resolution of aerobic vs. anaerobic catabolic pathways was shown via molecular monitoring. Interpretation of such scenarios is still complex, but a principal congruency of evidence resulting from different monitoring methods – stable isotopes and molecular approaches – show that the strategies ideally complement one another. Anyway, the different lines of evidence (geochemical, isotopic, microbiologic and molecular) for ongoing natural attenuation at the site are overlapping for the different points of observation. Such comprehensive, all-embracing analyses will help to strengthen on site monitoring and remediation strategies. The prediction of dominating degrader physiologies via molecular methods included in the routine site monitoring programs would be a beneficial development here.

5.4. An emerging new view of redox gradients in contaminated aquifers

In this thesis, a detailed and elaborate perspective of the initial impacts of a contamination event on a pristine aquifer model ecosystem is presented. The impacts of contamination on the spatial and temporal succession of overall bacterial communities and the establishment of contaminant-degrading populations were traced. Moreover, first spatially highly-resolved insights into distinct and partially unexpected quantitative distribution patterns of aerobic and anaerobic toluene degraders over the redox gradients and compartments of a real BTEX plume were provided. As shown previously (Winderl et al., 2008; Anneser et al., 2010), the highest *bssA* abundance correlated with maximal sulphide production as well as overlapping toluene and sulphate gradients at the lower plume fringe. In clear contrast, monooxygenase (*tmoA*) genes were found (and most abundant) in plume zones considered as highly reduced, i.e. the actual plume core, and did not increase in abundance towards the upper fringe. Consistently, putative degraders lineages related to potential aerobic hydrocarbon degraders within the *Burkholderiaceae* and *Comamonadaceae* were found abundant in the reduced plume core. Thus, aerobic degrader lineages were not positioned as expected at the redox gradient.

Moreover, the marker gene for anaerobic toluene degradation (*bssA*) was detected in groundwater of the indoor aquifer model system over the whole duration of the experiment, already upon initially low contaminant quantities. In contrast, an increased *bssA* quantity was detected in distinct sediment compartments at the highest toluene concentrations only. The anaerobic toluene exposure time was probably too short for an establishment of highly abundant *bss*-utilising microorganisms. The plume center was without oxygen only for a few days. Still, a simultaneous aerobic and anaerobic degradation potential developed and was observed.

Similarly, an unexpected distribution of catabolic potentials was detected also at the Düsseldorf-Flingern aquifer. Aerobic catabolic potentials were found most abundant in strictly reduced plume compartments. A possible explanation could be conceived based on the recent discovery of methane oxidation under absence of external oxygen by methanotrophs via self-sustained oxygenesis by NO-dismuation (Ettwig et al., 2010). Another new scenario for ‘aerobic’ degradation in absence of oxygen could involve electron flow at the cm-scale via conductive microstructures (El-Naggar and Finkel, 2013). At any rate, the anoxic-aerobic BTEX degradation scenario remains speculative, as long as no direct evidence for specific toluene oxidising activity is available. Still, this points

towards a potentially underestimated importance of unexpected 'aerobic' aromatics degraders in contaminated aquifers. This on its part suggests that the monitoring of distinct catabolic potentials across plume compartments may help to identify overlooked hot spots of biodegradation in-situ.

5.5. Heterogeneity of aerobic and anaerobic degradation in contaminated aquifers

Quantitative couplings between local aerobic and anaerobic degrader abundances and the importance of the respective degradation processes are difficult to substantiate. Interestingly, aerobic degraders with (toluene) monooxygenase enzymes are more effective in biodegradation under low oxygen concentrations than those with dioxygenases. Respective degradation rates decline less with decreasing oxygen availability. In both microaerophilic degradation and aerobic degradation under nitrate respiration, nitrate enhances the degradation only below a critical oxygen availability, which is dependent on the specific degraders and substrates (Leahy and Olsen, 1997). The amount of toluene degraded aerobically in the IGÖ flume can only be explained a strong replenishment of oxygen, including also the occurrence of aerobic degradation under denitrification.

However, also anaerobic degradation was demonstrated to occur, especially after the added catastrophic contamination event. In addition to *bssA* gene abundance, also the formation of nitrite was a clear (qualitative) confirmation of nitrate reduction in our aquifer model upon high toluene contamination. Nitrite is only a leaky intermediate in denitrification, therefore the small amount measured cannot be indicative of the reduced nitrate. The geochemical zonation can be very complex in aquifers with hydrocarbon contamination, with strong spatial and temporal heterogeneities related to the sedimentary matrix, hydraulic fluctuations as well as dynamic fluxes of contaminants and oxidants. Although groundwater ecosystems are often considered as steady-state, there is never a stable equilibrium between the distribution of electron donors and –acceptors.

Dynamics of intrinsic microbial diversity patterns in the indoor model aquifer following the toluene impact suggested the rapid development, within days to weeks, of a specialized bacterial community adapted to toluene degradation. Here, toluene and oxygen consumption occurred in parallel to nitrate reduction as indicated by nitrite production. The key lineages putatively involved in aerobic toluene degradation were especially *Pseudomonas*-related as well as members of the *Comamonadaceae* and *Rhodocyclaceae*.

