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Biodegradation and bacterial growth in a mixing-controlled organic contaminant plume

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ABSTRACT

One of the biggest problems of our environment is the repeated pollution with contaminants such as organics, which is why scientists all over the world are working on achieving a better understanding of the biodegradation potential of microbial communities. Petroleum hydrocarbons, which are constituents of crude oil and its refined variants, are especially prominent and dangerous. Their monoaromatic representatives are benzene, toluene, ethylbenzene and xylene isomers, in short the BTEX compounds. BTEX pollution of aquifers from a point source usually results in the formation of a contaminant plume which is constantly mixing with the surrounding water through transverse dispersion. Certain microorganisms are capable of degrading these toxic contaminants under different redox-conditions, both oxic and anoxic. A multitude of mathematical models has been developed which allow the simulation of biodegradation of a contaminant plume under steady-state conditions. However, steady-state conditions are scarce in nature because of hydraulic and chemical dynamics which may alter the lateral position of the plume or the composition of the microbial community, ultimately affecting its biodegradation potential. Most models for simulating bacterial growth and contaminant degradation were created using data obtained from batch experiments which don't mirror the conditions in the field, and therefore these models often fall short of predicting contaminant degradation and bacterial growth in the field. To improve these models and to be able to predict biodegradation potentials and growth kinetics of various microorganisms is the goal of this research. For this purpose it is important to gather as much information as possible about which factors influence biodegradation and bacterial growth in situ. The factors particularly considered in this study were: sediment properties, groundwater flow velocity, contaminant concentration, differences between attached and suspended bacteria, as well as starvation behavior and decay of microorganisms.

Another point of concern is the discrepancies between results obtained from batch and flow-through systems and why model accuracy will benefit from implementing data obtained from flow-through rather than batch systems. Analyzing samples from an organically polluted aquifer, we assessed the potential of organic contaminants as a selective force by analyzing the bacterial community composition attached to different types of sediments from zones with different a degree of contamination. We follow this up with a proposal of appropriate ways on how to design experimental setups resembling field conditions more closely using either one-dimensional sediment-filled mini columns or 2-dimensional flow-through microcosms. Using *Aromatoleum aromaticum* strain EbN1 and *Pseudomonas putida* strain F1 as model organisms, we then conducted experiments in batch and sediment-filled columns applying varying concentrations of BTEX compounds along with different electron acceptors in order to assess variation in growth and biodegradation behavior of the organisms between batch cultures, which are closed and perfectly mixed systems, and column systems which are open one-dimensional flow-through systems mimicking natural conditions more accurately. The effects of varying flow velocity and contaminant concentration on the bacterial growth rates, contaminant degradation rates, yield and carbon assimilation efficiencies were assessed in each experiment. Finally, starvation conditions were applied in individual experiments to assess decay rates and detachment of attached cells. The information obtained from these studies were used by colleagues from the hydrogeology workgroup at the University of Tübingen to develop an accurate bioreactive transport model which is able to simulate one-dimensional transport and biodegradation with regards to redox-conditions, growth and decay rates of the organisms used as well as biomass dispersal between sediment and pore water.

Our results show that in the presence of BTEX and/or PAHs the similarity of bacterial communities on different types of minerals increased considerably, which indicates that petroleum hydrocarbons render all other selective factors controlling bacterial community

composition mostly irrelevant. The growth behavior of our model organisms in the column systems differed considerably from the batch systems, with cells growing attached to the sediment until the system's carrying capacity dictates the release of all further newly grown cells into the pore water. Both flow velocity and contaminant concentration were shown to affect the toluene removal efficiency and maximum specific growth rate, with higher flow velocities and higher contaminant concentrations leading to lower growth rates and toluene removal efficiency, most likely because of mass transfer limitations. In all column experiments the vast majority (>98%) of cells was found attached to the sediment, with the highest ratio of attached to suspended cells at lower contaminant concentrations. Long-term starvation led to a strong reduction in cell volume and activity, indicating that the cells are losing much of their water during this period. Toluene degradation potential seemed unaffected, as the cells were able to resume degradation immediately at the following contaminant exposition. We therefore assume that changes of contaminant plume position or groundwater flow won't have a negative impact on the aerobic biodegradation potential of natural microbial aquifer communities. Our results give important insights into the effects of various factors on bacterial growth and degradation potential, yet there are several more factors remaining that weren't the focus of this study. Future research should therefore focus on evaluating the importance of factors like food-web interactions, competition for resources, and grazing.

ZUSAMMENFASSUNG

Eines der größten Probleme unserer Umwelt ist die wiederholte Verschmutzung mit Schadstoffen wie organischen Verbindungen, darum arbeiten Wissenschaftler überall auf der Welt daran, das Abbaupotential von mikrobiellen Gemeinschaften besser verstehen zu lernen. Erdöl-Kohlenwasserstoffe, die Bestandteile von Rohöl und seinen aufbereiteten Varianten sind, sind besonders weit verbreitet und gefährlich. Deren monoaromatische Vertreter sind Benzol, Toluol, Ethylbenzol und Xylol-Isomere, kurz gesagt die BTEX-Verbindungen. BTEX-Verschmutzungen von Grundwasserleitern durch eine Punkt-Quelle enden für gewöhnlich mit der Bildung einer Schadstofffahne, die durch transversale Dispersion immer mit dem umgebenden Medium vermischt wird. Bestimmte Mikroorganismen sind in der Lage, diese giftigen Schadstoffe unter verschiedenen Redox-Bedingungen, sowohl oxischen als auch anoxischen, abzubauen. Eine Vielzahl mathematischer Modelle wurde entwickelt, die die Simulation des Abbaus einer Schadstofffahne unter stationären Bedingungen erlauben. Allerdings sind stationäre Bedingungen in der Natur aufgrund von hydraulischer und chemischer Dynamik, die die räumliche Position der Fahne oder die Zusammensetzung der mikrobiellen Gemeinschaft verändern können und dadurch letztendlich den Schadstoffabbau beeinflussen, nur selten gegeben. Die meisten Modelle zur Simulation von bakteriellem Wachstum und Schadstoffabbau wurden mit Daten erstellt, die aus Batch-Experimenten gewonnen wurden, und diese spiegeln nicht die genauen Bedingungen im Feld wieder, darum versagen diese Modelle auch darin, bakterielles Wachstum im Feld vorherzusagen. Diese Modelle zu verbessern und Schadstoffabbaupotentiale und Wachstumskinetiken verschiedener Mikroorganismen besser vorhersagen zu können, ist das Ziel dieser Forschung. Zu diesem Zweck ist es wichtig, so viele Informationen wie möglich über die Faktoren zu sammeln, die Schadstoffabbau und bakterielles Wachstum in situ beeinflussen. Die Faktoren, die in dieser

Studie im Speziellen berücksichtigt wurden, waren: Sedimenteigenschaften, Fließgeschwindigkeit, Schadstoffkonzentration, Unterschiede zwischen festsitzenden und suspendierten Bakterien, sowie Hungerverhalten und Absterben von Mikroorganismen. Ein weiterer zu bedenkender Punkt ist es, auf die Diskrepanzen zwischen den Ergebnissen aus Batch- und Durchfluss-Systemen einzugehen und warum die Genauigkeit von Modellen eher von Daten aus Durchflusssystemen profitieren wird als von Daten aus Batch-Systemen. Indem wir Proben aus einem organisch belasteten Grundwasserleiter analysierten, haben wir das Potential von organischen Schadstoffen als selektiver Faktor ermittelt, indem die Zusammensetzung der bakteriellen Gemeinschaft, die auf verschiedenen Sedimenttypen festsitzt, aus Zonen mit je einem unterschiedlichen Kontaminationsgrad analysiert wurde. Darauf folgen wir mit einem Vorschlag über die passenden Wege, wie man ein experimentelles Setup entwirft, das den Bedingungen im Feld näher kommt, indem man entweder eindimensionale sedimentgefüllte Mini-Säulen oder 2-dimensionale Durchfluss-Mikrokosmen verwendet. Indem wir *Aromatoleum aromaticum* Stamm EbN1 und *Pseudomonas putida* Stamm F1 als Modellorganismen verwendet haben, führten wir dann Experimente im Batch und sedimentgefüllten Säulen mit variierenden Konzentrationen der BTEX-Komponente zusammen mit verschiedenen Elektronen-Akzeptoren durch, um die Unterschiede in Bezug auf Wachstum und Abbauverhalten des Organismus zwischen Batch-Kulturen, die geschlossene und perfekt gemischte Systeme sind, und Säulensystemen, die eindimensionale offene Durchflusssysteme sind und natürliche Bedingungen genauer widerspiegeln. Die Effekte von variierender Fließgeschwindigkeit und Schadstoffkonzentration auf die bakteriellen Wachstumsraten, Schadstoff-Abbauraten, Ertrag und Kohlenstoffassimilationseffizienzen wurden in jedem Experiment ermittelt. Zu guter Letzt wurden Hungerbedingungen in einzelnen Experimenten erzeugt, um Sterberaten und Ablösung von festsitzenden Zellen zu erfassen. Die Informationen, die aus diesen Studien

erworben wurden, wurden von Kollegen aus der Hydrogeologie-Arbeitsgruppe an der Universität Tübingen benutzt, um einen genauen Code für den bioreaktiven Transport zu entwickeln, der in der Lage ist, den eindimensionalen Transport und den Schadstoffabbau zu simulieren, mit Rücksicht auf Redox-Bedingungen, Wachstums- und Verfallsraten der eingesetzten Mikroorganismen sowie die Verteilung der Biomasse zwischen Sediment und Suspension. Unsere Ergebnisse zeigen, dass die Ähnlichkeit bakterieller Gemeinschaften auf verschiedenen Mineralientypen in Anwesenheit von BTEX und/oder PAKs beträchtlich anstieg, ein Indiz dafür, dass Erdölkohlenwasserstoffe alle anderen selektiven Faktoren, die die Zusammensetzung der bakteriellen Gemeinschaft kontrollieren, zum größten Teil irrelevant machen. Das Wachstumsverhalten unserer Modell-Organismen in den Säulensystemen unterschied sich beachtlich von den Batch-Systemen, da die Zellen auf dem Sediment festsitzend wachsen, bis die Tragfähigkeit des Systems die Freisetzung aller weiteren neugebildeten Zellen in das Porenwasser diktiert. Es wurde gezeigt, dass sowohl Fließgeschwindigkeit als auch Schadstoffkonzentration die Toluol-Entfernungseffizienz und die maximale spezifische Wachstumsrate beeinflussen, und zwar führen höhere Fließgeschwindigkeiten und höhere Schadstoffkonzentrationen zu geringeren Wachstumsraten und Toluol-Entfernungseffizienzen, sehr wahrscheinlich wegen Massen-Transfer-Limitationen. In allen Säulenexperimenten wurde die große Mehrheit der Zellen (>98%) am Sediment festsitzend gefunden, das höchste Verhältnis festsitzender zu suspendierter Zellen gab es bei niedrigeren Schadstoffkonzentrationen. Langzeithunger führte zu einer starken Verringerung von Zellvolumen und –aktivität, ein Indiz dafür, dass die Zellen während dieser Periode viel von ihrem Wassergehalt verlieren. Das Toluol-Abbaupotential schien unbetroffen, da die Zellen bei nachfolgender Schadstoffexposition den Abbau sofort wieder aufnehmen konnten. Wir nehmen daher an, dass Veränderungen der Position der Schadstofffahne oder des Grundwasserflusses keine negative Auswirkung auf das aerobe Abbaupotential natürlicher

mikrobieller Grundwassergemeinschaften haben wird. Unsere Ergebnisse geben wichtige Einblicke in die Effekte verschiedener Faktoren auf bakterielles Wachstum und Abbaupotential, doch gibt es noch mehrere weitere Faktoren, die nicht im Fokus dieser Studie standen. Zukünftige Forschungen sollten sich daher darauf konzentrieren, die Wichtigkeit von Faktoren wie Nahrungsnetzinteraktionen, Konkurrenz um Ressourcen oder Grasen zu evaluieren.

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ABBREVIATIONS

BTEX	Benzene, Toluene, Ethylbenzene, Xylene Isomers
C_6H_6	Benzene
C_8H_{10}	Ethylbenzene
C_7H_8	Toluene
$C_6H_4C_2H_6$	Xylene (any isomer)
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
FCM	flow cytometry
GC-MS	Gas chromatography coupled to mass spectrometry
HPLC	High performance liquid chromatography
OTU	Operational taxonomic unit
OD	Optical density
PAH	Polycyclic aromatic hydrocarbons
PCA	Principle component analysis
PCR	Polymerase chain reaction
rpm	rounds per minute
RT	room temperature
SI	Supporting Information
T-RFLP	Terminal restriction fragment length polymorphism
WSM	Widdel sweet medium

1. INTRODUCTION

1.1 Environmental pollution

The technological advancements that were achieved during the industrial revolution along with the emergence of factories and the discovery and subsequent usage of fossil fuels as energy sources have raised the standard of living in the industrial world, but it came at a high price. Different waste products derived from various chemical processes contaminated and still contaminate our environment (Iwamoto and Nasu, 2001), and some unusable side products like CO_2 created by fossil fuel combustion significantly contribute to global warming. Today, environmental pollution is one of our world's biggest problems, with unforeseeable consequences to the various terrestrial and aquatic ecosystems and the organisms living therein. The one type of contaminant that this work will be focusing on is petroleum hydrocarbon pollution in aquifers. These aromatics constitute a large fraction in crude oil and, consequently, all fuels derived from crude oil, like gasoline or jet fuel. Petroleum compounds belong to contaminants of high concern because of their long-time persistence in both soils and aquifers, and because remediation of the environment often fails (Anneser et al., 2010). There are multiple ways as for how these contaminants can find their way into the environment. One example would be that pipelines or underground storage tanks leak (Chapelle, 2000), or it could be due to accidents at oil-well derricks, leading to contamination of soils and aquifers. Disposal of petroleum products, or the deposition or dumping of waste such as tar oil are other factors contributing to environmental pollution (Heider, 2007; Foght, 2008). Also, simple combustion of fossil fuels allows petroleum hydrocarbons to enter the environment (Foght, 2008). Aside from these human-induced cases, there are also natural sources which bring petroleum hydrocarbons to land surfaces and into the aquatic environment, like tar pits (Heider, 2007), or natural petroleum seepage (Foght,

2008). Either way, contamination of groundwater ecosystems from a point source of petroleum hydrocarbons usually results in the formation of a contaminant plume of varying length in groundwater, ranging in size from several meters to several kilometers (Bauer et al., 2008a & 2009; Anneser et al, 2008 & 2010; Meckenstock et al, 2010). An example of such a contaminant plume is depicted in Fig. 1.1. The introduction of organic pollutants will have varying effects on the microbial communities in aquifers. There have been reports that the introduction of wastewater leads to an increase in microbial diversity, probably because energy and new microbes are introduced into the subsurface ecosystem (Cho and Kim, 2000; Griebler and Lueders, 2009; Meckenstock et al., 2010). In many cases also the opposite can be true. The contamination of aquifers with toxic compounds leads to a decrease in microbial diversity by killing species that cannot cope with the environmental change (Griebler and Lueders, 2009; Silva-Castro et al., 2013). Also, the type of contaminant is of major importance when it comes to effects on the fauna of the environment.

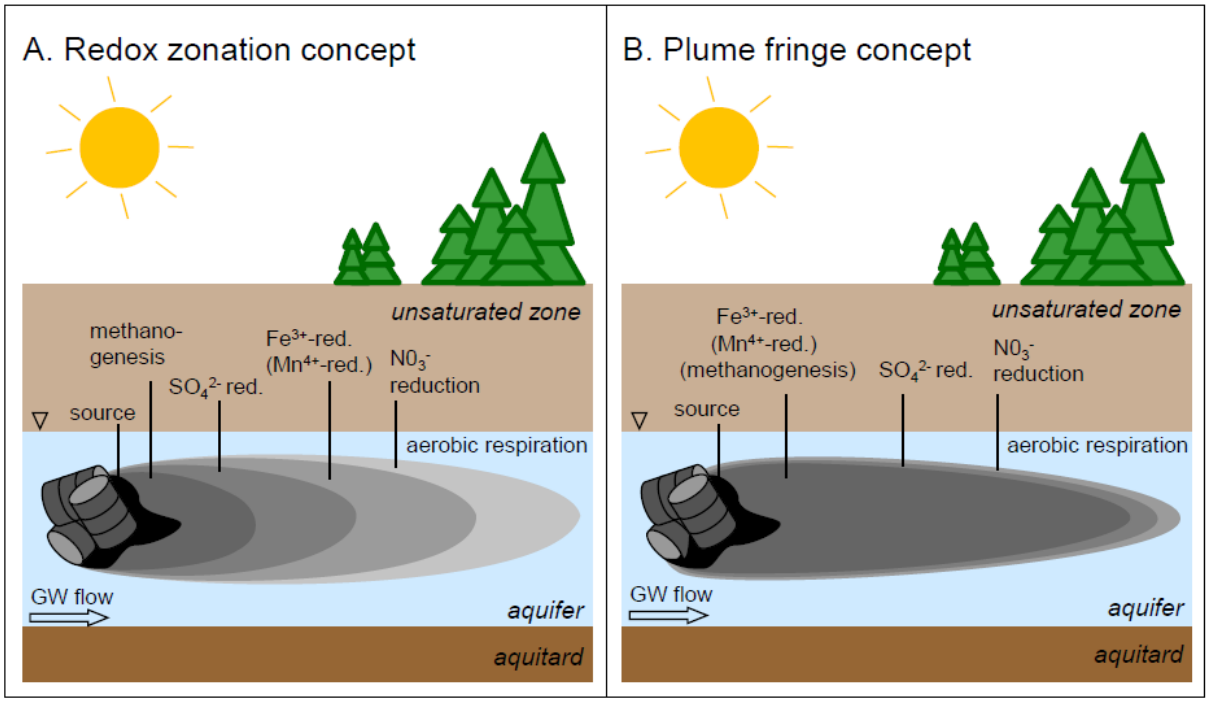

Figure 1.1: Classical example of aquifer contamination from a point source, e.g. waste disposal. (A) highlights the various redox-conditions relevant for aerobic and anaerobic biodegradation, (B) highlights the narrowness of these redox-zones at the plume fringe where transverse dispersion leads

to the mixing of the contaminant with various electron acceptors. (Figure taken Meckenstock et al., 2015)

1.2 Groundwater ecosystems

Our current understanding of groundwater ecosystems is still rather limited, as the idea of microbial life in the subsurface was considered absurd some decades ago (Gounot, 1994), until the discovery of microbes in the earth's underground during the 1970s and 1980s (Griebler and Lueders, 2009). Today we know that microorganisms can tolerate or even thrive under extreme environmental conditions, like extreme temperatures of up to 121°C, extreme pH values (both very acidic and very alkaline), high pressure, high salt concentrations, desiccation etc. and are therefore able to colonize pretty much any habitat on earth that lies within the limits of life, including the subsurface (Griebler and Lueders, 2009). The microbial communities found in the subsurface consist mainly of bacteria, archaea, protozoa, yeasts and fungi, and the total number of organisms per cm^3 of groundwater and sediment may vary by several orders of magnitude (Griebler and Lueders, 2009). Microbes in the subsurface play an important role in many biogeochemical processes (Humbert and Dorigo, 2005). In fact, the functioning of ecosystems is influenced by a variety of biotic and abiotic factors (Chapelle, 2000; Griebler and Mösslacher, 2003; Pilloni et al., 2011) including diversity or the activity and interaction of the microbial community. Abiotic factors are physicochemical conditions such as geology and water chemistry (Humbert and Dorigo, 2005). There are a few important differences between surface and subsurface microbial communities. Since groundwater reservoirs are located below the earth's surface, the microorganisms there cannot gain energy through photosynthesis, the constant temperature is expected to be low, and fresh organic carbon inputs are considered rare. Thus the microbial communities in the underground are considered to consist of mainly heterotrophic organisms adapted to the nutrient-poor environments where growth will be limited by the availability of degradable organic carbon,

nutrients, electron donors and acceptors, or a combination thereof (Bengtsson, 1991; Chapelle, 2000; Griebler and Lueders, 2009; Kappler et al., 2005; Marshall, 1988; Pilloni et al., 2011; Rüegg et al., 2007). Also, the majority of the microorganisms in aquifers are attached to the sediment or rock surfaces and form microcolonies and biofilms (Alfreider et al., 1997; Griebler et al., 2002; Lehman et al., 2001a; Lehman et al., 2001b). Attachment to the sediment offers a series of advantages to cells. Sediment grains can contain otherwise growth-limiting elements and nutrients, and organic compounds can adsorb to the sediment surfaces as well (Tuschewitzki et al., 1992). Rough sediment surfaces allow the bacteria to colonize fissures where they may be protected from protozoan grazing and direct shear forces (Korber et al.,1995). Some earlier studies have also shown that cells attached to sediments display higher activity, and that the microbial communities in groundwater and at the sediment can be composed differently and therefore can have a different biodegradation potential (Anneser et al., 2010; Flynn et al., 2008;). Groundwater ecosystems can be of varying size, since many underground areas are hydrologically connected (Wall-Freckmann et al., 1997).

Assessing the overall microbial diversity of a groundwater ecosystem is very difficult, because the vast majority of the organisms found in field sites cannot be cultured under laboratory conditions so far, and the only reliable method of investigating species is through molecular analysis, which is a cultivation-independent technique (Griebler and Lueders, 2009). Groundwater ecosystems appear in many different shapes or forms. One of these would be the pristine shallow aquifer, which provides drinking water and harbors different microbial communities than surface aquatic environments. The deep subsurface is again a totally different kind of ecosystem often isolated from the surface for thousands and millions of years (Fredrickson et al., 1991). Karstic systems and caves should also be mentioned here, even though we know much less about microbial life and activity in these kinds of

groundwater systems. Cave systems aren't isolated ecosystems, but are constantly influenced by runoff waters and airstreams from the surface or by animals migrating through the cave (Gounot, 1994). Finally, there's the transition zone between surface running water and a groundwater system which is called the hyporheic zone and influences both ecosystems (Fig. 1.2). The microbial communities of such hyporheic zones haven't been studied extensively, but contrary to microbial communities in aquifers, they show seasonal dynamics with the highest biodiversity in autumn (Feris et al., 2003).