Under successively anaerobic conditions, a denitrifying toluene degradation by *Azoarcus* spp. populations was apparent only for the sediments, intriguingly. In both natural aquifers and groundwater microcosms, benzene degradation has been linked to nitrate reduction, nitrite accumulation, and biomass growth (Burland and Edwards, 1999). Additionally, denitrification in conjunction with toluene degradation has also been observed in aquifers at different initial oxygen levels, up to saturation (Durant et al., 1999; Kim and Jaffe, 2008). Together with our results this indicates, that a simultaneous degradation of toluene with various electron acceptors does actually occur in such systems, or that processes are spatially separated on a very small scales.

Due to their widespread ability to respire both oxygen and nitrate, facultatively anaerobic, denitrifying bacteria typically occur in transition zones at redox gradients with with microaerophilic conditions. When oxygen drops below a certain concentration, these bacteria will switch to nitrate respiration; however, a common general oxygen threshold level for this switching has not been found yet (Wilson and Bouwer, 1997). Most probably there is a critical range of oxygen concentrations above which denitrification cannot proceed; depending on complex environmental factors including mass transfer and genetic regulation (Wilson and Bouwer, 1997). Under relatively high amounts of oxygen, limitations by blocking the synthesis of denitrification enzymes or inhibition of nitrate transport are postulated independently from nitrate concentration (Wilson and Bouwer, 1997). The interaction of oxygen and nitrate respiration in our study shows more a synergy of electrons acceptors usage than a competition. Also, in porous media aerobic and anoxic environments may co-exist.

Toluene degradation trough monooxygenase pathways and the ability of denitrification under aerobic conditions most probably are independent adaptations for the utilization of toluene by aerobic bacteria in environments with low oxygen (Leahy and Olsen, 1997). For our flume, it could be speculated that some *Pseudomonas* spp. could even have degraded toluene aerobically while respiring both, O_2 and/or NO_3^-. However, this requires further elucidation. Besides spatial heterogeneity, time was also an important driver of process allocation in the flume. The two assumed modes of toluene degradation, oxygenase-dependent and *bss*-dependent under denitrification, may have operated simultaneously, but in distinct micro-niches. It must be considered that the *BSS* enzyme is extremely sensitive towards oxygen. Therefore, *Azoarcus* spp. degraders could not have initiated their degradation biochemistry in the presence of oxygen. The fact that both populations were observed shows a surprising heterogeneity of the aquifer matrix at the

smallest scale, with oxygen consumption occurring in close spatial proximity of anoxic processes. Still, the detection of respective potentials on DNA level does not immediately imply activity. It may well be true that some lineages were able to switch their metabolism 'on' and 'off' within relatively short time intervals.

In sum, bacterial 16S rRNA gene information nicely matched to dominating biogeochemical processes ongoing in a given compartment of our indoor aquifer system over time. It was shown how ongoing reactive processes and involved degrader populations evolve simultaneously, adapting spatially to local contaminant and redox scenarios but staying highly variable in groundwater. Sediment and groundwater should be sampled together, for the most comprehensive picture of heterogeneous degradation processes occurring in natural aquifers. While free groundwater seems to inform better on temporal heterogeneity and fluctuations, sediment samples seem to give more meaningful detail on spatial successions and co-existence of degradation potentials. Together with the spatial distribution of potential toluene degraders reported here, this suggests that competitive niche partitioning between aerobic and anaerobic hydrocarbon degraders in the field may not yet be fully understood and that several available electron acceptors like oxygen and nitrate can be simultaneously used for toluene removal. Highly resolved definitions of local degrader communities showed a strong spatial separation. Hence, in addition to the concept of simultaneity and overlapping of processes, a strong temporal dynamic of microbial communities and processes is presented here, adding to the concept of temporal disturbance and dynamics for aquifer microbiota (Pilloni et al., 2011). A surprisingly fast reactivity of microbial communities and processes in a pristine aquifer to contamination was discovered, and a synchronicity of processes depending on different electron acceptors in space and time (Figure 3.2). This initial synchronicity of aerobic and anaerobic catabolic potentials could only have been proven experimentally, in a realistic mesoscale aquifer model system as used in this thesis and within the GOODWATER project.

5.6. Conclusion and outlook

In this thesis, the limitations and driving forces of biodegradation in groundwater systems were explored via spatial and temporal sampling, embracing a very strong ecological perspective of the involved microbes (e.g. extant catabolic potentials, key

players and populations, bacterial ecophysiology and also potential trophic connectivities). It becomes more and more clear, that the term 'redox condition' should be used to name the dominating redox process amongst several simultaneously occurring reduction processes (Christensen et al., 2000). This thesis substantiates two important novel aspects that expand the current ecological perception of hydrocarbon contaminant plumes in groundwater. It emphasises the importance of considering such plumes as 'holistic' system from a microbial perspective, including its distinct compartments, gradients, as well as dynamic temporal structure to comprehend the most prominent drivers of natural attenuation. Several synchronous lines of evidence for the spatially defined and dynamic development of specialised aerobic and anaerobic degrader communities involved in toluene degradation in groundwater were provided. The time scales over which such contaminant plumes are dynamic as a microbial ecosystem are thus now better understood, but clearly merit future scientific attention. This thesis provides primary field evidence that the competition, or better synergy between aerobic and anaerobic degradation processes may be controlled by more than just electron acceptor availability, and that some supposedly aerobic degraders may be physiologically more flexible than currently perceived.