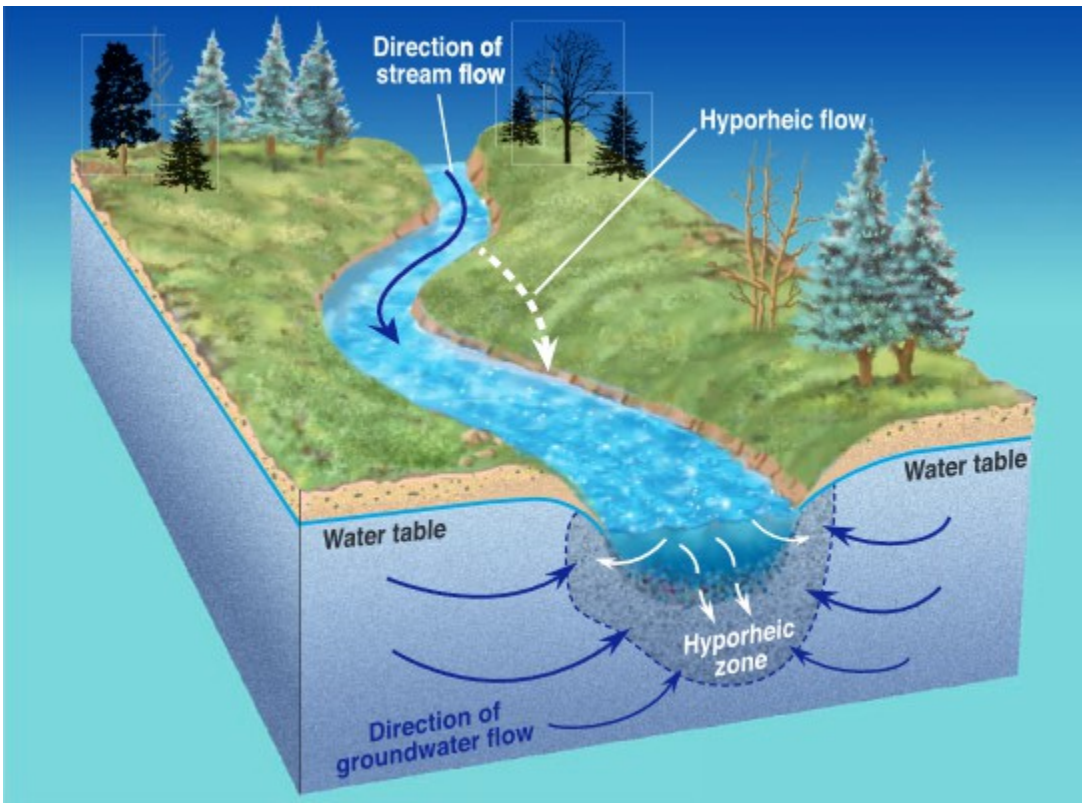

Figure 1.2: Schematic overview of the various types of water: the hyporheic zone forms the transition zone between the running surface water (e.g. rivers). Both matter and organisms are continuously exchanged between surface water and groundwater via the hyporheic zone. Figure taken from Alley et al, 2002.

Microbial diversity in groundwater is controlled by multiple factors. First of all, there are evolutionary aspects like population size and genetic flexibility to consider. Microorganisms can usually evolve faster than higher organisms because they have much shorter generation

times and useful genetic mechanisms like horizontal gene transfer (Allison and Martiny, 2008; Griebler and Lueders, 2009), allowing for swift adaptation to changing environmental conditions. Also, the rate of speciation is much faster than the rate of local extinction, leading to an ever increasing number of bacterial species (Dykhuizen, 1998). Habitat size and spatial heterogeneity are additional factors influencing microbial diversity. Generally, biodiversity increases with habitat size and structural complexity, because more ecological niches are available in a larger habitat (Griebler and Lueders, 2009). The spatial distribution of bacteria is also important, as many bacterial strains are unique to a certain depth in an aquifer (Griebler and Lueders, 2009).

Different functional groups have been found to be part of groundwater microbial communities, many of which can contribute to natural attenuation of various petroleum hydrocarbons by using a broad range of possible electron acceptors. The fringes of the contaminant plume are considered to be the zones with the highest microbial activity and thus the zones with the highest biodegradation potential because this is where electron donors and acceptors mix sufficiently due to transverse dispersion (Anneser et al., 2008; Bauer et al., 2008). The most well-known functional groups of microorganisms, apart from aerobes, include the denitrifiers, the manganese reducers, the iron reducers, and the sulfate reducers, which use nitrate, manganese(IV), iron(III) and sulfate as electron acceptors, respectively (see Fig. 1.1), along with the methane-oxidizers which can degrade certain chlorinated compounds, the lithoautotrophs which can use CO_2 as their sole source of carbon, and the methanogens which produce methane (Beller et al., 1996; Bombach et al., 2009; Coates et al., 2001; Edwards and Grbić-Galić, 1992; Foght, 2008; Fowler et al., 2011; Heider, 2007; Morasch et al., 2004; Rabus and Widdel, 1994; Rabus and Widdel, 1996; Weelink et al., 2010). The detection of these groups within a groundwater sample is usually accomplished by functional marker assays based on key enzymes, or catabolic marker gene assays (Griebler

and Lueders, 2009). Taking into account that some bacterial species prefer solid electron acceptors that are minerals or adsorb to the sediment, and others prefer dissolved electron acceptors suspended in the water, there's usually a difference between the community composition in groundwater and in the sediment (Anneser et al., 2010).

1.3 Bacterial growth

The doubling time of bacteria is different from species to species and highly depends on environmental conditions. Some bacteria like *E. coli*, for example, have doubling times of only 20 minutes, while others take hours or even days or months. The growth of bacteria under laboratory conditions can usually be divided into four phases as depicted in Fig. 1.3 (Chapelle, 2000; Janke, 2002): In the lag phase at the beginning of a new culture inoculation, the growth is slow due to the low number of starting cells and because the bacteria may need time to adjust to the new conditions or express the enzymes required for degrading the available carbon source. Subsequently, the exponential phase follows, marked by rapid growth, until the nutrients are depleted and/or toxic waste products become abundant. This is when either both the growth and death rates of bacteria become roughly equal or no more growth occurs. The culture enters the so-called stationary phase. The last phase is called the death phase, where the bacteria will die off due to starvation or due to the effect of a lot of toxic waste products.

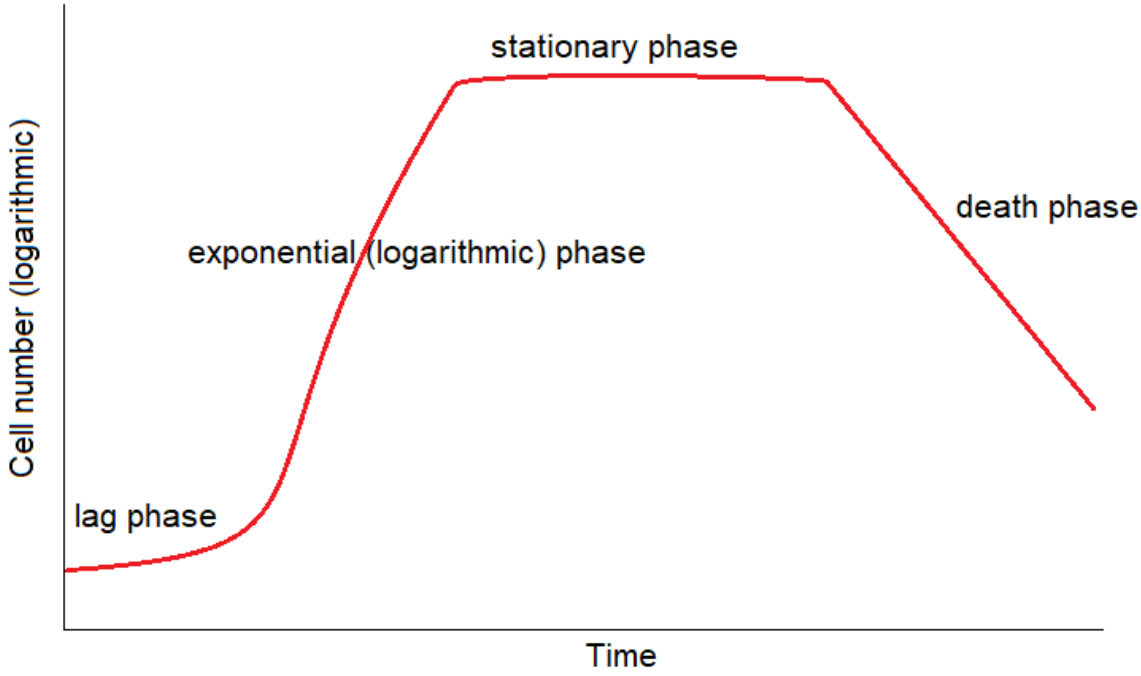

Figure 1.3: The four phases of bacterial growth in enclosed systems (e.g. batch culture bottles).

The growth of microorganisms depends largely on the substrate available and the energy that can be obtained. When a carbon source is consumed by bacteria, a certain percentage of the carbon is fueled through catabolic metabolism and released in the form of CO_2 (respiration), while part of the carbon is converted into new biomass (assimilation). The fraction of carbon converted to biomass is defined by the carbon assimilation efficiency (or carbon use efficiency or growth efficiency) which is different from species to species, and again dependent on the substrate that is consumed and the environmental conditions such as the availability of essential nutrients. Higher carbon assimilation efficiency means being able to build more biomass, leading to faster growth. Generally, anaerobic organisms grow less efficiently because the inorganic compounds they use as terminal electron acceptors have a lower reduction potential than oxygen. Apart from the availability of nutrients and terminal electron acceptors, there are several other environmental factors that influence bacterial growth, like temperature, pH, osmotic pressure, signaling, microbial food web interactions or just plain limited surface area (Chapelle, 2000).

1.4 Bioremediation

The continued pollution of the environment all over the world has evoked an increasing interest of scientists to study the self-purification potential of nature based on microorganisms that are capable of degrading organic pollutants. When groundwater or sediments are polluted by aromatic hydrocarbons or some other organic contaminant, the degradation activities of microorganisms may substantially contribute to the removal of these contaminants from the environment, a process called 'natural attenuation' (Iwamoto and Nasu, 2001; Foght, 2008). Using microorganisms to one's advantage in order to clean polluted environmental sites is what 'bioremediation' is all about. While *ex situ* bioremediation processes are performed at a separate treatment facility, *in situ* bioremediation takes place directly in situ. Monitored natural attenuation describes the natural process of biodegradation without human interference which is just monitored to observe the speed of degradation and to enable predictions about when the contamination will be diminished. However, since natural attenuation is a rather slow process, additional steps are often taken, usually in the form of biostimulation or bioaugmentation. Biostimulation means the injection of co-substrates, electron donors or acceptors, or additional nutrients into the aquifer in order to stimulate the microbial community and increase their biodegradation potential.

Lastly, there is the approach of bioaugmentation, which means the injection of bacteria into the aquifer, and often even new bacterial strains that possess the desired degradation abilities. However, this method has to be performed with caution, as the addition of new species or additional amounts of already present species changes the composition of the microbial community in the environment and can have unpredictable side effects on the ecosystem and possibly even on humans (Iwamoto and Nasu, 2001). That's why the effects of such bacteria on the habitat and the microbial community have to be carefully studied in advance. Moreover, in many field studies, bioaugmentation seemed not worth the trouble because the

injected microorganisms could not successfully compete with indigenous microbial populations for resources (Tchelet et al., 1999; Massol-Deyá et al., 1997), and because the cells often attach themselves to sediment grains too soon during the injection process and thus do not reach the area of the contaminated groundwater (Chapelle, 2000).

Previous studies have revealed that effective biodegradation requires favorable geochemical and hydrological conditions, and that biodegradation itself in turn influences the sediment and groundwater properties, because electron acceptors are depleted and the amount of metabolic intermediates or end products increases (Anneser et al., 2010). However, the current knowledge about the impact of bioremediation on the ecosystem is overall still limited because many bacteria that are found at a field site still could not be successfully cultured under laboratory conditions (Griebler and Lueders, 2009). Another problem is that even if biodegradation is observed in situ, it is difficult to distinguish whether the compounds are removed via degradation by microorganisms or via some abiotic mechanism including sorption, dilution or volatilization, so the biological contribution to natural attenuation processes is still unclear (Iwamoto and Nasu, 2001). One has to keep in mind also that different information is obtainable from groundwater and sediment analyses. While the presence and availability of solid electron acceptors like elemental sulfur and ferric iron can only be directly assessed in sediments, dissolved electron donors cannot be properly monitored in sediment samples, but in groundwater samples (Anneser et al., 2010). Reliable information about the contamination with polycyclic aromatic hydrocarbons (PAHs) can again only be obtained from water and sediment, because most PAHs have a high tendency to adsorb to the sediment matrix, unlike the lighter BTEX compounds (Anneser et al., 2010) (Meckenstock et al., 2010). Hence why it is best to analyze both groundwater and sediment samples from the same field site and compare the results in order to gain sufficient knowledge about the natural attenuation potential and actual activities at contaminated aquifers.

In order to distinguish between biotic and abiotic factors influencing degradation, it has been proposed that signature metabolites which are specific to the substrate's degradation pathway should be used as indicators. Some signature biomarkers, like benzylsuccinic acid produced during anaerobic toluene degradation or methylbenzylsuccinic acid produced during xylene degradation (Griebler et al., 2004; Jobelius et al., 2011) have been detected at various field sites. Neither of these two compounds is produced in other pathways, so they can be used as indicators for anaerobic biodegradation of toluene or xylene, respectively. Since the identification of these metabolites represents a qualitative indication only, determining the relation of new-produced metabolites to their parent compounds was evaluated in order to quantify biodegradation. This is quite difficult, however, as some metabolites like benzylsuccinic acid show only low concentrations which is probably due to their fast turn-over, thus a quantitative statement on biodegradation based on metabolite levels is unreliable (Jobelius et al., 2011). Nevertheless, this is still just the beginning of a wide field of research. We do know that the effectiveness of bioremediation is dependent on the type and concentration of the terminal electron acceptors that are available (Foght, 2008). For example, the addition of sulfate to an aquifer-derived microbial population greatly stimulated benzene degradation under anoxic conditions (Edwards and Grbić-Galić, 1992). Learning more about the effects of biostimulation (nutrient and electron acceptor addition) and bioaugmentation (bacteria addition) to a contaminant site might prove useful in developing strategies to purify contaminated sediments or aquifers.

1.5 Monoaromatic hydrocarbons

Hydrocarbons are organic compounds that occur mostly in crude oil and can be classified into several types. The basic structure of all hydrocarbons is the same. They consist solely of hydrogen and carbon. Aromatic hydrocarbons are also called arenes and have at least one

aromatic ring, which is a ring consisting of six carbon atoms with a number of delocalized electrons between them in the circle (Chapelle, 2000). Benzene (C_6H_6) is the simplest aromatic hydrocarbon, consisting only of the ring, while other derivatives of it have one or more substituents attached to it. In case of several substituents, their exact distance to one another is important, as can be seen with the three xylene isomers. Xylene has two methyl groups attached to it, and either in *ortho*-, *meta*-, or *para*-position (see Fig. 1.4).

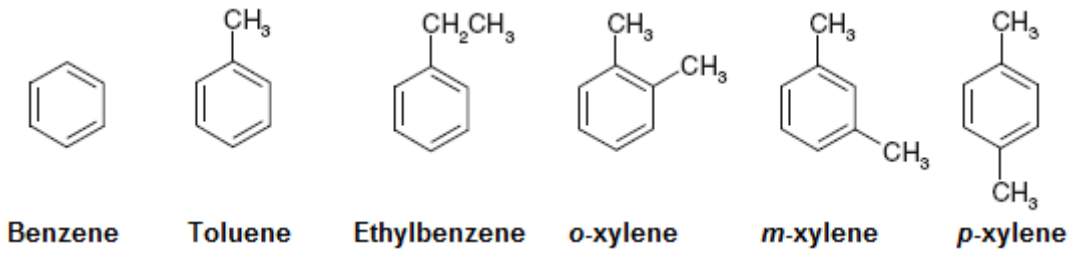

Figure 1.4: Structure of the various BTEX compounds.

Petroleum is the liquid form of hydrocarbons, and these petroleum compounds can be either monoaromatic hydrocarbons like the BTEX compounds (benzene, toluene, ethylbenzene and xylene isomers) or polycyclic aromatic hydrocarbons (PAHs) like naphthalene. These compounds can be found in oil, tar pits and coal. Refined gasolines are usually composed of around 16-25% aliphatic hydrocarbons, 35-55% cycloalkane hydrocarbons, 10-20% aromatic hydrocarbons, and up to 15% fuel oxygenates (Chapelle, 2000). The BTEX compounds are of special interest to researchers because they are parts of various fuels, even though they only comprise about 1-3% of gasoline (Chapelle, 2000). Still, their high toxicity to both humans and animals is what makes them one of the most serious environmental pollutants (Meckenstock and Mouttaki, 2011). They're carcinogenic and can lead to tumor formation (Vieth et al., 2004; Zink and Rabus, 2010). Finally, their high water solubility (Foght, 2008) makes them very mobile in situ. At the same time, they should be easier to biodegrade than non-soluble hydrocarbon compounds like branched alkanes and n-alkanes (Chapelle, 2000). It

also has to be noted that the various BTEX compounds can be of varying toxicity to certain microbial species, and every species' individual sensitivity or tolerance contributes to the alteration of the microbial community composition by inducing selective pressure on the community members (Anneser et al., 2010), influencing the overall effectiveness of biodegradation.

For many decades, it was believed that molecular oxygen is needed as a highly reactive co-substrate in order to activate aromatic petroleum hydrocarbons, and that their degradation can only occur under oxic conditions. This proved to be wrong, however, as studies in the last three to four decades have discovered several different species capable of degrading hydrocarbons under anoxic conditions. These microbial species cover a wide range of terminal electron accepting processes. Nitrate-reducing, manganese- and iron-reducing, sulfate-reducing and methanogenic bacteria have been identified as anaerobic hydrocarbon degraders through mostly unknown and novel biochemical reactions (Chapelle, 2000; Heider, 2007). However, due to the usually much lower energy yield of many anaerobic organisms, all reactions proceed slower and less efficiently. Since petroleum hydrocarbons serve as the electron donors, there is never a lack of electron donors in a contaminated aquifer, so the most limiting factor for bacterial growth in organically contaminated aquifers is probably the availability of sufficient amounts of electron acceptors and nutrients (Chapelle, 2000; Pilloni et al., 2011). During aerobic biodegradation, the oxygen which is inserted into ring structures is coming exclusively from molecular oxygen. Under anoxic conditions, however, there are far less possibilities to derive oxygen, and most likely the required oxygen comes from water in this case (Chapelle, 2000). Generally, oxygen seems to be important for biodegradation in subsurface environments only in the beginning, until the oxygen in the system is depleted, and aerobic growth ceases. This is the point where anaerobic bacteria take over and continue the

degradation using one or more of the above-mentioned electron acceptors (Bauer et al., 2008b and 2009; Meckenstock et al., 2010).

1.6 Aim of this work

The goal of this research was to shed some new light on some of the main questions concerning natural attenuation, namely:

1.) **Does aromatic hydrocarbon contamination influence microbial community composition in aquifers?** In this project we took contaminated and non-contaminated samples from a well-studied aquifer and compared the microbial community composition on different types of minerals via DNA fingerprinting to unravel the main selective forces responsible for shaping microbial communities.

2.) **What is the best way to mimic aquifer conditions in experimental setups in the laboratory?** For this we have written instructions on how to set up two different kinds of flow-through experiments for the lab which, albeit simplified, allow studying of some key factors influencing contaminant degradation on a micro-scale.

3.) **What are the key drivers of biodegradation and bacterial growth in flow-through systems?** We devised several mini sediment column experiments to clarify how flow velocity and contaminant concentration influence degradation and growth behavior of model organisms and natural aquifer communities, what the main differences and similarities between batch systems and flow-through systems are, and also how natural attenuation can be modeled more accurately.

4.) **How does substrate absence affect bacterial survivability and therefore their ability to perform biodegradation after long-term starvation?** This study focused on inducing starvation conditions in mini sediment column setups to observe bacterial behavior when exposed to substrate depletion over longer periods of time.

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2. Organic contamination versus mineral properties – Competing selective forces shaping bacterial community assembly in sediments

Aquatic Microbial Ecology **76:** 243-255.

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Abstract

Multiple factors have been shown to influence the assembly of sediment microbial communities. We hypothesized that in an organically polluted aquifer the degree of contamination controls bacterial distribution patterns, superimposing other selective forces such as sediment and mineral properties. Groundwater and sediment samples were analyzed from distinct zones of a petroleum hydrocarbon contaminated sandy aquifer that correspond to different degrees of contamination; Zone 1 with high concentration of dissolved contaminants (BTEX), Zone 2 with high concentrations of sediment-bound polycyclic aromatic hydrocarbons (PAHs), and Zone 3 with only a minor PAH contamination. Sediment analysis concentrated on two mineral fractions differing in many sediment properties, i.e. translucent quartz (TQ) and mica. Sediment bacterial communities were analyzed by DNA fingerprinting (T-RFLP) and total cell counts. While Zone 1 exhibited highly similar communities on TQ and mica, the selective sorption of PAHs to mica revealed sediment bacterial communities with hardly any taxonomic unit shared in Zone 2. Only in Zone 3, typical selective forces active in sediments of oligotrophic habitats, such as sediment mineral content and surface roughness, gained influence. Similarly, the least contamination revealed the most pronounced differences in Shannon diversity, Evenness and total cell counts, between the mineral fractions tested, with mica characterized by highest biomass and bacterial diversity. The role of contamination as a selective force is also underlined by the zone-specific dominance of key microbes involved in petroleum hydrocarbons degradation. Our results demonstrate that typical selective forces shaping aquifer sediment microbial communities are outcompeted by organic contamination.

Keywords: Aquifer microbiology, groundwater, petroleum hydrocarbons, sediment community assembly, selective forces, quartz, mica

2.1 Introduction

The majority of prokaryotic cells in aquifers are found attached to the sediment surface while only a minor portion (0.01-10%) are freely floating in the pore water (Alfreider et al. 1997, Geesey 2001, Griebler et al. 2002, Zhou et al. 2012). Numerous abiotic and biotic factors drive the colonization of sediment surfaces and the subsequent composition of communities as well as the distribution of cells between the pore water and the solid phase (Reardon et al. 2004, Fazi et al. 2008, Kanzog & Ramette 2009, Augspurger et al. 2010, Flynn et al. 2013, Rizoulis et al. 2013). In habitats poor in nutrients and/or organic carbon, sediment surfaces have frequently been suggested to be attractive habitats for microbes since they are offering access to limiting elements contained in the minerals (Marshall 1988, Banfield & Hamers 1997, Bennett et al. 2000, 2001, Rogers & Bennett 2004, Boyd et al. 2007, Mauck & Roberts 2007, Carson et al. 2009, Gadd 2010). Indeed, surfaces are sites of adsorption of organic molecules and thus may provide a reservoir of organic matter under limiting conditions (Bakker et al. 2003). Besides the mineral composition and accumulation of organic carbon, various other properties of sediments may play an important role in bacterial colonization, with, for example, grain size and shape influencing microbial community composition (Nickels et al., 1981; Llobet-Brossa et al, 1998; Edwards & Rutenberg 2001, Köster et al., 2005). Rough surfaces providing a higher surface area encourage bacterial settlement more strongly than smooth surfaces (Dubois et al. 2010). In this context, a point of discussion is that cells colonizing fissures and depressions at surfaces are protected from direct shear forces, as well as from direct protozoan grazing (Kölbel-Boelke et al. 1988). The same is argued for the embedment of individual cells in biofilms. Hydrodynamic conditions are thus another key-factor influencing the colonization of surfaces in water (Augspurger et al. 2010).

Suspended and attached prokaryotic communities in aquifers differ not only in terms of cell numbers. Early, it has been demonstrated that attached populations may exhibit distinct cell morphology (Hirsch et al. 1992) and physiology (Hirsch 1992, Kölbel-Boelke & Hirsch 1989, Watnick & Kolter 2000). More recently, it has been confirmed that suspended and attached prokaryotic communities most often show striking differences also in community composition (Reardon et al. 2004, Flynn et al. 2008, Griebler & Lueders 2009, Anneser et al. 2010, Flynn et al. 2013; Rizoulis et al. 2013, Hug et al. 2015). The factors responsible include those mentioned above, as well as additional ones such as the chemical composition of the groundwater, substrate and electron acceptor availability, the redox conditions, and explicitly the impact of contaminants (Griebler & Lueders 2009, Rizoulis et al. 2013). All of these factors act as selective forces on the prokaryotic communities dwelling on the sediment surfaces. To date, only a few studies have taken a comparative look at the importance of individual driving forces governing the characteristics of sediment prokaryotic communities.