Second, unprecedented details on the immediate response of a pristine aquifer indoor model system to toluene contamination were provided. The spatial and temporal succession of reactive processes and involved microbiota were traced for the first time. These outcomes provided unique new insights on the initial reaction of pristine aquifer microbiota to contamination, something that cannot be investigated in the field. It was shown how ongoing reactive processes and involved degrader populations evolve simultaneously, adapting spatially to local contaminant and redox scenarios but staying highly variable in groundwater.

In the future, molecular and isotopic analyses should be not only employed, but also synchronized in routine site monitoring, to obtain a comprehensive, integrated understanding of heterogeneous sites. This study may guide further efforts to this end. The development of optimized quantitative detection assays or platforms to fully trace and interpret the presence of distinct catabolic potentials in the field, especially at plume fringes and in plume cores is still an important task. Furthermore, to clearly identify dominating degrader lineages found *in situ*, meticulous labeling studies, i.e. via DNA-based stable isotope probing (SIP) studies, transcriptional approaches, and isolation/culturing strategies (for key-players not available in culture yet) are all of great

importance. This will be indispensable to truly unravel the ecology of contaminant plumes *in situ*, and to the design of 'tailor-made' bioremediation strategies and next-generation monitoring concepts for natural attenuation by stakeholders.

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Authorship clarifications

Chapter 2: "*Fine scale degrader community profiling over an aerobic/anaerobic redox gradient in a toluene contaminated aquifer*"

The idea and concept for the experimental design were from Tillmann Lüders and the PhD candidate. Bettina Anneser, Anne Bayer, Florian Einsiedl, and Christian Griebler performed on-site geochemical. The PhD candidate did all DNA extractions, gene quantifications, cloning and sequencing analyses, statistics, as well as the evaluation of the results (the latter together with Tillmann Lueders). Katrin Hörmann (Technical Assistant) helped with microbial community fingerprinting. The manuscript was written by me and Tillmann Lüders. The manuscript was accepted on September 21st, 2012, in Environmental Microbiology Reports.

Chapter 3: "*Fast microbial community response and development of aerobic and anaerobic degrader populations upon toluene contamination of a pristine indoor model aquifer*"

The concept and experimental design was developed between the PhD candidate and Tillmann Lüders, in close scientific interaction with Agnieszka Herzyk, Christian Griebler, and Rainer Meckenstock of the IGOE. The overall setup of the indoor aquifer was technically planned and realized by Piotr Maloszewski, Marko Hünninger, Susanne Schmidt and the technical staff of the IGOE. Sampling campaigns were planned and organised by the PhD candidate together with Agnieszka Herzyk, Sviatlana Marozava, Shiran Qiu, Michael Granitsiotis (the GOODWATER PhD students), and also practical help of the technical assistants Katrin Hörmann, Gabriele Barthel, Sigrid Kaschuba, Martina Höche, and Günter Hinreiner of the IGOE. Daily hydro-chemical measurements were performed by Günter Hinreiner and the PhD candidate (in turns), oxygen measurements by the PhD candidate and Sigrid Kaschuba. Geochemical measurements including nitrate and nitrite quantifications were done by the PhD candidate, partly with help from Michael Stöckl and Peter Knappet (IGÖ). Toluene in groundwater was measured by Shiran Qiu, with technical support of Martina Höche, from the Environmental Isotope Chemistry group. Nucleic acid extractions were done by the PhD candidate and Agnieszka Herzyk, with occasional help from Katrin Hörmann and Sigrid Kaschuba. Microbial community fingerprints and gene quantification were done by the PhD candidate. The candidate prepared and processed the tagged amplicons for pyrosequencing, which was

then technically performed by Marion Engel (EGEN) and also Michael Granitsiotis (IGÖ). The evaluation and interpretation of the results was performed by the PhD candidate and Tillmann Lüders, Agnieszka Herzyk assisted with multivariate statistics. The manuscript was written by the candidate together with Tillmann Lüders.

Chapter 4: "*Molecular monitoring strategies for the assessment of natural attenuation based on catabolic gene abundances in correlation to geochemical and isotopic field data*"

The concept for the field study was developed by the PhD candidate together with Tillmann Lüders and Heinrich Eisenmann (Isodetect). The company "mplan eG" performed groundwater sampling and hydro-geochemical analyses. Isodetect GmbH performed all laboratory incubations, biodegradation experiments and isotopic analyses. The PhD candidate performed sediment sampling, nucleic acid extraction, microbial community fingerprinting and gene quantification. The evaluation and interpretation of the results was performed by the PhD candidate and Heinrich Eisenmann. The manuscript was written by the candidate together with Tillmann Lüders and Isodetect.

Publications in peer reviewed journals or in preparation by the author during this thesis:

- Larentis, M. and Alfreider, A. (2011) Comparative evaluation of prokaryotic 16S rDNA clone libraries and SSCP in groundwater samples. J Basic Microbiol 51: 330–335. *[manuscript stems from the diploma thesis of the author and does not relate to the content of this thesis]*
- Larentis, M., Hoermann, K., and Lueders, T. (2013) Fine scale depth monitoring of aerobic vs. anaerobic toluene degradation potentials over a redox gradient in a contaminated aquifer. Environ Microbiol Rep 5: 225-234.
- Fast microbial community response and development of aerobic and anaerobic degrader populations upon toluene contamination of a pristine indoor model aquifer. *[in preparation based on chapter 3, to be submitted to Environ Microbiol]*

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

- VAAM & DGHM conference in Hannover (Germany), March 28-31, 2010.
- VAAM conference in Karlsruhe (Germany), April 3-6, 2011.
- ISSM conference in Garmisch-Partenkirchen (Germany), September 11-16, 2011.

Collaborations and scientific exchange conducted within the GOODWATER project

- Collaboration with E. Dogan in VITO (Belgium) for DGGE-screening of aerobic aromatic degradation genes, April 2010.
- Hosting of M.B. Pazarbasi (GEUS, Denmark) for tRFLP analyses, April 2011.
- Metagenomic library screening for catabolic marker genes with E. Bouhajja at UCL (Belgium), February 2012.
- Visiting internship by the candidate with Isodetect GmbH (Neuherberg), for a work experience in contaminated site monitoring and site management, June 2011.

Attendance of scientific workshops within the GOODWATER ITN.

- August 17-21, 2009: workshop nr.1 and general assembly 1 in Gilleleje, Denmark.
- December 7-11, 2009: introductory winterschool in Munich, Germany.
- September 6-10, 2010: workshop nr.2, general assembly 2, and EU-Midterm Review in Schliersbergalm, Germany.
- January 10-14, 2011: winterschool nr.2 in Gent and Mol, Belgium.
- October 10-13, 2011: workshop nr.3 and general assembly 3 in Leuven, Belgium.
- January 16-19, 2012: winterschool nr.3 in Copenhagen, Denmark.
- March 12-15, 2012: final project-workshop and conference in Munich, Germany.

Appendix

Table A1. Chemical parameters through the BTEX plume of the Düsseldorf-Flingern aquifer (Feb. 2006); as plotted in Fig. 2.1. Measurements performed by B. Anneser and A. Bayer of the Microbial Ecology group, IGÖ.

depth [m]	toluene [mg L^{-1}]	Std.Dev.	redox [mV]	NO_3^- [mg L^{-1}]	Std.Dev.
6.385	0.00		-20	1.82	0.00
6.435	10.72	0.65		1.10	0.02
6.460	21.09	1.62	-100	1.09	0.01
6.485	29.45	0.34		1.18	0.06
6.510	31.60	5.00		1.14	0.02
6.535	36.91	0.40		1.13	0.03
6.560	38.08	0.65	-150	1.17	0.00
6.585	42.12	0.26		2.69	0.02
6.610	44.75	1.39		1.90	1.17
6.635	45.38	0.88		2.71	0.02
6.665	44.66	0.46	-190	2.72	0.01
6.695	45.34	0.95		2.69	0.00
6.805	39.80	0.19	-235	1.96	1.21
6.905	31.97	0.19		2.71	0.00
7.105	4.08	0.08	-230	2.75	0.01

Table A2. Average of the extraction-bias corrected bacterial qPCR gene counts for contaminated sediment of the Düsseldorf-Flingern aquifer. Measurement values are given with application of the methodological correction factor of 5.5 for bacterial 16S rRNA genes and 2.7 for *tmoA* for the sedimentary DNA extraction and quantification bias (Larentis et al., 2013; Winderl et al., 2008).

depth [m]	16S [cop. g^{-1}]	Std.Dev.	*bssA* [cop. g^{-1}]	Std.Dev.	*tmoA* [cop. g^{-1}]	Std.Dev.
6.450	1.87×10^8	9.65×10^7	3.47×10^6	7.65×10^5	3.53×10^6	2.36×10^6
6.500	1.37×10^8	4.40×10^7	8.57×10^6	6.96×10^5	2.74×10^6	1.32×10^6
6.550	1.12×10^8	9.93×10^7	1.29×10^7	5.17×10^6	3.54×10^6	3.96×10^6
6.575	1.31×10^8	6.70×10^7	1.91×10^7	1.83×10^6	9.21×10^6	5.80×10^6
6.600	8.49×10^7	7.32×10^7	2.56×10^7	1.15×10^6	5.53×10^6	6.16×10^6
6.625	5.29×10^7	5.66×10^7	1.46×10^7	1.32×10^6	4.52×10^6	5.45×10^6
6.650	8.74×10^8	6.47×10^8	1.13×10^7	9.82×10^6	1.70×10^6	2.47×10^6
6.700	7.77×10^8	5.45×10^8	1.54×10^7	1.44×10^6	1.83×10^6	2.41×10^6
6.800	5.90×10^8	5.30×10^8	9.17×10^7	2.06×10^7	1.46×10^6	1.04×10^6

Table A3. Replicated qPCR measurements of bacterial 16S rRNA genes in the Düsseldorf-Flingern aquifer. Measurement values are given without application of the methodological correction factor (Winderl et al., 2008).