Here, we tested the working hypothesis that high concentrations of organic pollutants such as petroleum hydrocarbons act as dominant selective force on the prokaryotic communities attached to sediment surfaces, being toxic for some of their members, while providing others with a source of carbon and energy (Foght 2008, Heider 2007). We propose that at high contaminant concentrations, other selective factors such as sediment and mineral properties as well as physical-chemical conditions are of comparatively minor influence, but become more relevant once contaminant concentrations become low. We analyzed sediments from an organically polluted aquifer exposed to different concentrations of petroleum hydrocarbons. Samples were collected from (i) a zone with high concentrations of contaminants, *i.e.* monoaromatic (BTEX) and polycyclic aromatic hydrocarbons (PAHs), both dissolved in the groundwater and adsorbed to the sediment matrix (referred to as Zone 1), (ii) a zone dominated by adsorbed contaminants but poor in dissolved contaminants (referred to

as Zone 2), and (iii) a zone with only a low PAH background adsorbed to the sediment (referred to as Zone 3). Detailed investigations involved qualitative and quantitative analyses of the bacterial communities and concentrated exemplarily on two very different types of minerals contained in the natural Quaternary sandy sediment, namely particles of translucent quartz with smooth surfaces and rough black mica grains.

2.2 Materials and Methods

2.2.1 Field site and sampling of groundwater and sediments

Groundwater and sediments were collected at a former gasworks site in Düsseldorf-Flingern, Germany. The Quaternary shallow sandy aquifer, contaminated over several decades with tar oil and residues of gasification of coal, today, after individual remediation activities, harbors a vertically thin plume of aromatic hydrocarbons dissolved in groundwater consisting mainly of BTEX (benzene, toluene, ethylbenzene, and xylenes) and naphthalene. Additionally, polycyclic aromatic hydrocarbons (PAHs) are, locally distinct, found adsorbed to the sediment matrix (Fig. 2.1) (Anneser et al. 2008, 2010).

Water samples for the analysis of dissolved organic contaminants and water chemistry were collected using a special high-resolution multi-level well. Fresh sediment samples were obtained by drilling. Sediment cores were subsampled under a continuous stream of argon gas. A detailed description of groundwater and sediment sampling is described in Anneser et al. (2008). The samples processed for this study originated from a survey in June 2009 and represent three zones of different contamination. Zone 1 was located at the time of sampling within the plume of dissolved contaminants (BTEX and naphthalene) at a depth between 6.80 - 6.85 m below land surface (bls) (Fig. 2.1, Table 2.1). Zone 2 (at 7.20 – 7.25 m bls) represents an area characterized by low concentrations of BTEX but high concentrations of PAHs adsorbed to the sediment (Fig. 2.1, Table 2.1). Zone 3, at 10.65 – 10.70 m bls, was

located beneath the highly contaminated area with BTEX compounds below detection and low background concentrations of PAHs (Fig. 2.1, Table 2.1). Further biogeochemical characteristics of groundwater from the individual depths are summarized in Table 2.1.

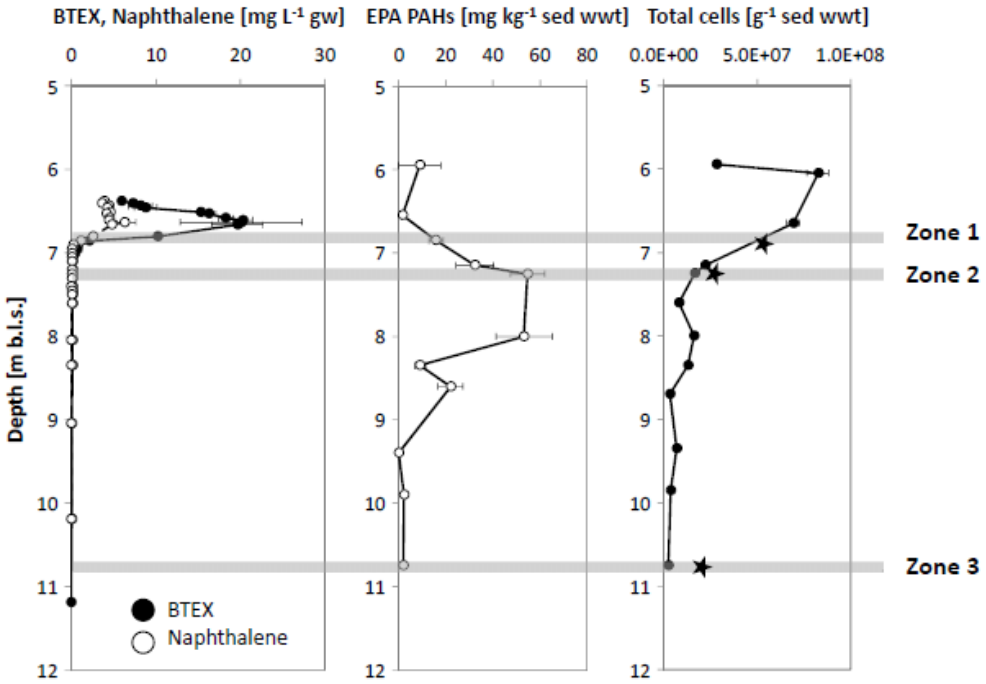

Figure 2.1: Vertical distribution of (a) monoaromatic hydrocarbons (BTEX) and naphthalene in groundwater, (b) the sum of EPA-PAHs sorbed to the sediment matrix, and (c) the total number of prokaryotic cells attached to the sediments at the test site in Düsseldorf-Flingern, Germany. Stars depict values of total cell counts in natural (mixed) sediment samples obtained after re-processing samples from the three selected depths (6.80-6.85m, 7.20-7.25m, and 10.65-10.70m); for further explanation refer to text. Values are means ± SD.

2.2.2 Groundwater analyses

Groundwater was withdrawn simultaneously from the three sampling depths, collected in 100 ml glass bottles and processed immediately for measuring electric conductivity, pH, and redox potential as well as for the analysis of dissolved iron and sulfide species, which were quantified *on-site*. Samples for the measurement of major anions and cations were cooled and analyzed back in the lab via ion chromatography (DionexDC-100, Idstein, Germany).

Groundwater samples dedicated to the analysis of dissolved mono- and polycyclic aromatic hydrocarbons were immediately split to glass vials of different volumes and treated with NaOH (0.1 M final concentration) to stop biological activity before being closed tightly. Later, BTEX concentrations were measured via GC-MS by headspace analysis. The less volatile polycyclic aromatic hydrocarbons (PAH) were extracted from 13 mL groundwater samples by adding 500 µL of cyclohexane and shaking intensively for 60 min. Contaminant concentrations in the cyclohexane phase were then determined by GC-MS via liquid injection. Detailed protocols are given in Anneser et al. (2008, 2010).

2.2.3 Analysis of sediment samples

Sediments from three depths were immediately processed for different subsequent analyses. Samples for community profiling and later mineralogical characterization were shock frozen on dry ice, while samples for cell counts were fixed with glutardialdehyde (2.5% final conc.). Concentrations of contaminants were determined from sediments after extraction (see below).

Table 2.1: Selected biogeochemical features of groundwater and sediment at three selected zones of varying contamination in a tar oil contaminated aquifer in Düsseldorf-Flingern, Germany.

Parameters	**Zone 1**	**Zone 2**	**Zone 3**
(Groundwater)	**6.80-6.85 m***	**7.20-7.25 m***	**10.65-10.70 m***
Cells mL^{-1} x 10^6	1.14	3.61	0.5
E_H [mV]	-80	-45	-44
EC [µS/cm]	1160	1240	1170
Alkalinity [mEq L^{-1}]	9.5	8.5	8.4
Nitrate [mg L^{-1}]	0.04	-	-
Sulfate [mg L^{-1}]	171	240	288
Sulfide [mg L^{-1}]	5.1	0.2	-

Fe(II) [mg L^{-1}]	1.1	0.2	3.4
DOC [mg L^{-1}]	8.3	3.85	2.5
Benzene [mg L^{-1}]	0.04	0.01	-
Toluene [mg L^{-1}]	4	0.08	-
Ethylbenzene [mg L^{-1}]	0.3	0.008	-
m/p-xylene [mg L^{-1}]	1.1	0.003	-
o-xylene [mg L^{-1}]	0.4	0.003	-
BTEX total [mg L^{-1}]	6.3	0.1	-
Dibenzofuran [mg L^{-1}]	0.5	0.2	-
Naphthalene [mg L^{-1}]	1.9	0.008	-
Indane [mg L^{-1}]	0.2	-	-
Indene [mg L^{-1}]	0.04	-	-
Fluorene [mg L^{-1}]	0.03	-	-
Acenaphthene [mg L^{-1}]	0.9	0.6	0.07
EPA-PAHs [mg L^{-1}]	4.8	0.5	0.05
Parameters	**Zone 1**	**Zone 2**	**Zone 3**
(Sediment)	**6.80-6.85 m***	**7.20-7.25 m***	**10.65-10.70 m***
Cells g^{-1} sed wwt x 10^7	6.9	1.6-2.2	0.21
Naphthalene [mg kg^{-1}]	11.7	18.7	0.03
Acenaphthene[mg kg^{-1}]	3.3	15.3	-
Fluorene [mg kg^{-1}]	0.6	20.6	1.7
EPA-PAHs [mg kg^{-1}]	15.6	54.6	1.7

* Depths of sediment origin are not absolute but may vary ± 2.5 cm. Thus groundwater features, in case of zone 1 and 2, represent mean values of two depths, and in case of zone 3, refer to values obtained for a depth of 11.20 m bls.

Where no value is given, the compounds could not be detected.

2.2.4 Analysis of sediment contaminant content

PAHs adsorbed to the sediment were extracted with acetone at the presence of an internal standard mixture containing deuterated acenaphthene, chrysene, perylene and phenanthrene species (Internal Standards Mix 25, Ehrenstorfer, Augsburg, Germany).

Aromatic hydrocarbons were determined with GC-MS applying the settings as described in Anneser et al. (2010).

2.2.5 Sorting of sediment mineral fractions

The samples from the three selected depths mainly consisted of medium-sized sand with a diameter of 0.2-0.63 mm. For sorting, frozen (dedicated to DNA extraction and mineral characterization) and fixed sediments (dedicated to total cells counting) were transferred to a sterile Petri dish of 6 cm diameter containing sterile PBS buffer (10 mM). Sorting was carried out using a sterile spatula and forceps under a stereo microscope with the petri dish being placed on an aluminum cooling block. For the following experiments, 4 fractions of sediment were considered: (1) natural mixed sediments, (2) translucent quartz grains, (3) mica grains, and (4) the remaining sediment (sediment fraction after the majority of translucent quartz and mica was removed by sorting; this sediment fraction was then mainly composed of coated quartz grains and a small content of silt and clay)(Fig. S2.1). After sorting, the fractions were transferred into separate 1.5 ml Eppendorf tubes and stored either in a solution of glutardialdehyde (2% final conc.) for total cell counting or at -22 °C for community analysis and mineral characterization.

2.2.6 Sediment surface area and roughness

Translucent quartz and mica particles were chosen because of their strongly differing properties. Quartz grains were characterized by flat smooth surfaces while the surfaces of mica particles were found comparably rough (Fig. S2.1). The surface area of both mica and quartz particles has been calculated using the equation for the external specific surface area (SSA_{ext}) as described in Dubois et al. (2010). The roughness factor λ applied for quartz was 34 and 126 for mica, as stated in Suarez & Wood (1998).

2.2.7 Sediment elemental composition

After drying of sediment particles, the elemental composition of individual grains was determined by scanning electron microscopy (SEM) in combination with energy-dispersive X-ray spectrometry (EDX) using a Leo 1450 VP SEM equipped with an Oxford INCA Energy 200 Premium Si (Li) SATW-Detector. An acceleration voltage of 20kV and a probe current of 221 pA were used. X-ray spectra for quantification were acquired on flat parts in the centers of the grains to avoid artifacts from sample topography. At least 10 spectra of individual particles were measured for each sample type. Elemental quantification was done using the Oxford INCA software package.

2.2.8 Cell detachment and total cell counting

To detach the cells, 0.1 to 0.5 g of the sorted sediment was transferred into a 2 ml Eppendorf tube. In case less than 0.5 g of sediment was chosen or available, sterile quartz grains were added to a final weight of 0.5 g. Subsequently, 1.5 ml of 10 mM PBS were added to each sample before they were placed on a swing mill (Retsch GmbH, Haan, Germany) and shaken for 3 min at 20 Hz. After 5 min of sedimentation, the supernatant (about 1.5 ml) was carefully transferred into ultra-centrifuge tubes on top of 5 ml cold Nycodenz-solution (1.3 g mL^{-1}). This mechanical dislodgement step was repeated; so that the total volume in the ultra-centrifuge tubes finally equaled 8 ml. Samples were centrifuged at 11,000 rpm and 4 °C for 1 hour. Subsequently, the top 6.5 ml of each tube containing the cells but no more bigger inorganic particles were transferred to test tubes containing 5 ml sterile filtered 10 mM PBS and 15 µL of the nucleic acid stain Sybr-Green I (1000x, Molecular Probes). After brief vortexing and incubation for 15 min at room temperature in darkness, samples were filtered through a 0.2 µm black polycarbonate filter (Ø 25 mm, Whatman, Kent, UK), embedded in

anti-fading reagents (50% glycerol in 20 mM phosphate buffer and 0.1% *p*-phenylenediamine), and placed on a microscope slide. A minimum of 500 cells per sample was counted under the epifluorescence microscope (Zeiss Axioscope) at 1000 x magnification.

2.2.9 DNA extraction and community fingerprinting

DNA extraction from aquifer sediment (both sorted fractions and natural sediment) and T-RFLP analysis were performed as described in Winderl et al. (2008) and Pilloni et al. (2011). Data evaluation was performed as reported elsewhere (Pilloni et al. 2012, Larentis et al. 2013). Data from 454 pyrosequencing as conducted in previous studies at this site (Winderl et al. 2008, Anneser et al. 2010, Pilloni et al. 2012, Larentis et al. 2013) were used to assign the most common T-RFs to specific taxonomic groups. Briefly, forward and reverse quality-trimmed pyrosequencing reads were assembled into short contigs (up to ~500 bp) using the software Seqman II (DNAStar). Contigs were then aligned into an ARB (Ludwig *et al*., 2004) database (version SSURef-95, July 2008) and T-RFs of amplicon contigs were predicted using arb_edit4. Deviations between predicted and measured T-RFs were eventually handled by referring to our previously analyzed cloned amplicons from the same contaminated site (Winderl *et al*., 2008). For community fingerprinting of the translucent quartz and mica fractions, we were unfortunately not able to analyze biological replicates but only technical replicates (duplicates and occasionally triplicates) due to the limited material available after sorting. Earlier studies on sediments from the same sites showed a low variability within biological replicates and a high reproducibility of technical replicates with our T-RFLP protocol (Pilloni et al. 2012). Moreover, key degrader populations identified match well findings from earlier studies (Winderl et al. 2008, Anneser et al. 2010, Larentis et al. 2013).

2.2.10 Data preparation and statistical analysis

An unconstrained correspondence analysis was used to analyze the community composition of the six different samples (mica and quartz in the three different zones). The dominant TRFs, having a relative abundance higher than five percent in at least one of the sample were picked, resulting in 17 TRFs. In this analysis distances between objects (samples) are preserved; therefore distances between samples represent their similarity. Onto this ordination, environmental vectors were fitted using the envfit function provided in the vegan package (Oksanen et al., 2013) implemented in the open-source platform R (version 3.1.0). Individual contaminants were summarized into the categories BTEX (benzene, toluene, ethylbenzene, *m*-/*p*-xylol, *o*-xylol), PAH in water (naphthalene, dibenzofuran, fluorine, and acenaphthylene), and PAH adsorbed to the sediment (naphthalene, acenaphthylene, and fluorene). The significance of the fitted vectors was assessed using 720 permutations setting the alpha level to 0.05.

A principal component analysis (PCA) was used to elucidate the main environmental variables separating the different samples. The variables (as described above) were standardized to z-scores.

Venn diagrams were drawn to show overlapping and unique TRFs. Information on the abundance was transformed to presence-absence data and TRFs of the three different zones were compared regarding their appearance on one of the four different sediment fractions (mica, translucent quartz, the remaining sediment fraction, and natural mixed sediment).

Differences between individual samples and treatments were evaluated using a rank sum test (Mann Whitney-U test).

2.3 Results

2.3.1 Distribution of contaminants and overall physico-chemical conditions

The environmental conditions in the three zones of the organically contaminated aquifer investigated were significantly different in many respects. Zone 1 was characterized by comparably high concentrations of BTEX and PAHs dissolved in groundwater, with concentrations of 6.3 mg L^{-1} and 4.8 mg L^{-1}, respectively (Tab. 2.1, Fig. 2.1). Values obtained for BTEX and PAHs match well the 8.3 mg L^{-1} of DOC when considering that part of the very volatile contaminants get lost during DOC-measurement. Polycyclic aromatic hydrocarbons adsorbed to the sediment accounted for 15.6 mg kg^{-1} sediment (wwt) with 75% represented by naphthalene (Table 2.1). Zone 1 was highly reduced (E_H = -80 mV) and exhibited strong evidence for active sulfate and iron reduction indicated by the concentrations of dissolved sulfide (5.1 mg L^{-1}) and ferrous iron (1.1 mg L^{-1}) (Tab. 2.1).

Zone 2 was characterized by considerably lower concentrations of dissolved contaminants, with only 0.1 mg L^{-1} of BTEX and 1 mg L^{-1} of PAHs, respectively. However, this zone contained 54.6 mg kg^{-1} sediment (wwt) of PAHs adsorbed to the sediment matrix, almost equally composed of naphthalene, acenaphthene, and fluorene (Tab. 2.1, Fig. 2.1). Concentrations of dissolved sulfide and ferrous iron were both in the range of 0.2 mg L^{-1} and the redox potential increased to E_H = -45 mV when compared to Zone 1 (Tab. 2.1).

Zone 3 was located 3.5 to 4 meters vertically distant to zones 1 and 2 outside the main area of contamination. Still, a minor PAH background of 1.7 mg kg^{-1} sediment (wwt) (mainly fluorene) adsorbed to the sediment was present. Monoaromatic hydrocarbons were below detection limit and PAHs dissolved in groundwater accounted for less than 0.1 mg L^{-1} (Tab. 2.1). While no sulfide was found in dissolved form, there was evidence for active iron reduction from ferrous iron concentrations of 3.4 mg L^{-1}. The redox potential was similar to the one measured in Zone 2 (Tab. 2.1).

Table 2.2: Mineral composition of sediment samples from three different depths.

	Depth [m]	Translucent quartz	Mica	Clay slate	Laminated quartz
Zone 1	6.80-6.85	10.4%	1%	12%	76.6%
Zone 2	7.20-7.25	14.1%	0.1%	12%	73.9%
Zone 3	10.65-10.70	11.1%	0.1%	15%	74%

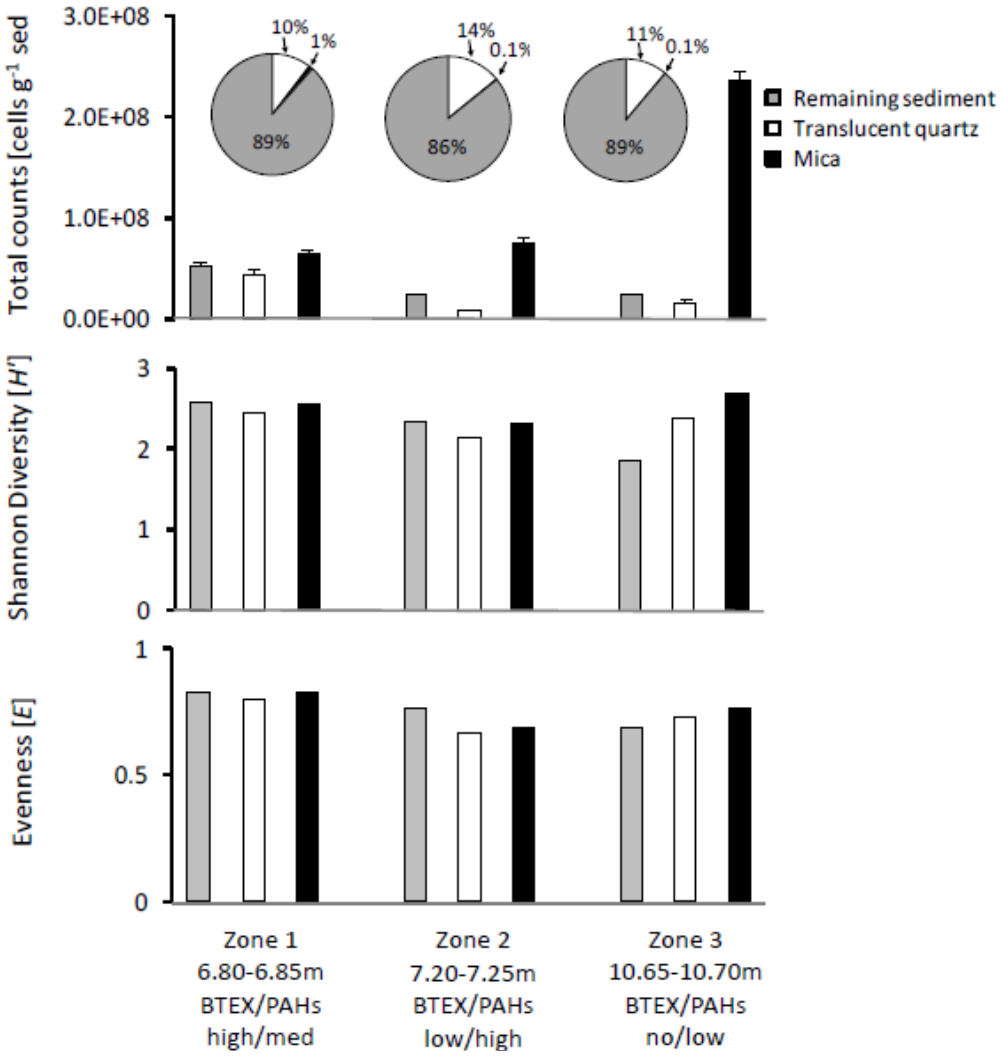

Fig. 2.2: Total cell counts, Shannon diversity [H'] and Evenness [E] of bacterial communities attached to different sediment mineral fractions from three depths of varying contamination in a sandy aquifer contaminated with petroleum hydrocarbons. Cake diagrams refer to the relative abundance of the sediment mineral fractions.

2.3.2 *Mineral composition of sediments*

The mineralogical examination of the selected sediments revealed a high content of quartz with 10-14% of translucent quartz grains and 74-77% of quartz particles coated with reddish and greenish precipitate. 12-15% of the sediments were clay and silt particles (Tab. 2.2). A small fraction of 0.1 to 1% of the sediments was constituted by dark mica particles (Tab. 2.2, Fig. 2.2). Only minor changes in sediment composition were observed with depth, i.e. the highest mica fraction was found in zone 1, while zone 2 contained a slightly higher fraction of translucent quartz (Tab. 2.2, Fig. 2.2).

The SEM-EDX analysis of the quartz fraction revealed a general elemental composition (mass percentage) of O (63-69%), Si (26-33%), and Al at a mass proportion of 0.8-1.3% in the translucent quartz and 1.3-3.3% in the coated quartz. Iron was a significant element in the fraction of coated quartz with 7% in Zone 1, 3% in Zone 2 and trace concentrations (0.1%) in Zone 3. The translucent quartz fraction contained Fe only at very low concentrations. Both quartz fractions, contained traces of Ca, K, and Mg. The elemental analysis of the mica fraction revealed an elemental composition (mass percentage) of O (54-63%), Si (22-27%), Fe (3-11%), Al (5-7%), K (0.7-2.4%), Na (0.5-1.8%), Ca (0.1-1.2%), Mg (0.5-0.7%), and traces of Mn, P, S, and Ti; a composition that very much refers to biotite.