16S [cop. g^{-1}]	Avg.	Std.Dev.	depth [m]	name
4.05×10^7				
3.19×10^7				
1.63×10^7				
4.28×10^7				
7.48×10^7				
2.28×10^7				
2.83×10^7				
2.15×10^7				
2.71×10^7	3.40×10^7	1.75×10^7	6.450	DV1
2.90×10^7				
2.38×10^7				
3.30×10^7				
1.84×10^7				
2.02×10^7				
2.97×10^7				
8.37×10^6				
3.19×10^7				
2.93×10^7	2.49×10^7	8.00×10^6	6.500	DV2
7.05×10^7				
1.52×10^5				
7.26×10^1				
7.27×10^6				
2.65×10^7				
3.59×10^7				
3.94×10^7				
3.75×10^7				
3.66×10^7	2.04×10^7	1.81×10^7	6.550	DV3
3.73×10^7				
3.87×10^7				
3.42×10^7				
8.29×10^6				
5.83×10^6				
2.02×10^7				
3.12×10^7				
1.98×10^7				
1.88×10^7	2.38×10^7	1.22×10^7	6.575	DV4
4.04×10^6				
2.74×10^6				
4.43×10^6				
3.35×10^7				
2.31×10^7				
2.48×10^7	1.54×10^7	1.33×10^7	6.600	DV5
7.19×10^5				
2.31×10^4				
1.35×10^5				
2.03×10^7				
1.71×10^7				
1.95×10^7	9.63×10^6	1.03×10^7	6.625	DV6
1.30×10^7				

1.44×10^7 1.03×10^7 2.19×10^8 2.07×10^8 1.42×10^8 2.78×10^8 2.70×10^8 2.76×10^8	1.59×10^8	1.18×10^8	6.650	DV7
1.72×10^7 1.78×10^7 1.01×10^7 1.44×10^8 1.79×10^8 2.38×10^8 2.09×10^8 2.15×10^8 2.42×10^8	1.41×10^8	9.92×10^7	6.700	DV8
2.26×10^8 2.70×10^8 1.92×10^8 1.48×10^7 1.42×10^7 8.67×10^6 7.31×10^7 7.11×10^7 9.63×10^7	1.07×10^8	9.63×10^7	6.800	DV9

Table A4. Replicated qPCR measurements of bacterial *bssA* and *tmoA* genes in the Düsseldorf-Flingern aquifer. Measurement values are given without application of the methodological correction factor for *tmoA* of 2.7 for sedimentary gene extraction and quantification bias as determined in this study.

name	**depth [m]**	***bssA* [cop. g^{-1}]**	***tmoA* [cop. g^{-1}]**
DV1	6.450	2.70×10^6	3.89×10^2
DV1	6.450	4.74×10^6	1.48×10^6
DV1	6.450	3.12×10^6	3.59×10^5
DV1	6.450	3.33×10^6	2.08×10^6
DV2	6.500	9.73×10^6	4.50×10^5
DV2	6.500	8.25×10^6	1.32×10^6
DV2	6.500	8.43×10^6	1.38×10^3
DV2	6.500	7.88×10^6	1.27×10^6
DV3	6.550	7.19×10^6	3.62×10^5
DV3	6.550	1.90×10^7	1.65×10^5
DV3	6.550	1.70×10^7	3.37×10^6
DV3	6.550	8.46×10^6	1.36×10^6
DV4	6.575	2.12×10^7	1.08×10^6
DV4	6.575	1.63×10^7	3.85×10^6
DV4	6.575	2.02×10^7	1.48×10^2
DV4	6.575	1.89×10^7	5.31×10^6
DV5	6.600	1.58×10^7	4.72×10^5
DV5	6.600	1.26×10^7	1.84×10^6
DV5	6.600	3.84×10^7	5.46×10^5

DV5	6.600	3.57×10^7	5.34×10^6
DV6	6.625	1.43×10^6	1.78×10^5
DV6	6.625	1.38×10^6	2.45×10^5
DV6	6.625	2.83×10^7	1.77×10^6
DV6	6.625	2.74×10^7	4.49×10^6
DV7	6.650	1.43×10^6	1.40×10^5
DV7	6.650	1.47×10^6	1.31×10^5
DV7	6.650	2.10×10^7	2.49×10^5
DV7	6.650	2.11×10^7	2.00×10^6
DV8	6.700	2.06×10^6	1.43×10^5
DV8	6.700	2.21×10^6	1.88×10^5
DV8	6.700	2.07×10^7	1.61×10^3
DV8	6.700	3.65×10^7	1.71×10^6
DV9	6.800	1.04×10^8	9.83×10^4
DV9	6.800	1.19×10^8	7.92×10^5
DV9	6.800	7.44×10^7	1.51×10^3
DV9	6.800	6.93×10^7	7.30×10^5

Table A5. Chemical parameters in the indoor model aquifer over time points as averages of the duplicate measurements. Toluene was measured by S. Qiu from the Environmental Isotope Chemistry group, IGÖ. Nitrate and nitrite were measured by the PhD candidate, partly with help from M. Stöckl and P. Knappet (IGOE).

t after toluene injection [d]	**flow distance [m]**	**depth [m]**	**O_2 [mg L^{-1}]**	**toluene [mg L^{-1}]**	**NO_3^- [mg L^{-1}]**	**NO_2^- [mg L^{-1}]**
0	0.35	0.49	n.m.	n.d.	5.15	n.d.
0	0.35	0.45	8.93	n.d.	5.11	n.d.
0	0.35	0.41	8.98	n.d.	5.07	n.d.
0	0.35	0.36	9.03	n.d.	5.06	n.d.
0	0.35	0.31	8.58	n.d.	5.09	n.d.
0	0.35	0.27	9.05	n.d.	5.09	n.d.
0	0.35	0.23	9.25	n.d.	5.11	n.d.
0	0.35	0.19	9.44	n.d.	5.09	n.d.
0	1.10	0.49	8.97	n.d.	6.38	n.d.
0	1.10	0.41	8.83	n.d.	6.15	n.d.
0	1.10	0.36	8.77	n.d.	6.16	n.d.
0	2.20	0.31	8.71	n.d.	6.21	n.d.
0	2.20	0.23	8.71	n.d.	6.26	n.d.
0	2.20	0.15	8.82	n.d.	6.14	n.d.
0	4.40	0.57	8.22	n.d.	5.17	n.d.
0	4.40	0.49	7.47	n.d.	5.00	n.d.
0	4.40	0.41	7.40	n.d.	5.38	n.d.
0	4.40	0.36	7.40	n.d.	4.96	n.d.
0	4.40	0.31	7.33	n.d.	5.06	n.d.
0	4.40	0.23	7.00	n.d.	5.15	n.d.
0	4.40	0.19	7.33	n.d.	5.11	n.d.
0	4.40	0.15	7.50	n.d.	5.08	n.d.
16	0.35	0.62	n.m.	n.d.	8.00	n.d.
16	0.35	0.45	7.74	n.d.	7.77	n.d.
16	0.35	0.41	7.82	n.d.	7.78	n.d.
16	0.35	0.36	7.91	n.d.	7.72	n.d.
16	0.35	0.31	6.01	0.097	7.42	n.d.

16	0.35	0.27	6.08	1.855	6.88	n.d.
16	0.35	0.23	7.10	n.d.	7.84	n.d.
16	0.35	0.19	8.12	n.d.	7.76	n.d.
16	0.35	0.15	n.m.	n.d.	7.84	n.d.
16	1.10	0.62	n.m.	n.d.	10.53	n.d.
16	2.20	0.53	8.64	n.m.	10.65	n.d.
16	2.20	0.49	9.26	n.d.	10.77	n.d.
16	1.10	0.45	8.51	n.m.	10.90	n.d.
16	1.10	0.41	7.76	n.d.	10.11	n.d.
16	1.10	0.36	6.07	n.m.	9.92	n.d.
16	1.10	0.31	4.38	n.d.	9.42	n.d.
16	1.10	0.27	4.07	n.m.	9.57	n.d.
16	1.10	0.23	3.76	n.d.	10.08	n.d.
16	1.10	0.19	5.78	n.m.	10.33	n.d.
16	1.10	0.15	7.81	n.d.	10.37	n.d.
16	4.40	0.62	n.m.	n.d.	11.17	n.d.
16	4.40	0.57	8.93	n.d.	11.25	n.d.
16	4.40	0.49	8.63	n.d.	11.32	n.d.
16	4.40	0.45	8.36	n.d.	11.14	n.d.
16	4.40	0.41	8.08	0.001	10.88	n.d.
16	4.40	0.36	7.81	0.016	10.43	n.d.
16	4.40	0.31	7.54	0.002	10.20	n.d.
16	4.40	0.27	7.36	0.014	10.36	n.d.
16	4.40	0.23	7.17	n.d.	10.28	n.d.
16	4.40	0.19	7.98	0.004	10.72	n.d.
16	4.40	0.15	8.33	0.013	10.87	n.d.
63	0.35	0.45	8.66	n.d.	6.35	n.d.
63	0.35	0.36	8.66	n.d.	6.25	n.d.
63	0.35	0.31	1.67	21.236	5.52	n.d.
63	0.35	0.27	7.21	1.342	5.94	n.d.
63	0.35	0.23	8.15	n.d.	6.11	n.d.
63	0.35	0.19	9.09	n.d.	6.36	n.d.
63	2.20	0.49	8.53	n.d.	8.40	n.d.
63	2.20	0.41	7.63	n.d.	8.44	n.d.
63	1.10	0.36	5.47	0.011	7.55	n.d.
63	1.10	0.31	3.31	1.401	5.26	0.30
63	1.10	0.27	2.60	0.558	6.80	0.05
63	1.10	0.23	1.89	n.d.	8.40	n.d.
63	1.10	0.19	3.88	n.d.	8.42	n.d.
63	2.20	0.15	5.87	n.d.	8.40	n.d.
63	4.40	0.57	8.23	n.d.	8.15	n.d.
63	4.40	0.49	7.69	n.d.	8.29	n.d.
63	4.40	0.36	5.07	0.075	7.34	n.d.
63	4.40	0.31	2.45	0.042	6.60	n.d.
63	4.40	0.27	2.35	0.190	6.18	n.d.
63	4.40	0.23	2.24	0.065	6.29	n.d.
63	4.40	0.19	4.57	0.057	7.04	n.d.
63	4.40	0.15	4.78	0.090	7.87	n.d.
137	0.35	0.45	8.25	n.m.	n.m.	n.m.
137	0.35	0.36	8.24	0.120	0.60	0.07
137	0.35	0.31	5.85	34.819	0.45	0.11
137	0.35	0.27	2.55	92.258	0.69	0.10
137	0.35	0.23	5.74	1.768	0.45	0.07
137	0.35	0.19	8.92	0.037	0.83	0.06
137	0.35	0.15	n.m.	0.248	0.99	0.06
137	2.20	0.49	n.m.	n.m.	n.m.	n.m.