2.3.3 *Sediment total cell numbers*

For the samples immediately fixed after sediment drilling, highest cell counts were found in Zone 1 ($6.9 * 10^7$ cells g sed wwt^{-1}), followed by Zone 2 ($1.6\text{-}2.2 * 10^7$ cells g sed wwt^{-1}), and Zone 3 ($2.1 * 10^6$ cells g sed wwt^{-1}) (Fig. 2.1). Water samples revealed a different picture with highest counts in Zone 2, followed by Zone 1 and Zone 3 (Tab. 2.1). Sediment samples re-processed for the current study, i.e. being influenced by the sorting procedure in PBS buffer, exhibited slightly different to significantly different values for total counts in the

mixed, natural fractions (Fig. 2.1 & Fig. 2.2). While the numbers of attached cells agreed fairly well for samples of Zone 1 and 2, total counts in the reprocessed mixed sediment samples from Zone 3 revealed tenfold higher cell numbers than determined in the parent samples. With regard to the individual mineral fractions, highest cell counts were always obtained for mica, independently of depth and degree of contamination (Fig. 2.2). The lowest cell numbers were always found with the translucent quartz fraction (Fig. 2.2). A Mann-Whitney-U test revealed that in Zone 1, there was no significant difference between the cell abundance on translucent quartz and the mixed sediment fraction (mainly coated quartz) ($P = 0.85$, $n_1 = n_2 = 3$), while all other pairwise comparisons between individual sediment/mineral fractions were different by trend from each other ($P = 0.01$, $n_1 = n_2 = 3$). The mica fraction of Zone 3 contained more than seven times the cells counted in other fractions (Fig. 2.2). However, due to the low overall content of mica in the natural sediments, the generally higher cell numbers with the mica fraction represented only a small portion of the total cells in the natural sediments (Tab. 2.3).

Table 2.3: Relative abundance of cells with different sediment mineral fractions.

	Depth [m]	Translucent quartz	Mica	Remaining sediment*
Zone 1	6.80-6.85	19.2%	2.7%	78.1%
Zone 2	7.20-7.25	7.4%	0.5%	92.1%
Zone 3	10.65-10.70	9.5%	1.2%	89.3%

*Cell numbers and relative abundance of cells on the remaining sediment fraction has been calculated based on direct counts with translucent quartz, mica, and mixed natural sediment samples.

2.3.4 Bacterial community patterns

Both bacterial Shannon diversity and evenness were slightly higher in Zone 1 than in Zones 2 and 3, the only exception being the diversity of the mica in Zone 3 (Fig. 2.2). In both Zone 1 (which contained a high amount BTEX and naphthalene) and Zone 2 (which contained a high amount of adsorbed PAHs), the diversity was quite similar on all three

mineral fractions analyzed, but in Zone 3, where hardly any contaminants were found, the highest diversity could be found on the mica fraction, while the community on the translucent quartz showed a lower overall diversity (Fig. 2.2). Likewise, Zone 1 revealed a roughly equal evenness with all three mineral fractions, whereas in Zone 2 the highest evenness was found on the remaining sediment fraction, and Zone 3 revealed a slightly higher evenness for the mica fraction (Fig. 2.2).

Although not much differences were observed with regard to the bacterial diversity, the bacterial community composition, as analyzed by DNA fingerprinting, revealed significant differences between the sediments from the three investigated zones (Fig. 2.3). In total, 113 distinct T-RFs were obtained, with 14 appearing exclusively in Zone 1, 48 exclusively in Zone 2 and 30 solely in Zone 3. Only 7% of all OTUs (operational taxonomic units) were commonly present in the three zones. The share of OTUs between individual zones was small ranging from 3 to 5% (Fig. S2.2). Looking in more detail into the composition of the bacterial communities with respect to the selected mineral fractions exhibited very distinct patterns for the three zones. While in Zone 1 the selected mineral fractions, *i.e.* translucent quartz (TQ), mica and remaining sediment (coated quartz plus silt and clay) shared 55% of all OTUs, only 18% of OTUs were common in Zone 3 and no OTU was commonly present in Zone 2 (Fig. S2.3). In Zone 2, the TQ fraction hardly shared any community members with the other mineral fractions. However, as can be seen from the correspondence analysis (CA), the TQ community was quite similar to the TQ fraction and mica fraction in Zone 3 (Fig. 2.4; see below).

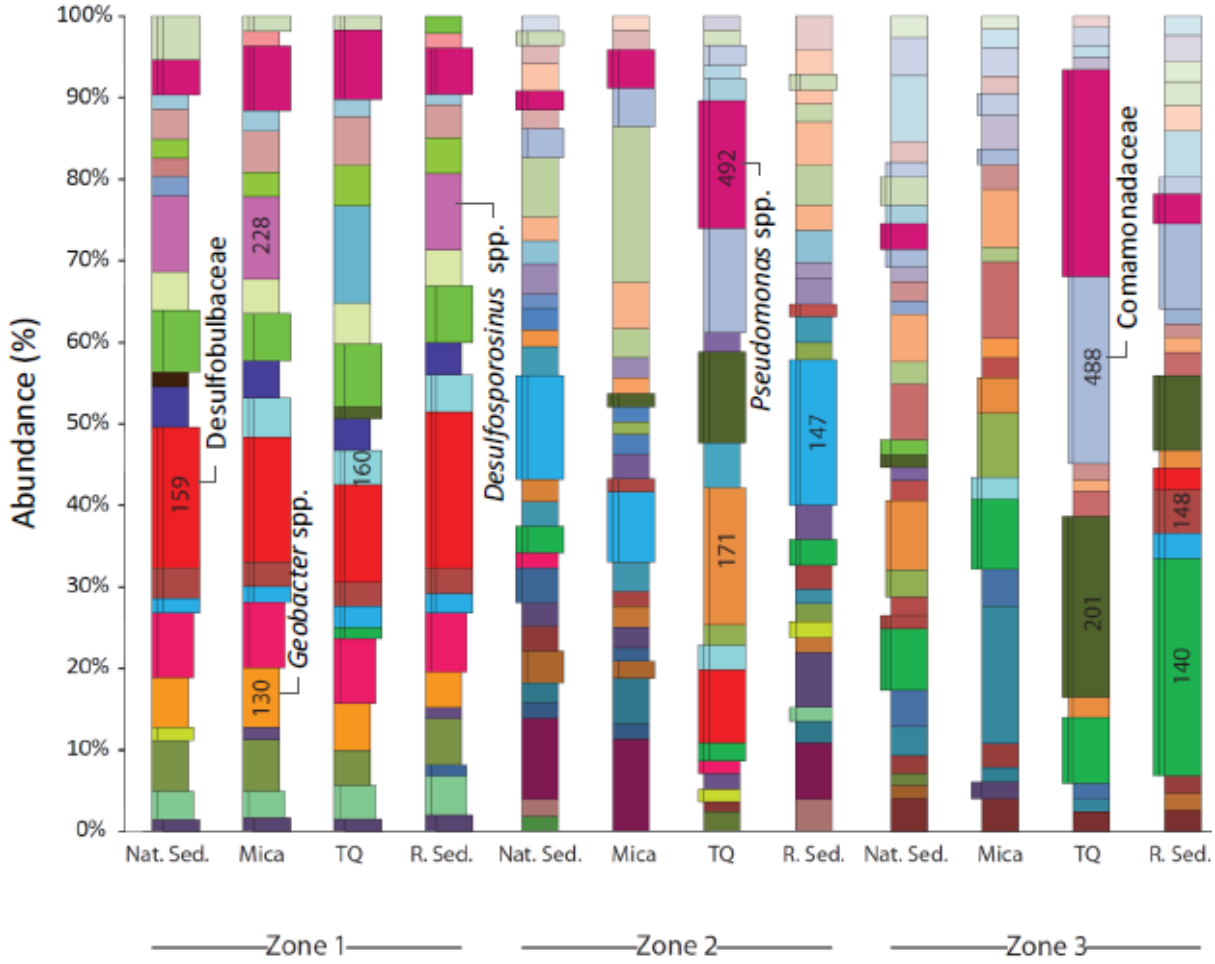

Fig. 2.3: Relative abundance of dominant operational taxonomic units (OTUs) found with the different sediment mineral fractions from three zones of varying contamination in a sandy aquifer contaminated with petroleum hydrocarbons. Values highlighted in individual pillars refer to specific TRFs.

The most dominant OTUs were represented by the T-RFs 130 bp, 140 bp, 147 bp, 159 bp, 160 bp, 171 bp, 201 bp, 211 bp, 228 bp, 488 bp, and 492 bp (Fig. 2.3). The T-RFs 130 bp, 159 bp (possibly together with 160 bp) and 228 bp are related to known key-degraders in the contaminated aquifer, i.e. bacteria affiliated to *Geobacter* spp., Desulfobulbaceae (e.g. *Desulfocapsa* spp.), and clostridial sulphate reducers (*Desulfosporosinus* spp.), respectively. The TRFs 130 and 228 were found exclusively restricted to Zone 1. TRF 159 (and together with 160) exhibited its highest relative abundance in Zone 1, however, has been found abundant in the bacterial community on TQ in Zone 2 as well (Fig. 2.3). T-RF 492 bp, affiliated to *Pseudomonas* spp., was found in all three different zones of the aquifer, but did

not show up in the mica fraction in Zone 3. T-RF 488 bp, affiliated to members of the *Comamonadaceae*, was present only in Zone 2 and 3, most prominent on the TQ fraction (Fig. 2.3). TRF 147 was mainly found in Zone 2, and TRFs 140, 201 and 492 were dominant in Zone 3 (Fig. 2.4). More detailed information on the affiliation of TRFs to specific bacterial lineages is provided in Pilloni et al. (2012) and Larentis et al. (2013).

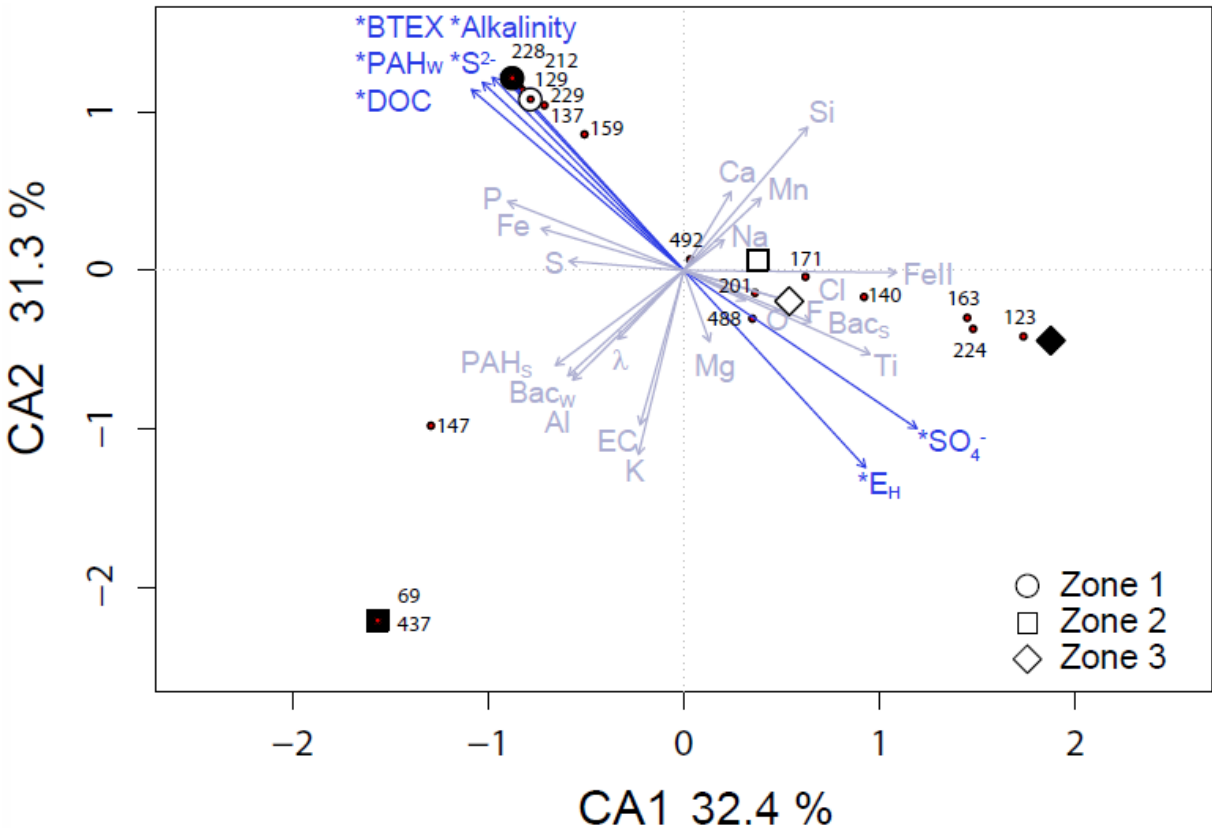

Fig. 2.4: CA plot evaluating sediment bacterial fingerprinting data together with sediment and mineral properties as well as the physical-chemical characteristics of groundwater. Open symbols refer to the translucent quartz fraction and black symbols to the mica fraction. Small dots represent specific OTUs with numbers referring to the respective TRFs length in base pairs. BTEX = sum of benzene, toluene, ethylbenzene and xylenes; S^{2-} = dissolved hydrogen sulfide; PAH = polycyclic aromatic hydrocarbons ('W' = in water or 'S' on sediment), DOC = dissolved organic carbon, EH = redox potential, SO_4^{2-} = dissolved sulfate; Bac = total prokaryotic cell counts ('W' = in water or 'S' on sediment); EC = electric conductivity; Fe(II)= ferrous iron dissolved in groundwater; all other letters stand for chemical elements identified during mineral analysis.

2.3.5 *Selective drivers of sediment bacterial community patterns*

To compare the similarity between the bacterial communities in the sediment fractions translucent quartz and mica of the three zones, and to evaluate major drivers of bacterial community composition with regard to physico-chemical conditions, contaminant chemistry, and mineral composition, individual multivariate analyses were performed. A principal component analysis (PCA) revealed a separation of the different translucent quartz (TQ) and mica fractions from the individual zones (depths) governed mainly by the contaminant loading, total amount of dissolved organic carbon, dissolved hydrogen sulfide, and alkalinity (vertical axis in the PCA plot; Fig. S2.4). The two mineral fractions separated from each other (horizontal direction in the PCA plot) by its different mineral composition and surface roughness. A subsequent correspondence analysis (CA) revealed attached bacterial communities from TQ and mica closely clustering together in Zone 1 with respect to bacterial community composition (Fig. 2.4). Key environmental factors associated were again the concentrations of dissolved contaminants (BTEX & PAHs) as well as sulfide, total DOC and alkalinity (Fig. 2.4). Samples from Zone 2 and 3 were clearly distant to the samples from Zone 1. Highest dissimilarity within communities between the two selected sediment fractions TQ and mica were observed with Zone 2, with the high concentration of adsorbed PAHs being the major driver, as indicated by the CA.

2.4 Discussion

There is a multitude of factors that may contribute to the selective assembly of microbial communities on sediment surfaces including physical-chemical factors, sediment properties, resources availability, hydrodynamics, and the local and regional pool of diversity (Griebler & Lueders 2009; Augspurger, et al., 2010; Stegen et al., 2012, Lindström & Langeheder 2012). Organic contaminants, such as petroleum hydrocarbons, are carbon and

energy source to a subset of the natural microbial community while being toxic to others. Moreover, a high load of organics switch aquifers from oxidized to reduced conditions. As such, petroleum hydrocarbons are considered key drivers of microbial community composition and dynamics (Bombach et al. 2009, Bordel et al. 2007, Castillo & Ramos 2007, Meckenstock et al. 2015, Meckenstock & Mouttaki 2011, Vecht et al. 1988). Groundwater ecosystem, in particular, are generally considered low-productivity systems characterized by comparably stable environmental conditions. As such, organic contamination may constitute an ecosystem disturbance with more pronounced effects to the intrinsic communities than in other, more productive and dynamic habitats (Griebler & Lueders 2009). In our study, we specifically investigated the influence of organic contamination, i.e. BTEX and PAHs, and water chemistry in comparison to individual sediment properties, such as mineral composition and surface roughness, onto the composition and cell density of attached microbial communities in a shallow sandy aquifer. Our working hypothesis assumed that sediment properties are indeed active selective factors shaping sediment microbial community patterns but are superimposed by organic contaminants at high loadings.

The shallow sandy aquifer at the former gasworks site in Düsseldorf-Flingern, Germany, was heavily contaminated locally by subsurface disposal of tar-oil several decades ago (Anneser et al. 2008). Still, petroleum hydrocarbons, i.e. mainly monoaromatic (BTEX) and polycyclic aromatic hydrocarbons (PAHs), partition from the contaminant source into groundwater and form a contaminant plume (Anneser et al. 2008, Prommer et al. 2009, Meckenstock et al. 2010). With respect to the contamination, we distinguished three zones in the vertical direction within the shallow aquifer, a zone of highly contaminated groundwater (Zone 1), a zone with high concentrations of contaminants adsorbed to the sediment matrix (Zone 2), and a zone with only minor contamination (Zone 3) (Fig. 2.1). Microbial community composition investigated in natural (‘mixed’) aquifer sediments collected from

different zones and depths of the respective aquifer have been studied in earlier work with respect to key contaminant degraders (Winderl et al. 2008, Pilloni et al. 2011, 2012, Larentis et al. 2013), redox gradients (Anneser et al. 2008, 2010), and sulfur cycling (Einsiedl et al., 2015). Here, we specifically evaluate individual selective forces responsible for shaping sediment microbial community composition and biomass including organic contamination and water chemistry. Moreover, two very different mineral fractions, i.e. translucent quartz (TQ) and mica, were selected and sorted from the natural sediments to test for the influence of sediment properties.

It was striking that the attached bacterial communities from the different depths of the contaminated aquifer face very different environmental conditions that actively shape the composition of the attached bacterial communities. Only 7% of all OTUs (operational taxonomic units) were found in common with the different zones. This core microbiome consisting of 8 TRFs (140, 147, 148, 159, 160, 201, 492, 509) displayed opposing abundances. TRF 159 was most abundant in Zone 1, whereas TRF 147 was mainly found in Zone 2, and TRFs 140, 201 and 492 were dominant in Zone 3 (Fig. 2.3 and 2.4). In Zone 1, exhibiting high concentrations of petroleum hydrocarbons (BTEX and PAHs) and dissolved hydrogen sulfide in groundwater as well as a considerable concentration of PAHs adsorbed to the sediment matrix, the organic contamination superimposed all other factors potentially contributing to the assembly of sediment bacterial communities. The share of 58% of OTUs between the TQ and the mica fraction, accounted for the highest similarity in community composition when compared to the other zones (Fig. S2.3). Moreover, bacterial Shannon diversity and Evenness were not significantly different and the two different minerals carried a similar number of attached cells per gram sediment. This picture changed completely in Zone 2. Here, with a share of only 3% of OTUs, the two mineral fractions were characterized by two completely different bacterial communities. Especially the mica community separated

from all others mainly driven by the high concentration of PAHs adsorbed to the sediment. This selectivity was reflected by the high number of OTUs (42%) that were found exclusively in Zone 2. Here it needs to be considered that petroleum hydrocarbons show a lower sorption to quartz than to mica (Kleineidam et al. 1999a, 1999b, Müller et al. 2007). Additionally, already at a low sediment organic carbon (OC) content PAHs effectively sorb to surfaces, with mica, the iron-coated quartz and silt and clay significantly exceeding the OC content of translucent quartz (Müller et al. 2007, Anneser et al. 2010). It thus can be assumed that the PAHs loading were much higher on mica and the 'remaining sediment fraction' (sediment that was depleted in TQ and mica) than on the translucent quartz. And indeed, between the mica fraction and the remaining sediment fraction, a significantly higher share of OTUs (27%) was found. These facts clearly point at adsorbed PAHs being the main driver for bacterial community composition in Zone 2. Opposite to what was expected for Zone 3, with its minor contamination, the pronounced differences in attached bacterial community composition, with a share of only 26% of OTUs, could not be explained by sediment properties. In fact, mineral composition and surface roughness only had a low and non-significant explanatory power in the correspondence analysis (CA) using the post-hoc analysis 'envfit'. While the CA shows the high similarity of the samples in zone 1, sharing several key taxa, which can be explained by the high levels of contamination, it also points at bacterial communities on mica to be highly different in the three zones. In comparison, the OTUs found on TQ did not separate as much in the ordination plot. Especially TQ samples from Zone 2 and 3 differed only marginally. This clearly indicates distinct drivers that influence the community composition on mica and TQ.

Within each of the three zones tested, the total numbers of attached cells were always highest on the mica fractions and lowest on the translucent quartz, indicating that surface roughness and thus specific surface area do play a role in microbial colonization of sediments.

This difference in cell numbers with TQ and mica was small, although of borderline significance, in Zone 1 and 2 with its high contamination, but well pronounced in Zone 3 (Fig. 2.2). A positive relationship between bacterial cell numbers and sediment surface area has been observed in many other studies (Meadows & Anderson 1967; Dale 1974; Nickels et al., 1981, DeFlaun & Mayer, 1983).

The important role of contamination as a strong selective force is also obvious from the bacterial community composition in terms of specific OTUs detected. In Zone 1, known key-degraders of BTEX, i.e. representatives of the taxa of *Geobacter* spp. (TRF 130 bp), Desulfobulbaceae (TRF 159 bp) and *Desulfosporosinus* spp. (TRF 228 bp) that have been identified in previous studies at this site (Winderl et al. 2008, Anneser et al. 2010, Pilloni et al. 2011, 2012, Larentis et al. 2013) were most abundant. Moreover, their relative abundance did not differ significantly between TQ and mica (Fig. 2.3). Specific BTEX degraders (TRFs 130 and 228) were not detected in Zone 2 and 3. The BTEX thus contribute to the assembly of bacterial communities being a priority organic carbon and energy source. However, the fact that other OTUs, abundant in Zone 2 and 3, are absent in Zone 1, e.g. TRF 488, may also point at BTEX being a toxicant to many microbes (Sikkema et al. 1995, Isken & de Bont 1998). A similar conclusion is drawn by Rizoulis and coworkers (2013) who studied microbial communities in a phenol contaminated sandstone aquifer.

In conclusion, our study underlines the overwhelming role of aromatic petroleum hydrocarbons, which are prominent pollutants in groundwater ecosystems, in shaping sediment bacterial communities. Organic contamination with the very mobile BTEX, as present in Zone 1, was shown to superimpose the selective power of sediment properties, such as mineral composition and surface roughness that were frequently shown to significantly influence colonization, establishment, and composition of attached microbial communities. However, with the more immobile and adsorbed PAHs, contaminating Zone 2, sediment

properties came indirectly into play because surface roughness and sediment coating are linked to organic carbon content which itself is linked to sorption capacity. There is strong indication that PAHs mainly adsorbed to sediment fractions other than the translucent quartz are responsible for the tremendous differences in sediment community composition found in Zone 2. Most pronounced direct influence of sediment properties onto attached bacterial community composition was revealed in Zone 3 where the organic contamination was minor.