137	2.20	0.41	8.37	n.m.	n.m.	n.m.
137	1.10	0.36	7.56	0.017	0.35	0.05
137	1.10	0.31	5.46	4.990	0.25	0.06
137	1.10	0.27	2.73	45.880	0.23	0.22
137	1.10	0.23	n.d.	39.262	0.82	0.11
137	1.10	0.19	1.09	2.809	0.24	0.04
137	2.20	0.15	2.17	0.136	0.32	0.04
137	4.40	0.57	n.m.	n.m.	n.m.	n.m.
137	4.40	0.49	7.69	n.m.	n.m.	n.m.
137	4.40	0.36	7.34	0.014	0.07	0.48
137	4.40	0.31	n.d.	14.048	n.d.	0.93
137	4.40	0.27	n.d.	18.538	n.d.	0.46
137	4.40	0.23	n.d.	21.776	n.d.	0.21
137	4.40	0.19	n.d.	15.513	n.d.	0.56
137	4.40	0.15	n.d.	2.044	0.02	0.86

n.m., not measured – sample analysis not possible due to technical problems.

n.d., not detectable (below detection limit).

Table A6. qPCR measurements of 16S rRNA, *bssA*, and *tmoA* genes from groundwater and sediments of the indoor aquifer at different time points after toluene contamination. The experimentally determined correction factors of 5.5 for bacterial 16S rRNA (Winderl et al., 2008) and 2.7 for *tmoA* genes (Larentis et al., 2013) for sedimentary gene extraction and quantification bias as were applied.

t after toluene injection [d]	**Flow distance [m]**	**Water depth [m]**	***tmoA* [cop. mL^{-1}]**	***bssA* [cop. mL^{-1}]**	**16S [cop. mL^{-1}]**
0	0.35	0.490	n.d.	n.d.	2.05×10^{4}
0	0.35	0.410	n.d.	n.d.	3.10×10^{4}
0	0.35	0.360	n.d.	n.d.	5.20×10^{4}
0	0.35	0.310	n.d.	n.d.	3.03×10^{4}
0	1.10	0.490	n.d.	n.d.	3.20×10^{4}
0	1.10	0.410	n.d.	n.d.	4.31×10^{4}
0	1.10	0.360	n.d.	n.d.	9.08×10^{3}
0	4.40	0.490	n.d.	n.d.	7.95×10^{4}
0	4.40	0.410	n.d.	n.d.	1.84×10^{4}
0	4.40	0.360	n.d.	n.d.	1.35×10^{6}
0	4.40	0.230	n.d.	n.d.	7.96×10^{4}
16	0.35	0.620	7.38×10^{0}	n.d.	n.m.
16	0.35	0.450	2.13×10^{1}	n.d.	1.71×10^{4}
16	0.35	0.410	1.61×10^{1}	n.d.	1.12×10^{4}
16	0.35	0.360	2.22×10^{2}	n.d.	1.59×10^{6}
16	0.35	0.310	2.07×10^{6}	n.d.	3.18×10^{6}
16	0.35	0.270	1.84×10^{5}	1.63×10^{-1}	5.35×10^{5}
16	0.35	0.230	1.47×10^{1}	n.d.	2.05×10^{4}
16	0.35	0.190	2.82×10^{2}	n.d.	2.34×10^{4}
16	0.35	0.150	1.43×10^{2}	n.d.	n.m.
16	1.10	0.620	2.15×10^{1}	n.d.	n.m.
16	1.10	0.450	8.92×10^{1}	n.d.	1.93×10^{4}
16	1.10	0.410	1.35×10^{1}	2.25×10^{-2}	1.58×10^{4}
16	1.10	0.360	2.88×10^{3}	n.d.	1.95×10^{5}