This study has provided a better understanding of factors driving the assembly of sessile microbial communities in a tar-oil contaminated porous sandy aquifer, revealing pronounced phylogenetic differences within sediment bacterial communities with respect to mineralogy and contaminant load. Without doubt, only a small selection of environmental parameters have been tested, while others, such as biological factors, i.e. top-down control on bacterial communities via protozoan grazing and viral lysis (Simek, et al., 2003, Kent, et al., 2004, Salcher, et al., 2005, Kent, et al., 2007; Wey et al., 2008) await evaluation. Also, the temporal dynamics of sediment bacterial communities caused by the continuous exchange of cells between the water and sediment phase needs to be tested (Zhou et al. 2012, Hug et al. 2015). Another point of interest refers to ecological theory with respect to microbial community assembly at a local and regional scale (Lindström & Langenheder, 2012). A highly contaminated zone within an aquifer characterized by highly specific microbial communities, as introduced in this paper, constitutes a continuous source of species and specific functions to down-gradient environmental compartments including surface waters and soils (Hug et al. 2015).

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3. Protocols for Hydrocarbon and Lipid Microbiology

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Mini sediment columns and two-dimensional sediment flow-through microcosms – versatile experimental systems for studying biodegradation of organic contaminants in groundwater ecosystems

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Abstract

Groundwater ecosystems are our most important source for drinking water supply. The increasing pressure to our groundwater reservoirs from anthropogenic contamination is a major threat not only to the ecosystem but also to human health. Microbial transformation of quantitatively important organic contaminants, such as petroleum hydrocarbons, in aquifers is an ecosystem service of ecological as well as economic importance. However, key controls and limitations of biodegradation *in situ* are still poorly understood. Facing the limited accessibility of the subsurface, the complex structural heterogeneity and the hidden temporal physical-chemical and biotic dynamics, bench-top experimental systems are necessary tools for a systematic and controlled investigation of key variables in contaminant removal processes at appropriate micro- and meso-scales. Here, we introduce mini sediment columns and two-dimensional sediment flow-through microcosms as complementary versatile experimental systems that offer a high degree of simplification, experimental control and replication.

3.1 Introduction

The continuous and ever increasing contamination of the environment constitutes a major threat not only to the various ecosystems but also to human health [1]. The need for safe water and food, as well as the enormous financial burden of remediation and sanitation of contaminated sites, make ecosystem services such as natural attenuation of organic contaminants an attractive option [2, 3].

Groundwater is one of the most important resources for human life, especially in its role as an essential source for drinking water supply. Thus, contamination of groundwater and consequently drinking water quality is of increasing societal concern [4]. In Europe, hundreds of thousands of groundwater bodies are lastingly contaminated with a point source, e.g. at

former gasification plants, at industrial or landfill sites and at sites of accidental contaminant spills. Moreover, most of our aquifers are increasingly contaminated by diffusive contaminants originating from extensive agriculture (e.g. nutrients and pesticides), waste water (e.g. pharmaceuticals), and manure (e.g. antibiotics, pathogens), as well as from atmospheric deposition.

Petroleum hydrocarbons are one class of groundwater contaminants of major concern. Besides some natural sources, such as tar pits and natural petroleum seepage [5], most petroleum hydrocarbon contaminations in groundwater are of anthropogenic origin, from disposal of industrial waste products such as tar oil, leaking pipelines and storage tanks, as well as accidental contaminant release [6–8]. Among the petroleum hydrocarbons frequently detected in contaminated groundwater are representatives of monoaromatic hydrocarbons (e.g. benzene, toluene, ethylbenzene), polycyclic aromatic hydrocarbons (e.g. naphthalene, acenaphthene) and NSO heterocyclic compounds (e.g. quinoline, benzothiophene, benzofuran) [9–11]. Some of these hydrocarbons are known or suspected to be carcinogenic or toxic to both eukaryotic and prokaryotic organisms [12, 13]. Dependent on their concentration, individual aromatic hydrocarbons pose a severe toxic stress to members of aquifer microbial communities [14, 15]. On the other hand, microbiological research of the past decades has uncovered the enormous natural potential for microbial degradation and the presence of aerobic as well as anaerobic key degrader of aromatic hydrocarbons in the environment, and groundwater in particular [16].

The microbial transformation of organic contaminants in nature is an ecologically and economically valuable process. Since ‘monitored natural attenuation’ (MNA) and ‘enhanced natural attenuation’ (ENA) are becoming accepted strategies for the remediation of organically polluted aquifers, a better understanding of key controls of microbial contaminant transformation *in situ* is required. However, the limited accessibility, structural and

physicochemical heterogeneity, and hidden temporal physical-chemical and biotic dynamics of the subsurface strikingly challenge the systematic and controlled investigation of contaminant removal processes at an appropriate micro- and mesoscale in the field. An intelligent design of series of inhouse experiments, particularly when combined with mathematical modelling, may allow testing theoretical concepts and hypotheses. The complex conditions in the field, instead, often lead to vague interpretations and speculations which then require a fitting of parameters, hampering the evaluation of conceptual models [17]. Microbial model systems, such as batch tests, sediment columns and bioreactors, constitute a meaningful complementary alternative to field investigations that offer a high degree of simplification, experimental control, and replication [18].

Microbial ecology and specifically contaminant microbiology have a long history of using micro-, meso-, and macroscosms. For studying microbial contaminant transformation processes in groundwater and aquifers, microbial model systems need to reflect some key groundwater ecosystem features. First, and most important, the test systems must contain a solid matrix, such as rock or sediment, which is partly or fully saturated with groundwater (liquid phase). In aquifers the majority of microorganisms ‘live and work’ associated to sediment surfaces, with only a small fraction suspended in the groundwater [19–21]. This is of specific importance since the microbial activity, physiology, and community composition can vary dramatically between the suspended and attached microbes [22–24]. Second, aquifers are open systems connected with other ecosystems through groundwater flow, which provides transport of energy (including contaminants), nutrients and organisms. A strong physical-chemical and biological interaction between the liquid and the solid phase is desired. Model systems that fulfill these most necessary requirements are sediment columns (one-dimensional), frequently applied in varying sizes, as well as two-dimensional and three-dimensional flow-through sediment micro- and mesocosms [25].

In this book chapter, we will focus on two very versatile microbial model systems applicable for studying abiotic and biotic processes in porous groundwater systems: (1) highly parallelized mini sediment columns and (2) 2-D sediment flow-through microcosms. Both systems are described with a focus on aerobic and anaerobic degradation of petroleum hydrocarbons. These model systems can, of course, be used for a broad range of further applications in microbial ecology.

3.1.1 Mini sediment column systems

Sediment-packed column systems resemble natural aquifer conditions for many applications and research questions. They contain sediment or an alternative solid matrix such as glass beads that supports the establishment of attached microbial populations or mixed communities. Furthermore, they are flow-through systems facilitating advective transport of dissolved and particulate compounds, such as dissolved organic carbon (including contaminants), electron acceptors (oxygen, nitrate, sulfate), nutrients, colloids and organisms. Sediment flow-through columns provide a high experimental flexibility (1). Columns can be run with sterile medium or natural groundwater (2). They can be inoculated with selected model microbial strains or packed with fresh sediments carrying the natural microbial communities and fed with active groundwater (3). Microbial food web interactions can be considered or excluded via selective pre-filtration of the liquid phase (17, 25, 38, 39, 42, 72). Independent control of the flow-through rate, size of the sediment column, grain size of the filling, the water residence time (which controls residence time of mobile contaminants), reactive surface and microbial biomass allow researchers to specifically arrange column systems according to their research questions.

Sediment columns have proved to be valuable for studying (i) transport characteristics of dissolved contaminants [26, 27], (ii) cells and particles (e.g. colloids) [28, 29], (iii) sorption

and/or desorption processes [30–32], (iv) the qualitative proof and quantitative estimation of biodegradation linked to individual redox processes [33–36], and (v) a determination of microbial growth rates [27, 34, 37–39] and active microbial migration [40]. On the other hand, sediment columns may have serious limitations. For example, since columns are typically fed with a well-mixed liquid phase, transport processes and bioreactive gradients can be investigated in one dimension only. Important processes such as transverse dispersion cannot be taken into account.

The mini sediment columns, introduced in Figure 3.1, overcome a typical limitation of sediment column set-ups, i.e. the temporally inflexible access to sediment samples. Maintained with a high number of parallels [41], replicate columns can be sacrificed for sediment analysis whenever needed [38, 39]. Additionally, the small size of the columns generally prevents the establishment of pronounced physical-chemical and microbial gradients in the longitudinal direction.

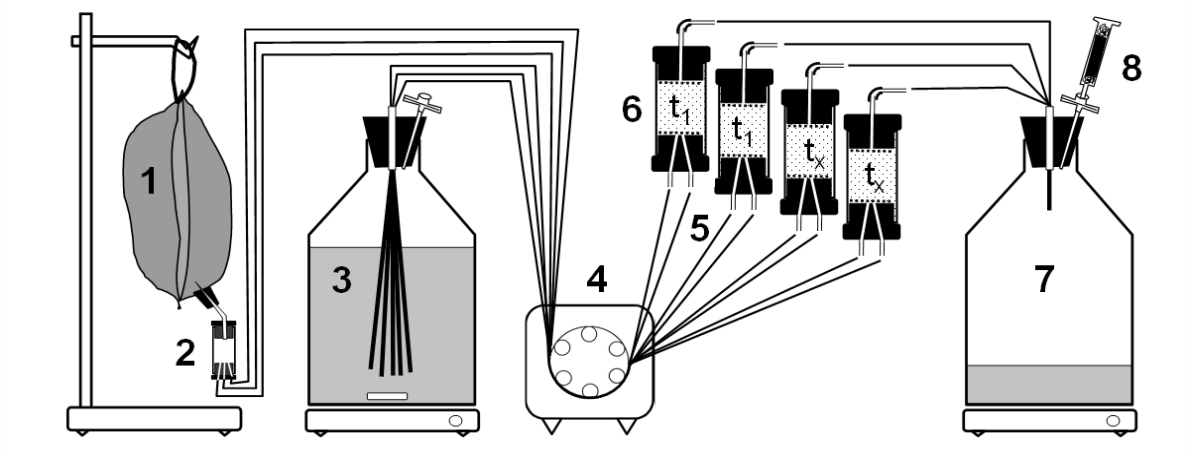

Figure 3.1: Setup of a mini sediment column experiment. (1) Gas-tight and inert Tedlar bag that contains the anoxic medium/groundwater amended with the contaminant (e.g. monoaromatic hydrocarbon compounds). Via a stainless steel capillary the medium is transported to (2) a splitter which feeds the many capillaries/tubes that supply the individual sediment columns. (3) Oxic medium/groundwater is supplied from a reservoir bottle that is closed by a rubber stopper and protected from atmospheric contamination by a filter that allows air to replace the continuously decreasing medium volume. This second medium reservoir ideally contains the electron acceptor (oxygen, nitrate, sulfate) and nutrients but no substrates. (4) Multi-channel peristaltic pump. (5) Both

media come together at the inlet of (6) the sediment columns. Columns are capped by Viton stoppers. At periods where there is not sampling of column outflow, the medium is transported to (7) a waste container. In case of volatile and toxic compounds, (8) a syringe stuffed with activated charcoal can be used to prevent escape of the contaminant(s). (9) Close-up of a mini sediment column showing three spots of oxygen sensitive foil (PRESENS, Regensburg, Germany) mounted to the inner wall of the glass cylinder **(Note 1)** for non-invasive monitoring of the oxygen content in the sediment pore water.

3.1.2 2-D sediment flow-through microcosm

Two-dimensional (2D) and three-dimensional (3D) model systems offer considerable advantages compared to 1D flow-through columns. In particular, they take into account the influence of transverse dispersive fluxes, which are of pivotal importance for contaminant transport and biodegradation. In case of a point source and a discrete contaminant plume, transverse dispersion is the most effective mixing mechanism between the groundwater carrying the dissolved contaminants (electron donor) and the surrounding non-contaminated groundwater carrying the dissolved electron acceptors (oxygen, nitrate, sulfate). Microbial activity and growth, which require both the electron donor and acceptor in sufficient amounts, therefore concentrates in the transition zone between contaminated and non-contaminated zones, such as the fringe of contaminant plumes [17, 42]. Due to their versatility, the use of 2-D flow-through systems of different sizes, ranging from millimeters [43] to several meters [44], has increased in recent years. Selected studies dealt with the conservative and reactive transport of tracers and dissolved as well as colloidal contaminants [27, 45–56], sorption effects [57], and the behavior of non-aqueous phase liquids (NAPLs) in saturated porous media [58, 59]. Research on biotic processes was directed to bacterial transport and motility in porous media [60, 61] and reactive microbial transport [62] as well as to microbial growth in micropores at mixing zones [63]. Studies on the fate of contaminant plumes subjected to microbial degradation were conducted either with surrogate substrates such as glucose or acetate, or with real contaminants such as chlorinated ethenes, phenol, toluene and

ethylbenzene [17, 37, 42, 50, 53, 64–72]. Recent 2D flow-through system experiments substantially contributed to the elucidation of key processes involved in natural attenuation, such as the plume fringe concept [17, 23, 42, 73, 74]. Besides 2-D microcosms, 3-D model systems of varying size have been applied in transport of contaminants and microbes and biodegradation studies [75–79]

The sediments flow-through system (tank) introduced here (Figure 3.2), allows the experimenter to study bioreactive transport of contaminants in porous media in two dimensions. Moreover, the structure and heterogeneity of the solid matrix can easily be varied, using either a homogeneous packing with glass beads and uniform quartz sand or natural sediments including defined zones of increased or decreased hydraulic conductivity (Figure 3.3). Contaminants and redox gradients transverse to the flow direction can be made visible using color tracers or redox indicator dyes.

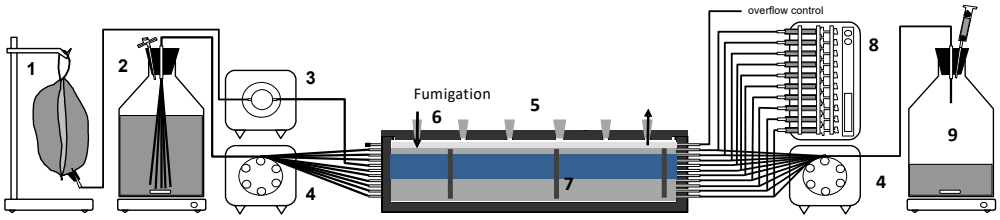

Figure 3.2: Setup of a 2-D sediment flow-through microcosm experiment. (1) Gas-tight and inert Tedlar bag containing the anoxic medium/groundwater amended with the contaminant (e.g. monoaromatic hydrocarbon compounds); (2) Glass bottle with the oxic medium/groundwater. This second medium reservoir ideally contains the electron acceptor (oxygen, nitrate, sulfate) and nutrients but no substrates. (3) Ceramic piston pump and (4) multi-channel peristaltic pump. Both media are introduced to (5) the 2-D microcosm, which can (6) be flushed by N_2/CO_2 or any other gas through stoppers in the lid if anoxic conditions are required (**Note 4**). (7) Stripes of oxygen-sensitive foil mounted to the inner side of the glass plate allow measurement of vertical oxygen gradients at distinct places **(Note 1)**. Capillaries at the outlet ports (**Note 7**) of the tank have a T-branch that allow connection of a (8) multi-channel syringe pump for sample collection. At periods where there is no sampling, the medium is transported to (9) a waste container. In case of volatile and toxic compounds, a syringe stuffed with activated charcoal is used to prevent escape of the contaminant(s).

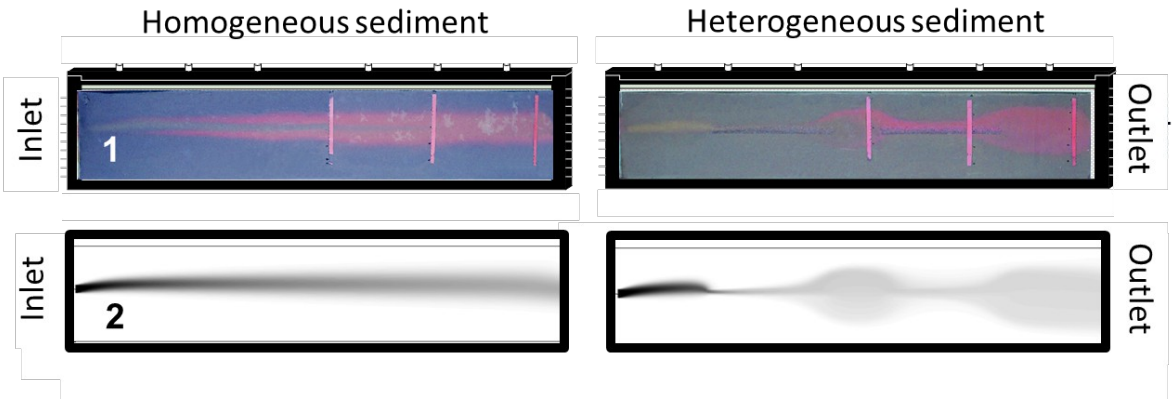

Figure 3.3: (1) Pictures of two microcosms during a biodegradation experiment, one run with a homogeneous sediment packing (left) and the other with two high-conductivity sand lenses embedded (right). The use of a redox indicator shows anoxic zones (white), oxic zones (blue), and the highly bioactive mixing zones (pink). (2) Numerical simulation of contaminant transport (for details see [17]).

In the following, the material requirements as well as the standard experimental set-up for mini sediment columns and 2-D sediment flow-through microcosms are described.

3.2 Materials

3.2.1 Material common to both flow-through systems

1. Multichannel peristaltic pumps (IPC-N Series, IDEX Health & Science, Switzerland)
2. Peristaltic pump tubing (Tygon SI 3350 Platin (sterile media) or Fluran HCA F-5500-A (solvent containing media, **Notes 2, 5, 6**), IDEX Health & Science, Switzerland)
3. Stainless steel capillaries (AD: 1/16 inch, ID 1 mm, CS-Chromatography service, Germany)
4. Syringe needles (B. Braun, Germany)
5. Tedlar gas sampling bags (Restek, Germany)
6. Glass bottles as medium reservoirs (Schott, Germany)

7. Brass T-Connectors (1/16 inch, Swagelok, USA)
8. Sediment (glass, quartz, natural or sterilized at 500°C (carbon free) for 16h)
9. fine metal gossamer with mesh size smaller than smallest matrix fraction (50 µm mesh width, Metallwaren Riffert, Austria)
10. Teflon tape

3.2.2 Specific material for the mini sediment columns

1. Glass columns (for technical specification see Table 3.1)
2. Stoppers for columns (Viton or Butyl, Fisher scientific, USA, Notes 2, 5)
3. Mounting parts for fitting the columns
4. Optional: Oxygen sensitive foil for non-invasive monitoring of oxygen in sediment pore water (PRESENS, Germany, **Note 1**)
5. **Table 3.1: Typical dimensions of mini sediment columns.**

Characteristics	**Dimension**
Diameter [cm]	1.34
Total length [cm] Length without stoppers [cm]	3 to 10 1 to 8
Column sediment volume [mL]	1.4 to 11.3

Table 3.2: Technical specifications of a 2-D sediment flow-through microcosm.

Characteristics	**Dimension**	
Tank dimensions [cm]	Outer dimensions: 100 x 18 x 2 Inner dimensions (experimental space): 95 x 15 x 1	
Inlet ports	11	
Outlet ports	12	
Sediment filling [dm^3]	Approx. 1.3	
Bore size of inlet & outlet ports	Inner bore [mm]	1.6
	Outer bore [mm]	6.3
Microcosm wall	Glass plates [cm]	95.0 x 16.5 x 0.5

3.2.3 *Specific material for 2D-tank*

1. 2-D flow-through microcosm (see technical specifications in Table 3.2 & Figure 3.4, inhouse construction)
2. Stainless steel needles (cannulas, B.Braun, Germany)
3. Butyl stoppers (1/4 CYLINDRICAL SEPTA 100, Grace, USA)
4. Silicone glue (Aqua-dicht, Knauf, Germany)
5. Aluminum foil for covering the tank.
6. Optional:
    α. Teflon lid to cover the tank (inhouse construction, **Note 5**)
    β. Multi-channel syringe pump for sampling (ISMATEC, Switzerland)
    χ. Glass syringes (Carl Roth, Germany)
    δ. Oxygen sensitive foil and optode device (e.g. PRESENS, Germany)
    ε. Cooling device (inhouse construction)

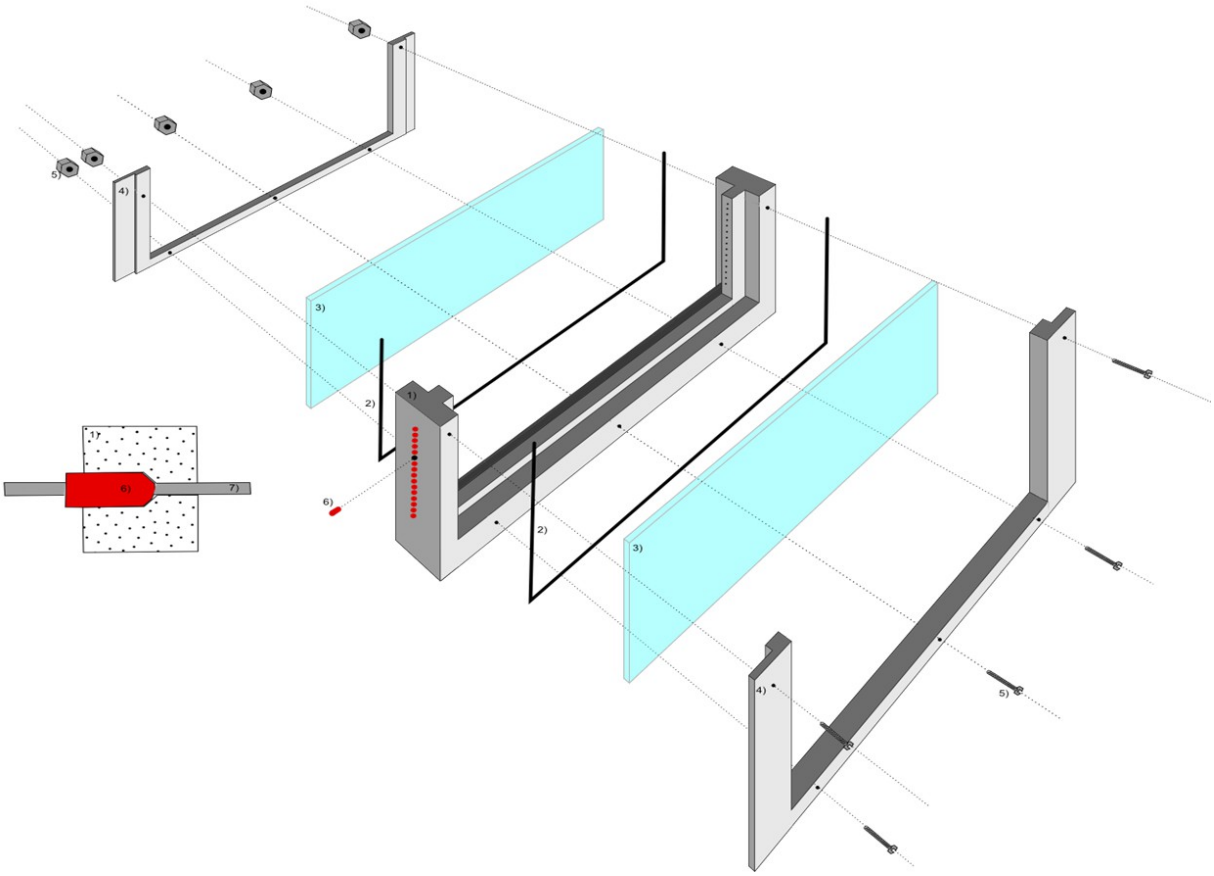

Figure 3.4: Explosion view of the 2-D microcosm. The system consists of a middle part from Teflon (1), to which a silicone seal (2) is inserted on each side before the glass plates (3) are mounted. These are fixed with two aluminium frames (4). The Teflon profile has numerous bores at the inlet and outlet side. Each bore is closed with a butyl stopper (6) that is penetrated by a stainless steel capillary (7) (see close-up).

3.3 Methods

This section covers all necessary steps to prepare and conduct experiments with mini sediment columns and 2-D sediment flow-through microcosms. Due to the wide spectrum of possible applications, no detailed description of liquid media composition and methods for the chemical and microbiological analysis of samples collected from the two systems are provided. Here, we refer to other relevant literature. In principle, there are no restrictions concerning media, microbes and or analytical methods, besides the generally small sample volumes obtained.

3.3.1 Common methods for 1D and 2D experiments

3.3.1.1 Sterilizing the experimental equipment

After cleaning, most of the individual parts of the experimental set-up of the 1-D and 2-D systems can be autoclaved. All tubing and plastic parts should be autoclaved soaked in water. Do not autoclave Teflon parts. The glass and metal parts can alternatively be heated. Afterwards, the model systems are set up as needed for the later experiment (**Note 3**). If the microcosms need to be sterile, they are rinsed with sodium hydroxide (NaOH 100 mM final concentration). Allow the NaOH filled set-up to stand overnight. Later, carefully rinse the set-up with distilled water or autoclaved liquid medium/groundwater (**Note 9**).

3.3.1.2 Sediment preparation

According to the design of the experiment the solid matrix can consist of glass beads, (sterile) quartz sand or natural, biologically active material from the field. Either individual grain size fractions are used or mixed sediment with grain size <0.5 cm. In most of our experiments we removed the clay fraction and grains larger than 2 mm by wet sieving. For sterilization, sediment can be either repeatedly autoclaved in small portions, or dried and heated to temperatures >180°C (at temperatures >250°C ATP is destroyed and at >450°C all organic matter is burned). Before packing the microcosms, dry sediments (that may have hydrophobic surfaces) need to be rewetted in sterile medium or groundwater. To avoid the inclusion of gas bubbles during packing of the columns and the microcosms, columns are filled with sediment under water, and with the microcosms a funnel that releases the wet sediment under the water table is used.

3.3.1.3 Medium preparation and supply

Separation of electron donor and acceptor

A commonly encountered problem is that once the experiment runs, the microbes inside the model systems start migrating and growing against the flow direction into the inlet tubing, the capillaries, and the pump tubing to the medium reservoir, following towards the source of energy and nutrients. In order to prevent microbes from growing into the reservoir, it is necessary to separate the electron acceptor (e.g. oxygen, nitrate, and sulfate) and the donor (e.g. organic contaminant). Thus two media must be prepared that are combined only at the inlet of the columns and microcosms, at a ratio that delivers the desired final composition.

Anoxic medium

All anoxic liquid media or groundwater should be transferred to and stored in flexible gas-tight, inert, sterile teflon gas sampling bags (Tedlar bags) avoiding the inclusion of a gas phase. During medium supply, the bags continuously collapse and can easily be replaced when empty. A cannula is inserted through the septum of the bag's screw cap and connected with a stainless steel capillary to the tubing of the peristaltic pump (**Note 6)**. In the case that several columns or inlet ports of a 2-D microcosm are supplied, a splitter must be used.

Oxic medium

Oxic medium that does not contain any volatile compound can be prepared and kept in glass bottles. Withdraw the medium from the sealed bottle with flexible tubing passing through a stopper or septum in the bottle's screw cap. To avoid low pressure, insert an additional needle with a 0.22 μm sterile syringe filter to prevent microbial contamination from the room atmosphere. To keep the medium oxygen-saturated, you may place the reservoir bottle on a magnetic stirrer (Figure 3.1 & 3.2).

3.3.2 Column experiments

3.3.2.1 Column setup

The anoxic and oxic media are transported via steel capillaries, tubing and a peristaltic pump to the inlet of the mini glass columns. The columns consist of a glass cylinder (of varying dimension), two stoppers (Viton or Butyl), a stainless steel mesh, and three steel cannulas (Figure 3.1). The cannulas are pushed through the stoppers to create an inlet and outlet of the column (two at the inlet, one at the outlet). The cannulas are connected with the steel capillaries using a short piece of tubing (Figure 3.1). If needed, oxygen sensitive foil can be glued to the inner wall of the glass cylinder (**Note 1**, there is an autoclaveable foil available, PRESENS, Regensburg, Germany). Take care to use suitable glue which is inert to sorption of organic solvents and does not leak organics such as softeners into the medium. For packing the column with sediment, the stopper with the inlet channels is inserted to the glass cylinder. Then a small piece of tubing is connected to the inlet capillaries, before the column is put under water in a big beaker to remove all air from the column and inlet. Subsequently, mesh is placed to the bottom of the column before the sediment is filled in keeping the column under water (or medium). When packed with sediment, again a mesh is placed on top before the second stopper is inserted, pushing the water overlaying the sediment out through the outlet tube. Before the column is lifted above the water table, the short piece of tubing at the inlet is closed with a clip. Fixed in a column holder, the packed and water saturated columns are then ready to be connected with the medium supply. The outlet of the column still need to be connected by a short piece of tubing and steel capillary with a waste collecting bottle of appropriate vials collecting samples from column outflow (Figure 3.1). The columns are infiltrated from bottom to top, which allows gas bubbles accidentally introduced or formed due to microbial activity to leave the columns.

3.3.2.2 Inoculation of the sediment columns

Once the source medium or media are connected with the packed columns, flush the columns several times to replace the sediment pore water. If desired, inoculate the columns with a 1x sediment pore volume of the bacterial culture of choice by disconnecting the column from the steel capillary at the inlet and injecting the inoculum through the inlet at the bottom of the column. It is recommended to inoculate with a considerably low initial concentration of bacteria (10^4 to 10^5 cells mL^{-1}), a concentration similar to that found in aquifers. Let the inoculum stand for 10 min in the column before the infiltration with source medium is continued.

3.3.2.3 Sampling

Collection of liquid samples

The collection of liquid samples at the column outlet is performed as follows:

- Disconnect the steel capillary at the outlet of the column
- Insert a syringe needle (cannula) into the connector-tubing
- Place a vial or tube of appropriate volume (2-15 mL) closed with a septum underneath the outlet and pierce it with the syringe needle that is connected to the outlet. The medium will now drop from the outlet into the sampling tube
- Protect the sampling tube from light
- If the column outflow contains volatile compounds make sure to collect the liquid in an pre-evacuated sealed vial, which can directly be used for later analysis (e.g. GC or HPLC vials)

The collection of liquid samples at the column inlet is performed as follows:

- Close the column inlet using a hose clamp

- Disconnect the steel capillary at the inlet of the column
- Add a short piece of connector-tubing to the steel capillary and a syringe needle
- Sample collections follows the same protocol as already described above
- Remove the vial and reconnect to the sediment column

Collection of sediment samples

At desired time points during the course of an experiment, sediment columns (in duplicates or triplicates) are disconnected from the system to analyze the solid matrix fraction. This allows for analysis of attached microbes as well as compounds adsorbed to the sediment matrix. Handling and analysis of sediments should follow microbiological and chemical standard operation procedures.

3.3.3 2D-tank experiments

3.3.3.1 Assembling the 2-D microcosm (tank)

The following description refers to a tank set-up which was designed for bioreactive transport experiments with organic solvents (e.g. monoaromatic hydrocarbons) made up of mainly glass, metal and Teflon material, strictly avoiding the use of plastics and other synthetic material to exclude discharge of undefined compounds. A sketch on the composition of the 2-D system is given in Figure 3.4. When putting the parts together, first the Teflon profile is cleaned carefully with ethanol and then the outer sides of the profile are covered by a thin layer of silicon glue. Before drying of the glue, insert the sealing and the glass pane and fix the setup with the screws (see Figure 3.4). Take care that the glue is sealing the space between the glass plates and the Teflon profile to prevent preferential flow channels.

3.3.3.2 Packing the tank

- Autoclave a funnel connected to a tube long enough to reach the bottom of the tank, a small beaker and a rod (glass or steel)
- Make sure the tank is half-filled with sterile medium or groundwater
- Connect all inlet and outlet ports to medium supply and turn on pumps to pump medium into tank
- Use the funnel to fill the wet sediment into the tank by gently rinsing the sediment in the funnel into the tank with medium/groundwater
- Make sure the end of funnel tube is always below the water table and water is overlaying the sediment
- From time to time use a bar to stir the sediment for homogenization and to release accidentally entrapped air bubbles from sediment
- If necessary remove part of the rising water with a syringe to prevent flooding of the lab bench
- Fill the tank to 9/10 of its height and finally smoothen the sediment surface
- Reverse the flow direction of the pump at the tank outlet and allow the tank to equilibrate.
- The top layer (uppermost 0.5 cm) of the sediment must be unsaturated. If sediment packing is not high enough, do not use the upper inlet and outlet ports
- To get a constant flow field, use X inlet ports and X+1 outlet ports
- Set the pumping rate at the individual ports in a way that the total rate of medium introduced at the inlet side (= X times the rate of the individual inlet ports) is slightly less than the total rate of medium pumped out at the outlet side (= X+1 times the rate at the individual outlet ports) (**Note 7**).

- Constant flow conditions are reached or will be reached when all inlet ports transport medium/groundwater, but the uppermost outlet port partially carries medium and air.
- Make sure that there is a continuous supply of medium. Once unsaturated conditions accidentally establish in the sediment you will have to restart the experiment by emptying and re-filling the tank.
- Cover the open top of the microcosm with a Teflon lid or aluminum foil

3.3.3.3 Inoculation of the 2-D sediment flow-through systems

Inoculation of the tank systems can be performed in many ways.

- Inoculation of the sediment can be achieved by mixing the bacterial culture into the sediment before it is packed into the tank. This way of inoculation leads to a more or less homogeneous distribution of cells all over the sediment.
- Punctual inoculation at the inlet: Injection of inoculum through selected inlet ports while tank inflow and outflow is shortly interrupted. After inoculation turn medium inflow and outflow on again. This way of inoculation allows the localized introduction and wash-through of cells.
- Punctual inoculation near outlet: Injection of inoculum through selected outlet ports while tank inflow and outflow is shortly interrupted. Allow the introduced cells to settle for 15 min before restarting in- and outflow. This way of inoculation allows studying the growth of microbes from the outlet towards the substrate (contaminant) source.
- Punctual inoculation somewhere else in the tank can be achieved via penetration of the sediment with a suitable, long needle and injection of the bacterial culture. Remove the needle carefully to minimize sediment disturbance and spreading of the inoculum.

- For a visual control, the inoculum can be amended with a color tracer (e.g. uranine).

3.3.3.4 System cooling or heating

In the ideal case the experiments are conducted in a room with the desired temperature (e.g. climate room or chamber). Heating foil can be mounted to the glass plates of the microcosms. For cooling, we mounted a self-made device composed of two copper plates (in the dimension of the glass plates) with copper tubing fixed to it to the glass plates of the microcosm and connected it to a cooling water supply.

3.3.3.5 Sampling

Collection of liquid samples

Collection of samples at the inlet of the 2-D system is done as already described for the sediment column. At the outlet of the tank system, individual ports can be sampled using a syringe (plastic or glass) to collect small amounts of liquid manually. For a regular sampling of all outlet ports, we recommend to establish a sampling line separated from the waste collection line. As shown in figure 3.2, all capillaries at the outlet ports are connected to a T-branch, with one line connected to the waste container via a peristaltic pump. A second line is equipped with Luer-Lock fittings that allow connecting to glass syringes. In case of sampling, these sampling line (maximum of 10 capillaries) are connected to glass syringes held by a multi-channel syringe pump, which replaces the peristaltic pump for the period of sample collection (Figure 3.2), i.e. the peristaltic pump is turned off and the syringe pump, maintaining the exact same pumping rate, is turned on. Be aware that collection of several mL of sample from individual outlet ports may take several hours, dependent on the flow rates established in the model system.

Sediment sampling

During the course of an experiment there is only limited access to sediment samples. Main sediment sampling is done after the microcosm experiment is finished. If necessary, small sediment volumes can be obtained from the top zone of the sediment layer, which can be sampled with a sterile spatula or a syringe with the head part cut off. After sampling the excavation needs to be re-filled by new sediment. This kind of sampling may seriously disturb the flow regime.

At the end of the experiment, sediments in the 2-D microcosm can be obtained for the spatially resolved analysis of biotic and abiotic variables of interest. Sediment cores can be taken by sterile plastic syringes with the head part cut off or by removal of one glass plate; in this case the old glue needs to be removed from the glass and the Teflon frame and sealed again for next round of experiments.

3.4 Notes

1. The oxygen-sensitive foil should be handled with care. Do not touch it with bare hands or expose it to light. It is recommended to use a forceps to place a drop of silicon glue to the inner wall of the glass column and place the oxygen-sensitive foil on top. If possible, no gas bubbles should remain between the foil and the glass wall of the column (Figure 3.1). In a similar way, spots or stripes of the foil are mounted to the inner side of the glass plates of the 2-D systems (Figure 3.2 & 3.3). Caution, the foil has two different sides, of which only one can be read by the optode sensor (PRESENS, Germany).
2. When working with organic solvents and volatile compounds such as monoaromatic hydrocarbons, the tubing used for the peristaltic pumps and as connectors of

capillaries should be carefully selected. Based on our experience, fluran tubing (IDEX Health & Science, Switzerland) is gas-tight and soon saturated by the hydrocarbons without significant loss through the tubing wall and change in tubing performance. Make sure to change the tubing regularly; the official live time of the fluran tubing is only a couple of days. However, in practice, tubing is changed the moment flow rates start to deviate.

3. To avoid irregular source concentrations entering the column and tank systems, the peristaltic pump tubing should be conditioned prior to the start of the experiment. Pump the medium containing the hydrocarbon(s) through the tubing overnight before connecting to the model systems.
4. When performing experiments under strict anoxic conditions, microcosms can be closed with a lid made of Teflon or metal. Through openings in the lid, closed by rubber stoppers, the headspace of the microcosms can be continuously flushed with N_2 or N_2/CO_2 gas (Figure 3.2).
5. Especially low molecular weight hydrocarbons are able to penetrate most kinds of plastic. It is thus necessary to use glass and stainless steel parts as much as possible. As synthetic material, mainly Teflon and Viton are used.
6. Make sure to continuously check for clogging of capillaries at the columns and microcosms inlets and outlets. Often precipitation reactions take place (Figure 3.5) producing considerable amounts of particles that may plug the outlet ports. Groundwater oversaturated in calcium carbonate releases calcite when the CO_2 partitions to the atmosphere (Figure 3.6). Similarly, sulfide produced by microbial sulfate reduction reacts with iron forming iron sulfide precipitation. One should also be aware that high concentration of sulfide may lead to corrosion of the iron parts.

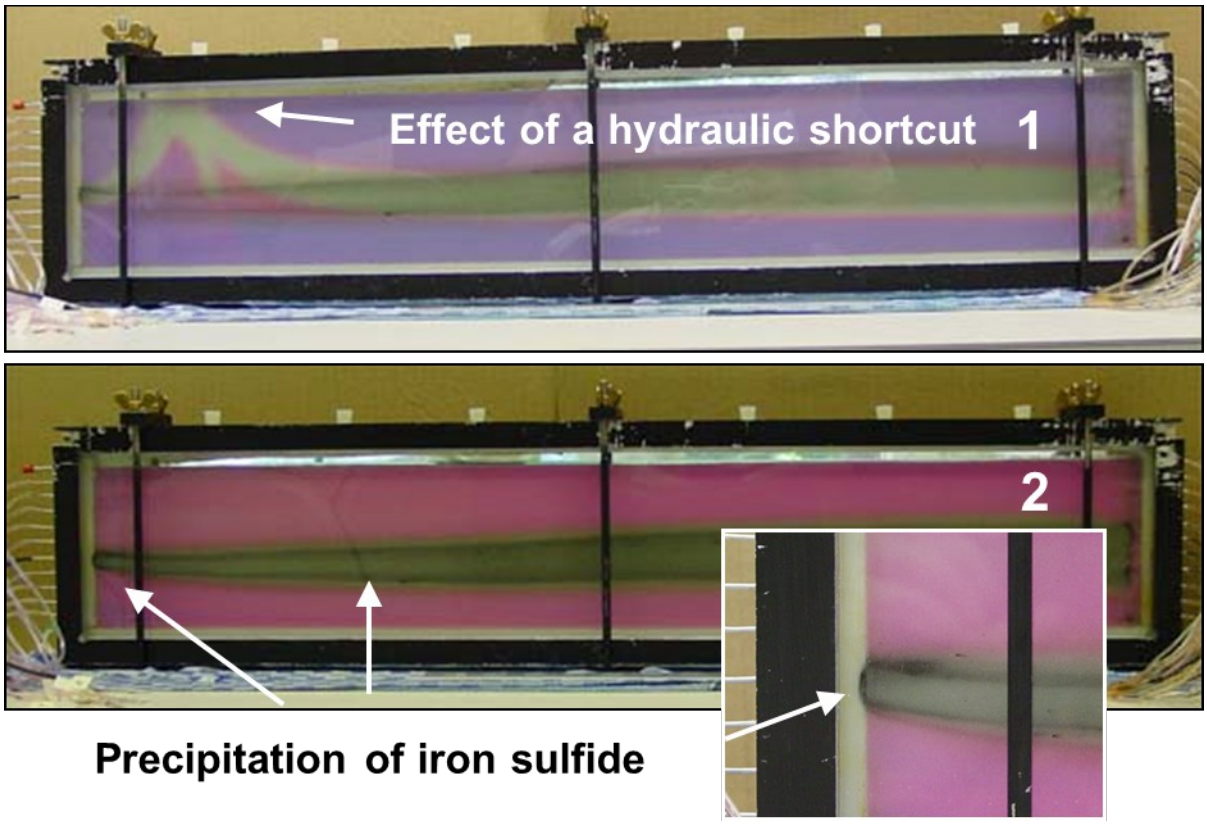

Figure 3.5: (1) The pictures show the effect of a hydraulic short-cut on the flow regime in the 2-D microcosms. The moment the sediment is flooded (loss of the unsaturated zone), the overlying water constitutes a hydraulic short-cut. The blackish precipitation indicates the position of the contaminant plume before the hydraulic short-cut, which forced the inflowing medium to rise above the sediment surface, shown be the whitish area. (2) Precipitation of iron sulphide along the fringes of the contaminant plumes (for further details [25]).

7. The outflow pump should be adjusted by the following formula:

$$\frac{(Inletvolume * numberofinletports)}{(numberofinletports + 1)}$$

8. Strictly avoid water rise in the 2-D microcosms that leads to loss of the unsaturated zone and water overlaying the sediment. In such a case, a hydraulic shortcut is generated and most of the medium infiltrated into the tank system will take the way up immediately after entering the microcosm flowing on top of the sediment (Figure 3.5) before it is forced back into the sediment at the end of the tank.

9. Always keep in mind that neither the mini sediment columns nor the 2-D microcosm systems can be maintained under sterile conditions for long. Even when putting the true sterile individual parts together, the systems when maintained are not perfectly closed; this is especially true for the 2-D tank systems. Moreover, replacing the medium reservoir and regular changing of the tubing, as well as sampling, represent sources of contamination. However, since biodegradation of hydrocarbons, to give one example, in most cases need microbial specialists, the risk for a fast establishment of invading microbes is low. We were able to successfully conduct experiments on aerobic as well as anaerobic degradation of monoaromatic hydrocarbons (e.g. toluene and ethylbenzene) [42, 53] or pristine aquifers lasting for several months.

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4. Contaminant Concentration versus Flow Velocity – Drivers of Biodegradation and Microbial Growth in Groundwater Model Systems

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Abstract

Aromatic hydrocarbons belong to the most abundant contaminants in groundwater systems. They can serve as carbon and energy source for a multitude of indigenous microorganisms. Predictions of contaminant biodegradation and microbial growth in contaminated aquifers are often vague because the parameters of microbial activity in the mathematical models used for predictions are typically derived from batch experiments, which don't represent conditions in the field. In order to improve our understanding of key drivers of natural attenuation and the accuracy of predictive models, we conducted comparative experiments in batch and sediment flow-through systems with varying concentrations of contaminant in the inflow and flow velocities applying the aerobic *Pseudomonas putida* strain F1 and the denitrifying *Aromatoleum aromaticum* strain EbN1. We followed toluene degradation and bacterial growth by measuring toluene and oxygen concentrations and by direct cell counts. In the sediment columns, the total amount of toluene degraded by *P. putida* F1 increased with increasing source concentration and flow velocity, while toluene removal efficiency gradually decreased. Results point at mass transfer limitation being an important process controlling toluene biodegradation that cannot be assessed with batch experiments. We also observed a decrease in the maximum specific growth rate with increasing source concentration and flow velocity. At low toluene concentrations, the efficiencies in carbon assimilation within the flow-through systems exceeded those in the batch systems. In all column experiments the number of attached cells plateaued after an initial growth phase indicating a specific "carrying capacity" depending on contaminant concentration and flow velocity. Moreover, in all cases, cells attached to the sediment dominated over those in suspension, and toluene degradation was performed practically by attached cells only. The observed effects of varying contaminant inflow concentration and flow velocity on biodegradation could be captured by a reactive-transport model. By monitoring both attached and suspended cells we could quantify the

release of new-grown cells from the sediments to the mobile aqueous phase. Studying flow velocity and contaminant concentrations as key drivers of contaminant transformation in sediment flow-through systems improves our system understanding and eventually the prediction of microbial biodegradation at contaminated sites.

4.1 Introduction

Groundwater is one of the most important resources of drinking water, accounting for 70% of public water supply in Germany. It is increasingly threatened by pollution (Bauer et al., 2007; Foght, 2008; Rabus and Widdel, 1994; Silva-Castro et al., 2013; Vieth et al., 2004). For the design of reliable and cost-efficient bioremediation methods we need to understand the controls and limitations of the biodegradation potential of natural microbial communities in aquifers (Meckenstock et al., 2015).

Petroleum hydrocarbons belong to the most abundant contaminants in aquifers (Rüegg et al., 2007; Meckenstock et al., 2004; Vieth et al., 2004; Meckenstock et al., 2010). Among them, the monoaromatic compounds benzene, toluene, ethylbenzene, and xylene (BTEX) are of major concern due to their toxicity (Bombach et al., 2009; Meckenstock and Mouttaki, 2011), relatively high solubility and mobility (Chapelle, 2000; Foght, 2008), and broad use. BTEX compounds such as toluene have repeatedly been used as model chemicals in lab and field studies, since aerobic and anaerobic degradation pathways are known and bacterial cultures of key degraders are easily available (Meckenstock et al., 2004; Fischer et al., 2006; Mak et al., 2006; Foght, 2008).

The biodegradation of BTEX in aquifers has frequently been observed. Both monitored and enhanced natural attenuation (MNA and ENA, respectively) are applied as sole remediation strategy for these compounds. Nonetheless, the ecology of the degrading microorganisms is hardly understood and thus the real biodegradation potential under in-situ

conditions remains unknown. Recent studies on the biodegradation of aromatic hydrocarbons in flow-through lab-studies and in the field shed some light on the limitation of biodegradation by transverse dispersive mixing (Anneser et al., 2008; 2010; Bauer et al., 2008; 2009; Eckert et al., 2015). Only if both the electron donor and a favorable electron acceptor are available, bacteria can degrade the contaminant. As a result, biodegradation activities are concentrated along the fringes of contaminant plumes at quasi-steady state (Anneser et al., 2008; Bauer et al., 2008). However, even if mixing does not control biodegradation, the interdependencies between contaminant transport, microbial transformation of the contaminants, microbial growth, and microbial transport hamper the predictability of biodegradation (Meckenstock et al. 2015).