16	1.10	0.310	4.09×10^{3}	n.d.	5.85×10^{5}
16	1.10	0.270	2.88×10^{3}	n.d.	1.66×10^{5}
16	1.10	0.230	2.10×10^{3}	n.d.	3.66×10^{4}
16	1.10	0.190	5.70×10^{2}	n.d.	1.31×10^{4}
16	1.10	0.150	8.85×10^{1}	n.d.	n.m.
16	4.40	0.620	1.31×10^{1}	n.d.	n.m.
16	4.40	0.450	1.88×10^{0}	n.d.	2.18×10^{4}
16	4.40	0.410	7.03×10^{2}	n.d.	2.77×10^{4}
16	4.40	0.360	8.42×10^{2}	n.d.	3.57×10^{4}
16	4.40	0.310	3.44×10^{2}	3.34×10^{-2}	4.36×10^{4}
16	4.40	0.270	8.36×10^{2}	1.03×10^{-1}	2.98×10^{4}
16	4.40	0.230	2.39×10^{3}	n.d.	5.04×10^{4}
16	4.40	0.190	9.45×10^{3}	n.d.	8.22×10^{4}
16	4.40	0.150	1.43×10^{3}	n.d.	n.m.
63	0.35	0.360	4.84×10^{4}	6.12×10^{-2}	1.41×10^{6}
63	0.35	0.310	8.23×10^{5}	4.01×10^{-2}	2.07×10^{6}
63	0.35	0.270	1.69×10^{3}	7.74×10^{-2}	3.20×10^{5}
63	0.35	0.230	1.36×10^{4}	n.d.	4.74×10^{5}
63	1.10	0.360	6.05×10^{4}	1.85×10^{-1}	2.01 x106
63	1.10	0.310	7.37×10^{4}	2.60×10^{-1}	6.65×10^{5}
63	1.10	0.270	2.41×10^{4}	3.34×10^{-1}	5.12×10^{5}
63	1.10	0.230	5.27×10^{3}	n.d.	5.30×10^{4}
63	1.10	0.190	1.53×10^{5}	n.d.	n.m.
63	4.40	0.360	1.13×10^{4}	1.64×10^{-1}	4.08×10^{5}
63	4.40	0.310	5.24×10^{2}	4.21×10^{-2}	3.05×10^{5}
63	4.40	0.270	4.19×10^{4}	3.69×10^{-2}	9.56×10^{5}
63	4.40	0.190	4.47×10^{4}	n.d.	5.94×10^{5}
137	0.35	0.360	4.01×10^{3}	3.70×10^{-2}	6.59×10^{5}
137	0.35	0.310	2.26×10^{4}	3.61×10^{-2}	6.35×10^{5}
137	0.35	0.270	5.38×10^{4}	1.49×10^{-2}	1.08×10^{5}
137	0.35	0.230	5.06×10^{5}	n.d.	7.63×10^{6}
137	0.35	0.190	2.43×10^{5}	n.d.	3.69×10^{5}
137	1.10	0.360	4.99×10^{3}	1.72×10^{-1}	7.18×10^{4}
137	1.10	0.310	4.43×10^{4}	4.77×10^{-1}	5.55×10^{4}
137	1.10	0.270	8.35×10^{4}	2.53×10^{-1}	1.07×10^{7}
137	1.10	0.230	1.09×10^{6}	4.53×10^{-2}	2.46×10^{6}
137	1.10	0.190	8.12×10^{5}	7.44×10^{-2}	5.70×10^{6}
137	4.40	0.360	n.m.	2.56×10^{0}	1.29×10^{7}
137	4.40	0.310	1.33×10^{4}	5.14×10^{-2}	4.70×10^{7}
137	4.40	0.270	7.96×10^{3}	n.d.	6.32×10^{6}
137	4.40	0.230	1.68×10^{4}	n.d.	8.20×10^{6}
137	4.40	0.190	1.75×10^{6}	1.29×10^{-1}	4.15×10^{7}
t after toluene injection [d]	**Flow distance [m]**	**Sediment depth [m]**	***tmoA* [cop. g^{-1}]**	***bssA* [cop. g^{-1}]**	**16S [cop. g^{-1}]**
16	0.35	0.375	n.d.	n.d.	2.04×10^{6}
16	0.35	0.325	n.d.	n.d.	4.12×10^{6}
16	0.35	0.275	n.d.	n.d.	2.47×10^{6}
16	1.10	0.375	1.21×10^{4}	n.d.	5.10×10^{5}
16	1.10	0.325	1.53×10^{6}	n.d.	4.58×10^{6}
16	1.10	0.275	1.37×10^{5}	n.d.	1.24×10^{6}
16	1.10	0.225	7.87×10^{4}	n.d.	7.09×10^{5}
16	1.10	0.175	3.02×10^{1}	n.d.	4.25×10^{5}
16	4.40	0.425	9.69×10^{3}	n.d.	3.78×10^{5}
16	4.40	0.325	4.44×10^{4}	n.d.	5.33×10^{5}
16	4.40	0.225	1.21×10^{4}	n.d.	2.67×10^{5}

16	4.40	0.175	n.d.	n.d.	3.21×10^{5}
63	0.35	0.400	n.d.	n.d.	2.10×10^{6}
63	0.35	0.325	n.d.	n.d.	1.40×10^{7}
63	0.35	0.275	1.75×10^{4}	n.d.	1.75×10^{7}
63	1.10	0.375	n.d.	n.d.	4.05×10^{6}
63	1.10	0.325	4.22×10^{5}	n.d.	6.75×10^{6}
63	1.10	0.275	1.31×10^{5}	1.14×10^{3}	3.81×10^{6}
63	1.10	0.225	n.d.	n.d.	5.71×10^{5}
63	4.40	0.425	3.05×10^{1}	n.d.	2.14×10^{5}
63	4.40	0.375	9.71×10^{4}	n.d.	1.36×10^{6}
63	4.40	0.325	9.93×10^{4}	n.d.	1.39×10^{6}
63	4.40	0.225	1.18×10^{3}	n.d.	8.63×10^{5}
63	4.40	0.175	6.97×10^{2}	n.d.	1.35×10^{6}
137	0.35	0.325	n.d.	n.d.	1.50×10^{7}
137	0.35	0.275	1.51×10^{5}	n.d.	3.34×10^{7}
137	0.35	0.225	n.d.	n.d.	2.34×10^{7}
137	1.10	0.375	n.d.	2.78×10^{2}	1.70×10^{7}
137	1.10	0.325	n.d.	2.72×10^{1}	6.17×10^{6}
137	1.10	0.275	3.85×10^{6}	5.75×10^{0}	1.54×10^{7}
137	1.10	0.225	6.98×10^{3}	n.d.	3.08×10^{6}
137	4.40	0.400	n.d.	n.d.	4.48×10^{5}
137	4.40	0.325	n.d.	n.d.	8.97×10^{5}
137	4.40	0.275	n.d.	1.31×10^{2}	1.35×10^{7}
137	4.40	0.225	5.34×10^{4}	n.d.	1.12×10^{6}
137	4.40	0.175	n.d.	n.d.	2.13×10^{7}
137	4.40	0.150	n.d.	2.79×10^{2}	2.69×10^{7}

n.m., not measured – sample analysis not possible due to technical problems.

n.d., not detectable (below detection limit).

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