Biodegradation coupled to bacterial growth can be simulated using analytical and numerical models. The biokinetic model parameters are commonly derived from batch experiments. Batch reactors are perfectly mixed closed systems with large water-to-solid ratios. Typically, comparably high contaminant concentrations are applied in incubation experiments. The substrate is usually the only limiting factor (Hofmann et al. 2016). In comparison, porous aquifers are open systems with small water-to-solids ratio and incompletely mixed. They are often affected by transient flow conditions and a dynamic contaminant load. While the activity of bacteria is supposed to be high and growth is fast in liquid batch systems due to the excess of the chosen electron acceptor and nutrients as well as continuous mixing, it is currently unclear how flow-through conditions, such as the flow velocity, and the sediment matrix influence biodegradation and microbial growth. The yield (i.e. the substrate carbon converted into biomass carbon) has been reported to be as high as 0.5 to 0.8 in batch and chemostat cultures dependent to the substrate applied (Ho and Payne, 1979; Payne and Wiebe, 1978, and references therein), while data from natural aquatic systems hint at considerably lower values (del Giorgio and Cole, 1998). The sigmoid growth

curve in batch cultures reflect exponential microbial growth followed by a plateau in cell density, mainly governed by the depletion of the substrate. In sediment flow-through systems, bacteria suspended in the mobile aqueous phase and attached to the sediment surfaces coexist and partition. In fact, in aquifers the majority of bacteria (>99%) are usually found attached to the sediments (Griebler and Lueders, 2009). Dependent on the continuous substrate load, a balance of microbial biomass between sediment and pore water is expected to establish (Griebler et al. 2002; Zhou et al. 2012). Various other factors, such as temperature, pH, availability of an energy source, quality of the substrate, toxicity, availability of terminal electron acceptors, and microbial food web interactions influence in-situ microbial growth and contaminant degradation (Chapelle, 2000; Meckenstock et al. 2015).

The discrepancy in conditions between flow-through and batch systems consequently raises the question how representative batch-derived rate coefficients of biodegradation and microbial growth are. Empirical findings regarding the comparability of biodegradation in batch and flow-through systems are ambiguous. While some studies reported that batch-derived biokinetic parameters adequately described biodegradation in flow-through systems (e.g., Kelly et al., 1996; Schirmer et al., 2000), others observed significant deviations (e.g., Simoni et al., 2001; Ballarini et al., 2014). In order to clarify the influence of flow conditions on biodegradation and microbial growth, we conducted a series of growth experiments using toluene as a model contaminant in batch systems and flow-through sediment microcosms applying different toluene concentration and different flow velocities with the aerobic toluene degrader *Pseudomonas putida* strain F1, the anaerobic denitrifier *Aromatoleum aromaticum* strain EbN1, and a natural microbial community from aquifer sediments. Regular measurements included the concentrations of toluene, oxygen, and cell numbers. By performing experiments in numerous replicated mini sediment columns that were successively sacrificed in the course of the experiments, we could also follow growth of the

attached microbes over time. All experimental data were analyzed by reactive-transport modeling considering mobile (pore-water) and immobile (sediment) bacteria. We chose toluene as the model contaminant because well characterized toluene-degrading bacterial strains are available, but we expect that the qualitative findings of this study are applicable to the degradation of other aromatic hydrocarbons too.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Media

We used the toluene-degrading strains *Pseudomonas putida* F1 (aerobic) and *Aromatoleum aromaticum* EbN1 (denitrifying) as model organisms. Pre-cultures of both strains were grown in 100 mL serum bottles at room temperature (20°C) in the dark with 70 µM toluene as the sole carbon and energy source. The groundwater medium was a bicarbonate-buffered freshwater medium (Widdel and Bak, 1992) prepared oxic – for experiments with *P. putida* – or anoxic – for experiments with *A. aromaticum* as described elsewhere (Bauer et al. 2008, 2009). For batch experiments, we amended the respective medium with varying concentrations of toluene in closed serum bottles (100 mL) before inoculation with the bacteria. For *A. aromaticum* strain EbN1, the medium was autoclaved under N_2 atmosphere and cooled down flushing the headspace with N_2/CO_2 (80:20). The medium was then transferred to serum bottles avowing oxygen penetration. Again the headspace in the serum bottles was flushed and replaced by N_2/CO_2 (80:20) before capped with Viton stoppers. Toluene (99.5%; Aldrich, USA) was injected with a sterile glass syringe through the Viton stoppers to obtain concentrations between 10 µM and 1 mM in the liquid phase.

In the sediment column experiments, we provided two media, one containing the electron donor (toluene) and the other the electron acceptor (oxygen or nitrate). They were mixed directly at the column inlet to avoid growth of bacteria back into the medium reservoirs

(Hofmann et al., 2016). In experiments with *P. putida* F1, one medium was oxygenated while the other was anoxic but contained toluene. With *A. aromaticum* EbN1, both media were oxygen-free, one containing toluene and the other nitrate. The media were contained in gastight and inert 5 L Tedlar bags (SKC, PA, USA) without headspace and protected from light.

4.2.2 Batch Experiments

Both strains were inoculated at a ratio of 1:10 from pre-cultures into 100 mL serum bottles carrying 70 mL of fresh medium amended with toluene (10 µM to 1 mM) as sole carbon and energy source, either saturated with oxygen and an oxic headspace or with anoxic medium amended with nitrate (10 mM) and oxygen-free (N_2/CO_2) headspace. Aerobic degradation experiments were incubated at a shaker (120 rpm) to ensure replenishment of oxygen from the headspace into the medium. We conducted incubations at room temperature in the dark and regularly collected samples for the analysis of toluene (GC-MS analysis), total cell counts (OD measurements, FACS analysis), and measurement of cell size (epifluorescence microscopy). Measurements were obtained by aseptically subsampling the liquid phase with a syringe through the Viton stopper.

4.2.3 Column Experiments Using Sterile Aquifer Sediments

We packed mini sediment columns (material: glass, total length: 3.5 cm, active inner length: 1.6 cm, inner diameter: 1.34 cm; Fig. 4.1) submerged in water with sterile natural aquifer sediment with a grain size ranging from 200 to 630 µm and closed them by Viton stoppers. Packed, the columns had a sediment volume of about 2.3 mL. The in- and outflow occurred through stainless-steel capillaries in the stoppers. The flow direction was from the bottom to the top. We ran twelve columns in parallel for each treatment and maintained flow-

through by means of multi-channel peristaltic pumps (Ismatech, Wertheim, Germany) using Fluran tubing. All columns carrying sterile sediment were inoculated once with the same pre-cultured strain containing a cell density of approximately 10^4 to 10^5 cells mL^{-1}. We used 1 mL of the pre-cultured strain as inoculum in each column and left it to stand in the column for 10 min before turning on the supply of cell-free medium from the reservoir. Toluene concentrations continuously supplied to the columns ranged from 30 to 100 µM. The standard flow rate was set to 3.2 mL h^{-1}. Because the porosity of the sediment was 0.3, the flow rate corresponded to a water residence time of 12.7 min and a flow velocity of approximately 1.8 m d^{-1}. Overall, we tested flow rates ranging from 1 mL h^{-1} to 6.6 mL h^{-1}. We collected water samples for the analysis of toluene (GC-MS analysis), total cell counts (FCM analysis), and occasional cell size measurements (epifluorescence microscopy) directly at the column inlet and outlet into small HPLC vials sealed with Teflon coated septa. The oxygen concentration within the columns was monitored by an optode technique using three spots of oxygen-sensitive foil glued to the inner wall of the glass columns. At various time points, we sacrificed columns to analyze the abundance and size of bacterial cells attached to the sediment. We determined the length and width of the cells via epifluorescence microscopy and subsequently calculated the biovolume of the cells. We divided sediments from the columns into three fractions of equal size using a sterile spatula, resulting in a bottom, a middle, and a top fraction, each representing 1/3 of the column volume (Fig. 4.1).

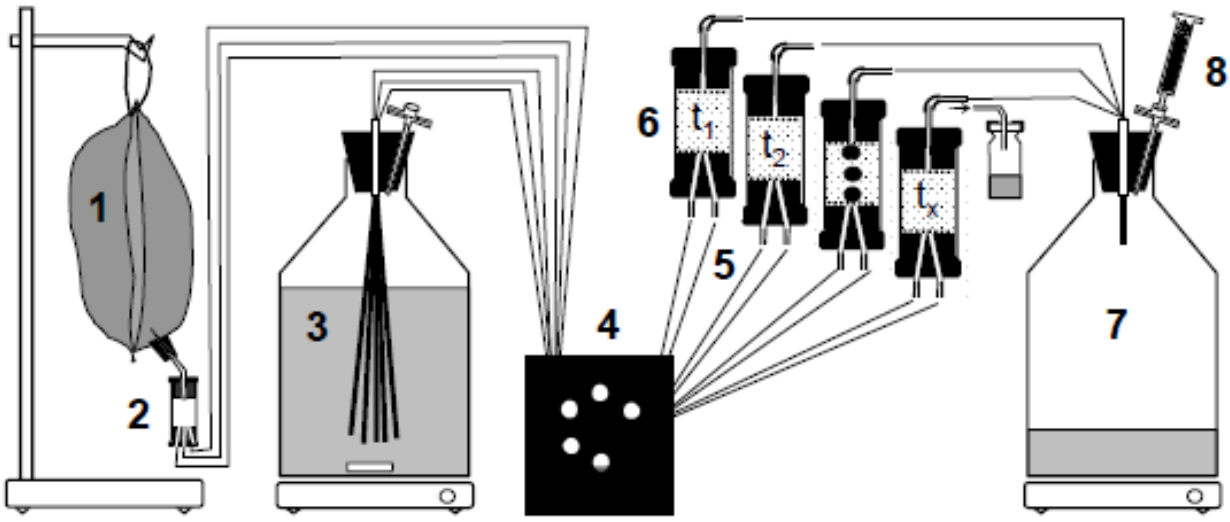

Figure 4.1: Setup of a mini sediment column experiment. (1) Gas-tight and inert Tedlar bag with anoxic medium/groundwater amended with toluene. (2) Transport of the medium via a stainless steel capillary to a splitter which feeds the capillaries/tubes that supply the individual sediment columns. (3) Oxic medium/groundwater is supplied from a reservoir bottle. (4) Multi-channel peristaltic pump. (5) Mixing of the two media come at the inlet of (6) the sediment columns. Columns are capped by Viton stoppers. At periods where there is not sampling of column outflow, the medium is transported to (7) a waste container. Mini sediment column 3 shows three spots of oxygen sensitive foil (PRESENS, Regensburg, Germany) mounted to the inner wall of the glass cylinder for non-invasive monitoring of the oxygen content in the sediment pore water (modified from Hofmann et al. 2016).

4.2.4 Column Experiments with Active Aquifer Sediment

In order to compare the growth kinetics of the selected model strains to those of a natural consortium, we performed flow-through experiments in columns packed with fresh natural aquifer sediment, which we infiltrated on-line by oxygen-saturated natural groundwater. The medium containing toluene was filter-sterilized groundwater, purged anoxic with N_2/CO_2. The toluene concentration supplied to these columns was about 70 µM. The sampling followed the same protocols as described above.

4.2.5 Chemical and Microbiological Analyses

Samples for toluene measurement collected at the column inlets and outlets were transferred to close GC vials (Fig. 4.1) containing NaOH to terminate bacterial activity.

Ethylbenzene was spiked prior to analysis as internal standard. We determined concentrations of toluene via headspace analysis by GC-MS following the protocols described in Anneser et al. (2008, 2010). Concentrations of nitrate were determined by ion chromatography (Dionex AS3500, Idstein, Germany).

Bacterial cell numbers in water and sediment samples were determined by flow cytometry (FCM). For water samples, 1 mL of sample was placed into an Eppendorf tube and fixed with 100 µl of glutardialdehyde to a final concentration of 2.5%. With sediment samples, a 0.5 mL aliquot was placed in a 2 mL Eppendorf tube and fixed with 1 mL of 2.5% glutardialdehyde solution. Fixed samples were stored at 4°C until further analysis. Later, sediment samples were further processed as described in Bayer et al. (2016). We stained water samples as well as the samples containing bacteria detached from sediment in triplicates with SybrGreen I (1000x, Molecular Probes, Invitrogen Life Sciences, 1 µl/mL) and determined cell densities in a Cytomics FC500 flow cytometer (Beckman Coulter System). The instrument settings for our experiment was: forward scatter 350 mV, sideward scatter 300 to 370 mV, bandpass filter 530 nm 500 to 580 mV and green fluorescence, bandpass filter 610 nm 650 mV and red fluorescence. The signal threshold was adjusted to 200 mV for both scatters to minimize background noise (Bayer et al. 2016).

4.2.6 Modeling of Batch Experiments

4.2.6.1 Direct Utilization of Toluene for Growth

In the standard model, we assume that the bacteria directly grow on the degradation of toluene. The electron acceptor is considered available in excess, and biomass decay is neglected. Then the standard Monod equations read as:

$$\frac{dX}{dt} = \mu_{max} \cdot \frac{c_{tol}}{c_{tol} + K_{tol}} \cdot X \tag{1}$$

$$\frac{dc_{tol}}{dt} = -\frac{1}{Y} \cdot \frac{dX}{dt} \tag{2}$$

in which μ_{max} [s^{-1}] is the maximum specific growth rate constant, c_{tol}, [µM] and X [cells L^{-1}] are the concentration of toluene and bacteria, respectively, whereas K_{tol} [µM] and Y [cells/µmol] are the half-saturation concentration of toluene and the yield coefficient. This system of ordinary differential equation are subject to initial values of the two concentrations and was solved with the ode-solver ode45 of Matlab, which is an explicit Runge-Kutta solver of fourth order.

4.2.6.2 Consideration of a Metabolite

In a second model, we assume that the bacteria first transform toluene to a metabolite without growth, and then grow on the degradation of the metabolite. A suitable candidate metabolite is methyl-catechol. The modified equations read as:

$$r_{tol} = r_{tol}^{max} \cdot \frac{c_{tol}}{c_{tol} + K_{tol}} \cdot X \tag{3}$$

$$r_{met} = r_{met}^{max} \cdot \frac{c_{met}}{c_{met} + K_{met}} \cdot X \tag{4}$$

$$\frac{dc_{tol}}{dt} = -r_{tol} \tag{5}$$

$$\frac{dc_{met}}{dt} = r_{tol} - r_{met} \tag{6}$$

$$\frac{dX}{dt} = Y \cdot r_{met} \tag{7}$$

in which r_{tol} [µM s^{-1}] and r_{met} [µM s^{-1}] are the transformation rates of toluene and the metabolite, r_{tol}^{max} [µmol cells^{-1} s^{-1}] and r_{met}^{max} [µmol cells^{-1} s^{-1}] are the corresponding maximum specific rates, and c_{met}, [µM] is the concentration of the metabolite with the corresponding half-saturation concentration K_{met} [µM].

4.2.7 Reactive-Transport Modeling

4.2.7.1 Governing Equations

We simulate microbial growth in the column systems coupled to one-dimensional reactive-transport with a numerical model that considers three mobile components, namely toluene (electron donor and carbon source), oxygen (electron acceptor), and suspended bacteria as well as the attached bacteria as immobile component. We model microbial growth of attached and suspended bacteria, depending on the simultaneous presence of toluene and oxygen, by dual Monod kinetics:

$$r_{growth}^{att} = \mu_{max} \cdot \frac{c_{tol}}{c_{tol} + K_{tol}} \cdot \frac{c_{ox}}{c_{ox} + K_{ox}} \cdot X_{att} \tag{8}$$

$$r_{growth}^{mob} = \mu_{max} \cdot \frac{c_{tol}}{c_{tol} + K_{tol}} \cdot \frac{c_{ox}}{c_{ox} + K_{ox}} \cdot X_{mob} \tag{9}$$

in which μ_{max} [s^{-1}] is the maximum specific growth rate constant, c_{tol}, c_{ox} [µM], X_{att} [cells L_{sed}^{-1}] and X_{mob} [cells L^{-1}] are the concentration of toluene, oxygen, attached, and mobile bacteria, respectively, whereas K_{tol} and K_{ox} [µM] are the half-saturation concentrations of toluene and oxygen, respectively. The concentration of attached cells X_{att} is expressed in number of cells per bulk volume of the sediments. Initially, we applied the same kinetic rate coefficients to the mobile and attached bacteria as expressed in equations 8 and 9. However, due to the short residence time of mobile bacteria in the 1.6 cm long columns, growth of mobile bacteria was found to be insignificant, and was neglected in the further mathematical analysis.

One-dimensional transport of toluene and oxygen in the column system and their consumption due to growth of attached bacteria can be described by a system of coupled advection-dispersion-reaction equations (in which we have neglected sorption):

$$\frac{\partial c_{tol}}{\partial t} = -v \cdot \frac{\partial c_{tol}}{\partial x} + D \cdot \frac{\partial^2 c_{tol}}{\partial x^2} - \frac{1}{Y} \cdot \left(r_{growth}^{att} + r_{growth}^{mob} \right) \tag{10}$$

$$\frac{\partial c_{ox}}{\partial t} = -v \cdot \frac{\partial c_{oc}}{\partial x} + D \cdot \frac{\partial^2 c_{ox}}{\partial x^2} - \frac{f_{ox}}{Y} \cdot \left(r_{growth}^{att} + r_{growth}^{mob} \right) \tag{11}$$

with the linear transport velocity v [m s^{-1}], the longitudinal dispersion coefficient D [m^2 s^{-1}], the microbial growth yield Y [cells μmol_{tol}^{-1}] and the ratio of stoichiometric coefficients of oxygen and toluene f_{ox} [μmol_{tox} μmol_{tol}^{-1}].

Results from the column experiments showed that the number of attached bacteria stopped increasing beyond a maximum value, indicating that there was a maximum carrying capacity of attached bacteria (X_{att}^{max} [cells L_{sed}^{-1}]) in the system. However, even when X_{att}^{max} was reached, the attached bacteria continued to replicate. In the model, the new-grown cells are released to the mobile aqueous phase and finally flushed out of the column. This release of new-grown cells from the sediment surface to the mobile aqueous phase has already been observed in earlier studies on microbial transport under growth conditions (e.g., Clement et al., 1997; Murphy et al., 1997; Yolcubal et al., 2002; Jordan et al., 2004; Mellage et al. 2015). We accounted for this process in the model by the dynamic detachment rate $r_{daughter}$ [cells L_{sed}^{-1} s^{-1}]:

$$r_{daughter} = r_{growth}^{att} \cdot \frac{X_{att}}{X_{att}^{max}} \tag{12}$$

If X_{att}^{max} is not yet reached, new-grown cells partially stay attached and partially are released to the mobile aqueous phase. When the carrying capacity is approached, the term X_{att} / X_{att}^{max} approaches unity and all new-grown cells are released to the aqueous phase.

Attachment of suspended bacteria to the sediment surface is described by the modified first-order attachment rate r_{attach} [cells L^{-1} s^{-1}]:

$$r_{att} = k_{att} \cdot X_{mob} \cdot \left(1 - \frac{X_{att}}{X_{att}^{max}} \right) \tag{13}$$

in which k_{att} [s^{-1}] is the first-order attachment rate coefficient and the term $\left(1 - \frac{X_{att}}{X_{att}^{max}}\right)$ is

introduced to account for the carrying capacity (Ding, 2010). The rate of change of attached X_{att} [cells mL_{sed}^{-1}] and mobile X_{mob} [cells mL^{-1}] bacteria is described by:

$$\frac{\partial X_{att}}{\partial t} = r_{growth}^{att} + n \cdot r_{att} - r_{daughter} \tag{14}$$

$$\frac{\partial X_{mob}}{\partial t} = -v \cdot \frac{\partial X_{mob}}{\partial X} + D \cdot \frac{\partial^2 X_{mob}}{\partial X^2} - r_{att} + \frac{1}{n} \cdot r_{daughter} \tag{15}$$

Note that the carrying capacity X_{att}^{max} is a prescribed model parameter that needs to be obtained by fitting the model to data. The model itself does not explain the mechanisms determining the carrying capacity.

4.2.7.2 Numerical Methods

We discretized the coupled system of one-dimensional reactive-transport equations in space by the cell-centered Finite Volume Method with a spatial discretization of Δx = 0.5 mm. We applied upwind differentiation of the advective term and set the dispersion coefficient to 1.95×10^{-8} m^2 s^{-1}. The coupled system of spatially discretized reactive-transport equations was integrated in time by an implicit Euler method with adaptive time stepping and a maximum time-step size of 600s. The resulting system of coupled non-linear algebraic equations was linearized by the Newton-Raphson method, and the UMFPACK solver implemented in Matlab was used to solve the resulting system of linear equations. The code was written as a Matlab program.

4.3 Results

4.3.1 *Batch Experiments*

Figure 4.2 shows measured and simulated concentrations of toluene and cell numbers in the batch experiments of aerobic toluene degradation with *Pseudomonas putida* F1. The lines show fitted model results, where the dashed lines represent the standard model, in which

a given fraction of toluene is immediately used for biomass growth, and the solid lines represent the model with an intermediate metabolite (shown as dotted line) that can be further utilized for assimilation. It is obvious that the standard model fails at reproducing the data because the decrease in toluene concentrations precedes the increase in cell numbers. In the standard model, the fitted maximum specific growth rate μ_{max} is 4.25 ± 0.24 d^{-1} and the fitted Monod constant K_{tol} is 10.9 ± 2.83 µM, with a yield coefficient of $Y = 2.83\times10^{8}$ cells μmol_{tol}^{-1}. In the model with the metabolite, the first reaction is considerably faster than the second one (r_{tol}^{max} /Y = 24.06 ± 0.01/d vs. r_{tol}^{max} /Y = 4.19 ± 0.03/d, in which the scaling with the yield is chosen to make the numbers comparable to μ_{max} of the standard model), and the Monod constant K_{tol} of 1.45 ± 0.002 µM is much better constrained.

In Figure S4.1 (supporting information) we show that the substrate carbon converted to biomass carbon over the entire experiment increased with the initial toluene concentration but plateaued for initial toluene concentrations of about 150 µM and higher. At the end of all experiments, toluene was completely degraded. The finding of a maximum biomass concentration indicates decreasing carbon-assimilation efficiency with increasing carbon and electron-donor supply.

To fit the toluene and cell data for the batch experiments under nitrate-reducing conditions with *Aromatoleum aromaticum* EbN1, it was not necessary to consider a metabolite (see the model fit assuming direct utilization of toluene for growth in Figure S4.2 of the supporting information). The fit of the classical Monod-growth-model to all toluene and cell data revealed a μ_{max} of 0.35 d^{-1}, K_{tol} = 21.7 µM, and $Y = 1.38\times10^{8}$ cells μmol_{tol}^{-1}. The maximum specific growth rate of the aerobe *P. putida* F1 was about 10 times higher than that of the denitrifier *A. aromaticum* EbN1, and K_{tol} was 15 times larger for the denitrifyer.

4.3.2 Sediment Column Experiments

We performed experiments with different toluene concentrations in the inflow and different flow velocities with *P. putida* F1, *A. aromaticum* EbN1, and a natural aquifer microbial community. Table 4.1 summarizes key results of the individual experiments with regard to toluene degradation, oxygen consumption, and microbial growth.

Table 4.1: Comparison of column experiments with *P. putida* F1, *A. aromaticum* EbN1, and a natural toluene degrading community at different flow rates and toluene source concentrations. C_{tol}^{in} : toluene concentration in the inflow (target concentrations are listed first, actual concentrations given in brackets), *v*: velocity, ΔTol: difference in toluene concentration between in- and outflow, ΔO_2: difference in oxygen concentration between in- and outflow, f_{ox}: stoichiometric ratio between oxygen and toluene, New Cells: increase in cell numbers.

Exp	C_{tol}^{in} [µM]	*v* [m/d]	ΔTol [µM]	ΔTol [%]	ΔO_2 [µM]	f_{ox} [μmol_{O2}/ μmol_{Tol}]	New cells [$\times 10^8$ cells]	Cells flushed out [%]	Yield [$\times 10^7$ cells/ μmol_{Tol}]	Cells attached [%]
P.putida F1										
A	70 (66.9)	0.6	66.9	100	207	3.1	4.4	70	3.6	99
B	70 (65)	1.8	59,6	92	203	3.4	14	76	4.0	99
C	70 (73.5)	3.6	51	69	261	5.1	28	79	5.3	99
D	30 (26.3)	1.8	26.3	100	104	3.95	6.6	72	5.8	99
E	100 (113)	1.8	67.2	59	247	3.7	53	93	11.7	98
A. aromaticum EbN1										
F	70 (85.4)	1.8	63.1	73	-	-	10	20	3.0	99
Natural microbial community										
G	70 (73.1)	1.8	73.1	100	256	3.5	11	50	3.5	99

4.3.2.1 Toluene Degradation

The reduction in toluene concentration (ΔTol) in the individual column experiments with *P. putida* F1 (Exp A to Exp C) showed a linear decrease with increasing flow velocity at identical inflow concentration (Table 4.1, Fig. 4.3B & 4.3C). The total toluene transformation within 192 hours ranged from 12.2 µmol in Exp A (v = 0.6 m d^{-1}) to 53.6 µmol in Exp C (v = 3.6 m d^{-1}) exhibiting a positive trend, i.e. an increase with increasing velocity and thus increasing toluene mass flux. With respect to the toluene removal efficiency, the experiment

with the lowest flow velocity, Exp A, showed 100% toluene removal followed by Exp B and C. At the highest flow velocity, only 69% of the toluene could be degraded aerobically by *P. putida* (Table 4.1). With regard to the inflow concentration, ΔTol increased with increasing C_{Tol}. However, at the highest toluene concentration in the inflow (100 µM; Exp E) the data clearly hint at an oxygen limitation and degradation efficiency dropped to 59% (Fig. 4.4; Table 4.1). After establishment of full biodegradation activity, also experiments D (*P. putida* at lower source concentration) and G (natural aerobic consortium) revealed 100% toluene removal efficiency (Table 4.1; Fig. 4.3C). Further relationships between toluene source concentration, flow velocity, and biodegradation efficiency are depicted in Figures 4.3A – C.

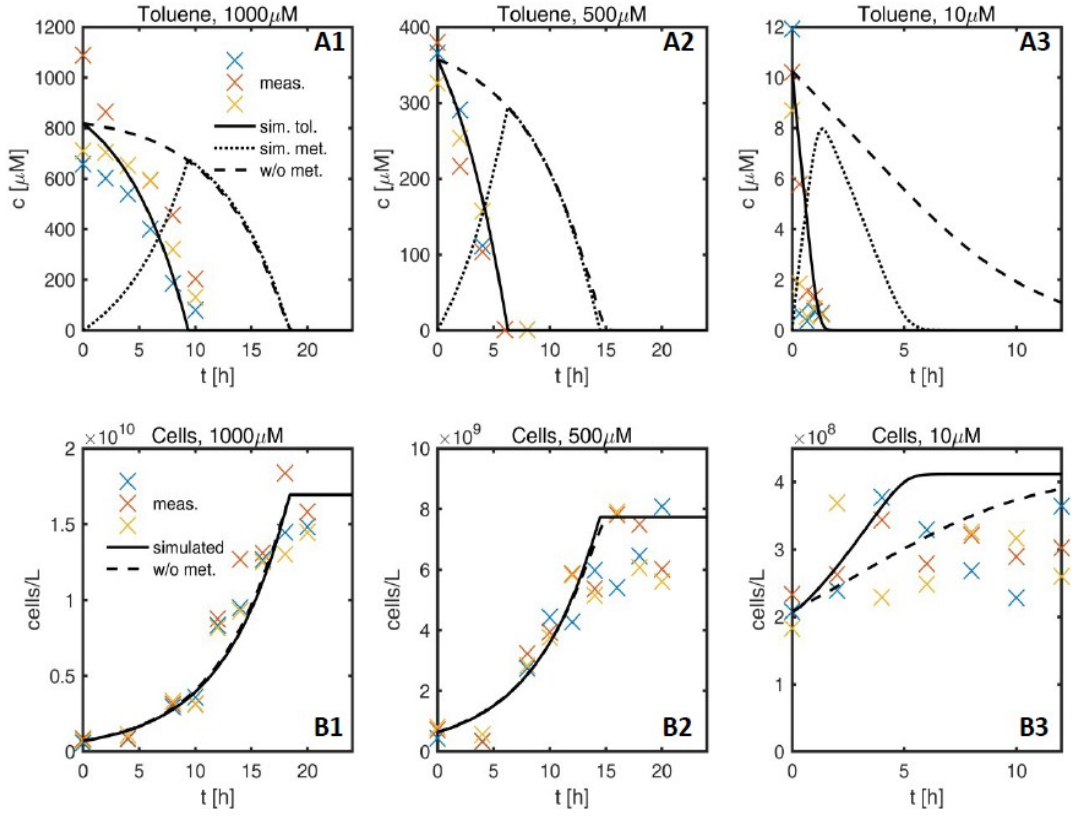

Figure 4.2: Measured and simulated toluene concentrations and bacterial growth of *P. putida* F1 over the course of batch experiments with different substrate starting concentrations. The color coded data points represent measured values of toluene and cells/ml, respectively, from batch triplicates. Two scenarios were considered, (1) with the formation of the central metabolite 3-methyl catechol (solid back lines - simulated toluene, dotted back lines - simulated metabolite), and (2) without metabolite (black dashed lines).

Figure 4.4 exemplarily depicts the time series of concentrations and cell numbers for one set of experimental conditions (Exp. E: *P. putida*, c_{tol} = 100 µM, v = 1.8 m d^{-1}). As can be seen, the concentration of toluene in the column outflow as well as the oxygen concentration within the sediment column immediately started to decline and both leveled off after 1-2 days at concentrations of approximately 45 µM toluene and <0.2 µM dissolved oxygen, respectively (Fig. 4.4). No differences in the oxygen values were found between the bottom, middle, and top column observation points indicating that oxygen was readily consumed in the bottom (inflow) part of the column. Experiments at lower inflow concentrations of toluene and varying flow velocities exhibited similar patterns (see Supplementary Information, Fig. S4.3-S4.6).

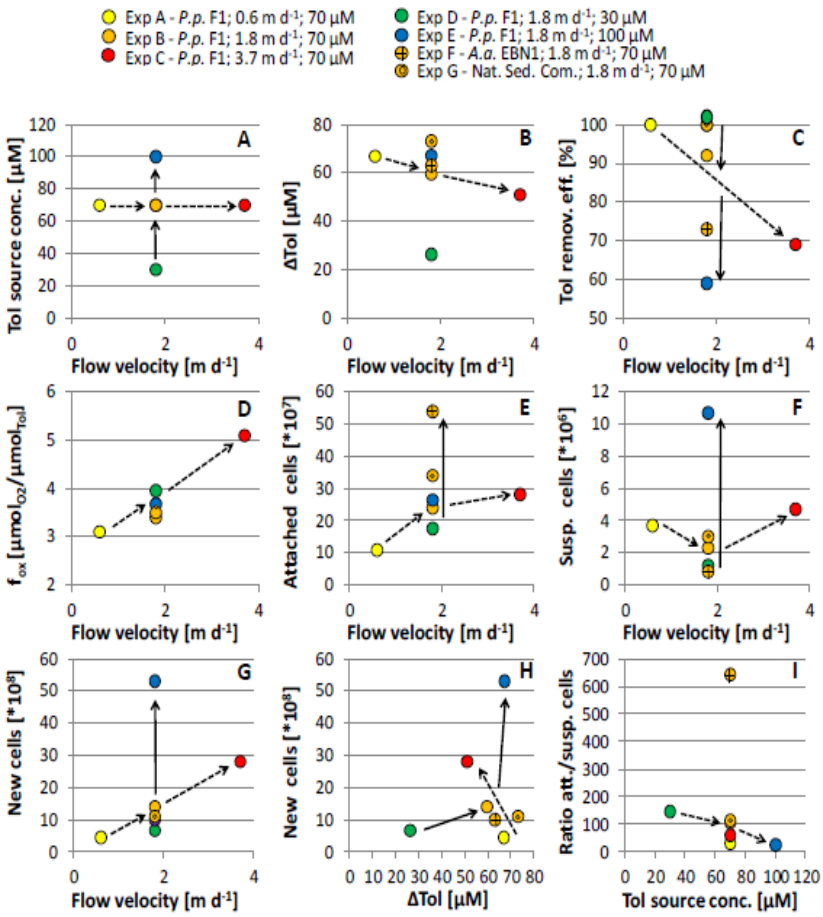

Figure 4.3: Influence of toluene inlet concentration (solid arrows) and flow velocity (dashed arrows) on biodegradation, growth and yield, as well as the distribution of bacterial cells on the sediment and

in porewater.

In the column experiment F, which is similar to that depicted in Figure 4.4, toluene degradation was examined under nitrate reducing conditions by the strain *A. aromaticum* EbN1. Here, the bacterial population took 3 days to establish the full toluene degradation capacity, which was considerably slower than the aerobic culture *P. putida*. We chose a nitrate concentration of 500 µM because this concentration would be sufficient for the complete transformation of the foreseen 70 µM toluene in a perfectly mixed solution. Unfortunately, the actual inflow concentration of toluene was about 80 µM and thus a bit higher than intended. However, the columns outflow still contained about 20µM of toluene, pointing at a nitrate limitation under the flow-through conditions in the mini sediment column (Fig. S4.7).

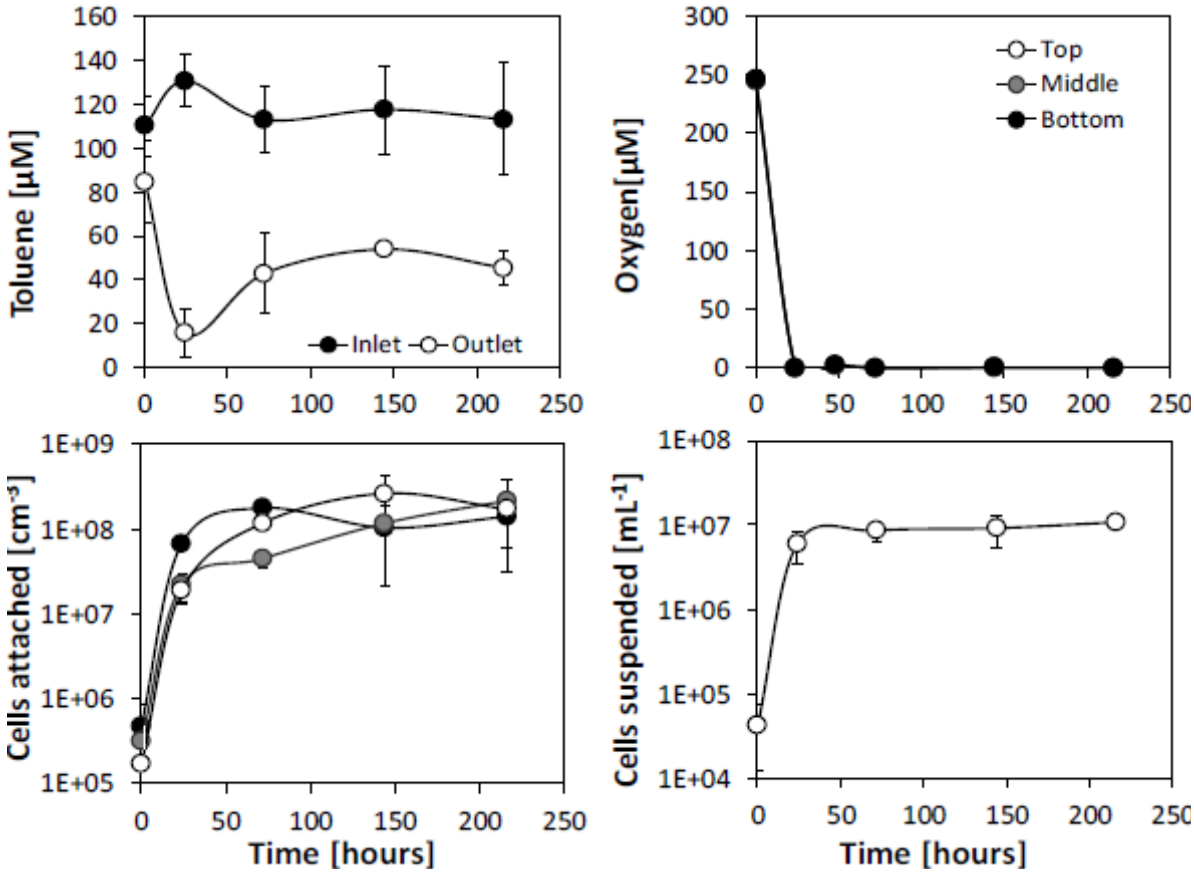

Figure 4.4: Aerobic toluene degradation and growth of P. putida F1 at a continuous source concentration of 100 µM and a flow velocity of 1.8 m d^{-1} (Exp E). Values are means of triplicate measurements ± SD.

The natural microbial sediment community fed by oxic groundwater containing 70µM toluene (Exp. G) was able to readily degrade toluene, albeit the maximum degradation efficiency was reached much later than with the specific degrader strains applied, *i.e.* after 6 days compared to 1-2 days with the *P. putida* F1 and 3 days with *A. aromaticum* EbN1. After establishment of quasi steady-state conditions in the sediment columns, the change of toluene concentration between the in- and outlet under similar experimental conditions (c_{tol} = 70 µM, v = 1.8 m d^{-1}) by *P. putida* F1 (59.6 µM), *A. aromaticum* EbN1 (63.1 µM) and the natural microbial community (73.1 µM) were in a similar range.

Based on toluene and oxygen measurements (ΔO_2 and ΔTol at fully established biodegradation activity; Table 4.1) conducted for the column experiments with *P. putida* F1 and the natural aquifer community, we calculated the empirical stoichiometric ratio f_{ox} between oxygen consumption and toluene degradation, as well as bacterial growth. The f_{ox}-values obtained for the different experiments fell in the range of 3.1 to 5.1 $\mu M_{Tol}\ \mu M_{O2}^{-1}$. We found the highest stoichiometric ratio with the highest flow velocity (Exp C) and thus the highest toluene mass flux. f_{ox} decreased together with the flow velocity (Fig. 4.3D). No pronounced differences in f_{ox} nor a consistent trend were observed with varying inflow concentration (Fig. 4.3D). In the experiment with the natural aquifer community a similar f_{ox}-value (3.5) was found as with *P. putida* F1 under comparable environmental conditions (Table 4.1).

A high stoichiometric ratio f_{ox} translates into low carbon biomass yield. The highest yield under quasi steady-state conditions was thus with the lowest toluene mass flux. However, dissecting the column experiments into an initial phase of rapid cell production and the subsequent phase at quasi-steady state revealed a very dynamic biomass yield. In almost all experiments, carbon assimilation was extremely high in the initial phase of growth and

then decreased within two, maximal three days to a lower constant value when the carrying capacity for the cell density was reached (see the carbon assimilation efficiency in Fig. S4.8).

Due to technical problems, nitrate concentrations could not be measured in the column experiment F with *A. aromaticum* EbN1. Therefore, we could not evaluate the stoichiometry between toluene degradation and nitrate consumption under nitrate-reducing conditions.

4.3.2.2 Microbial Growth

In all column experiments the number of bacterial cells attached to the sediment substantially increased within the first 1-3 days. As exemplarily depicted in Figure 4.4, the number of attached cells per volume sediment, here at an inflow concentration of 100 µM and a flow velocity of 1.8 m d^{-1}, increased by more than three orders of magnitude within 72 hours and then stayed rather constant for the remaining time of the experiment. The maximum density of attached cells reached $2.6\times10^{8} \pm 1.6\times10^{8}$ cells mL_{sed}^{-1}. The number of cells suspended in the pore-water collected at the column outflow also increased by two orders of magnitude reaching a constant value of about 1×10^{7} cells mL^{-1} already after 48 hours. This constant outwash of 1×10^{7} cells mL^{-1} following day two, pointed at an actively growing attached bacterial population, releasing its daughter cells into the mobile water phase. Since 99% of the cells per sediment volume were found attached to the sediment surface (see Table 4.1) and the water residence time in the columns was considerably short (12.7 min), toluene degradation and microbial growth could be fully attributed to the attached populations in the column system. However, over the entire phase of the experiment (192h), 97% of all newly produced cells were transported out the columns in experiment E.

We observed similar patterns for the other column experiments (Table 4.1). For *P. putida* F1, the final number of cells associated to the sediment showed a positive trend with increasing flow velocity as well as with increasing toluene inlet concentration (Fig. 4.3E). The

highest abundance of attached cells was obtained in the experiments with the denitrifyer *A. aromaticum* (Fig. 4.3E). In the pore-water, patterns of cell numbers were less clear. While, increasing toluene concentrations in the inlet pushed the cell numbers in the pore-water from 1.2×10^6 cells mL^{-1} in Exp D (30 µM) to 1×10^7 cells mL^{-1} in Exp E (100 µM), we could not observe a conclusive dependence of cell-numbers in the pore water and flow velocity (Fig. 4.3F). The ratio of attached to suspended cells ranged from 14 to 643, with the highest ratio for the denitrifyer *A. aromaticum* (Fig. 4.3I). For the aerobic strain *P. putida* F1 and in the natural aerobic consortium, we observed a lower ratio of attached to suspended cells.

Similar to the maximum total cell numbers observed in the pore-water and attached to the sediment, the number of newly grown cells, as determined by direct cell counts, systematically increased with the increase of toluene concentration in the inflow as well as with the flow velocity (Table 4.1; Fig. 4.3G & 4.3H). Consequently, we observed a similar trend for the microbial growth yield Y [cells μmol_{tol}^{-1}].

The estimated growth yield for the column experiments with *A. aromaticum* EbN1 under denitrifying conditions and the natural community, with values of 3.0×10^7 and 3.5×10^7 cells μmol_{tol}^{-1}, respectively, were in a similar range as for *P. putida* F1 (of 4.0×10^7) under similar experimental conditions (70µM toluene, v = 1.8 m d^{-1}) (Table 4.2 & Fig. 4.3G & 4.3H).

Even though a large fraction of the newly grown cells in the flow-through experiments conducted with *P. putida* F1 were flushed out of the column over time (60 to 93%), at any given time point the majority of cells per volume of water-saturated sediment was found to be attached to the sediment surface, regardless of the experiment (≥ 98%, Table 4.1). The experiment using the natural aquifer community showed a considerably lower percentage of washed-out cells (50%), and the experiment using *A. aromaticum* EbN1 had the lowest percentage of washed-out cells (20%, Table 4.1). While the measurements of attached cells

showed a distinct gradient in cell numbers along the length of the column in Exp B, with the highest cell numbers in the bottom (inlet) part of the column, no pronounced spatial gradient along the bottom, middle, and top parts of the columns was found in the other experiments (Fig. S4.3-S4.8).

We estimated the growth yield in our experiments in two ways: First, by converting the f_{ox}-values (see above) to yield coefficients, and second by comparing the amount of newly grown cells to the toluene mass degraded. The range of f_{ox}-values between 2.6 and 5.5 μmol_{Tol} μmol_{O2}^{-1} (Table 4.1), indicates carbon assimilation efficiencies of 0.39 to 0.72. Summing up all newly formed cells within the individual experiments led to similar carbon assimilation efficiencies of 0.35 to 0.7. The bacterial cell size of *P. putida* F1, sporadically determined via fluorescence microscopy, was found to be rather constant in the column experiments, with an average length of 1.6 µm and an average width of 0.8 µm. This corresponds to a biovolume of roughly 0.5 μm^3 per cell. Consequently, a mean cell carbon content of 130 fg was used for the conversion of cell numbers into cell carbon.

4.3.2.3 Reactive-Transport Modeling

Figure 4.5 depicts concentration and cell-count time series of the column experiments A-C, performed with *P. putida* F1 at different flow velocities, as well as the experiment performed with the denitrifier *A. aromaticum* EbN1 (Exp F) and the natural microbial community (Exp G), together with the corresponding time series obtained by reactive-transport simulations. Table 4.2 lists the individual model parameters. The maximum specific growth rate μ_{max}, the stoichiometric coefficient for oxygen consumption f_{ox}, and the attachment rate coefficient k_{att} were fitting parameters. All remaining parameters were either calculated from the batch and column data prior to the simulations or taken from the literature.

The model reproduces the observed breakthrough curves of toluene and suspended

cells at the column outlet, as well as the temporal evolution of the number of attached cells in the bottom, middle, and top parts of the columns very well, with exception of dissolved oxygen. The largest discrepancy between experimental and simulation results was found for the experiment with the natural microbial community (Exp G), for which the measurements indicated complete oxygen depletion already in the bottom (inflow) part of the column, whereas the simulation results showed a continuous gradient in oxygen concentrations along the length of the column.

Table 4.2: Model parameters**.** Parameters given in bold were fitting parameters. The maximum number of attached cells X_{att}^{max} was set to be the highest number of cells/ml obtained from sediment analysis in each experiment. The yield coefficient Y in the model was calculated from the column data by comparing the number of newly formed cells to the amount of degraded toluene. K_{tol} was taken from the preceding batch experiments, and K_{ox} was obtained from the literature.

Parameter		Exp A	Exp B	Exp C	Exp F	Exp G
μ_{max}	**[1 d^{-1}]**	**4.5**	**4.5**	**3.0**	**2.0**	**0.5**
K_{tol}	[µmol L^{-1}]	1.1	1.1	1.1	21.7	1.1
K_{ox}	[µmol L^{-1}]	10[a]	10[a]	10[a]	-	10[a]
f_{ox}	**[µmol$_{O2}$ µmol$_{Tol}^{-1}$]**	**2.6**	**4.0**	**4.9**	**-**	**3.7**
k_{att}	**[1 d^{-1}]**	**50**	**50**	**50**	**50**	**50**
	[cells mL_{Sed}^{-1}]	0.9×10^8	2.0×10^8	2.7×10^8	5.4×10^8	3.4×10^8
Y	[cells µmol$_{Tol}^{-1}$]	3.6×10^7	4.0×10^7	5.3×10^7	3.0×10^8	3.5×10^7

[a] *Bauer et al. (2009)*

While the concentration of suspended cells in the outflowing water was in the same range in all experiments conducted with *P. putida* F1 at a toluene concentration of 70 µM, the flux of cells leaving the column was increasing with higher flow rates. The leveling off in the number of attached cells at a maximum value is enforced in the model by introduction of the maximum number of attached cells X_{att}^{max} in equations 12 and 13. Without this term, we would have achieved higher biomass concentrations, particularly close to the column inlet. The

model also captures the continuous outflow of 2 to 4×10^6 cells mL^{-1} in all experiments conducted with *P. putida* F1 and 70 µM toluene well because it simulates the release of new-grown cells from the sediment (immobile phase) to the mobile aqueous phase by the dynamic growth-depended detachment rate $r_{daughter}$ (equation 12). This term balances biomass growth once the carrying capacity of the system for attached cells is reached.

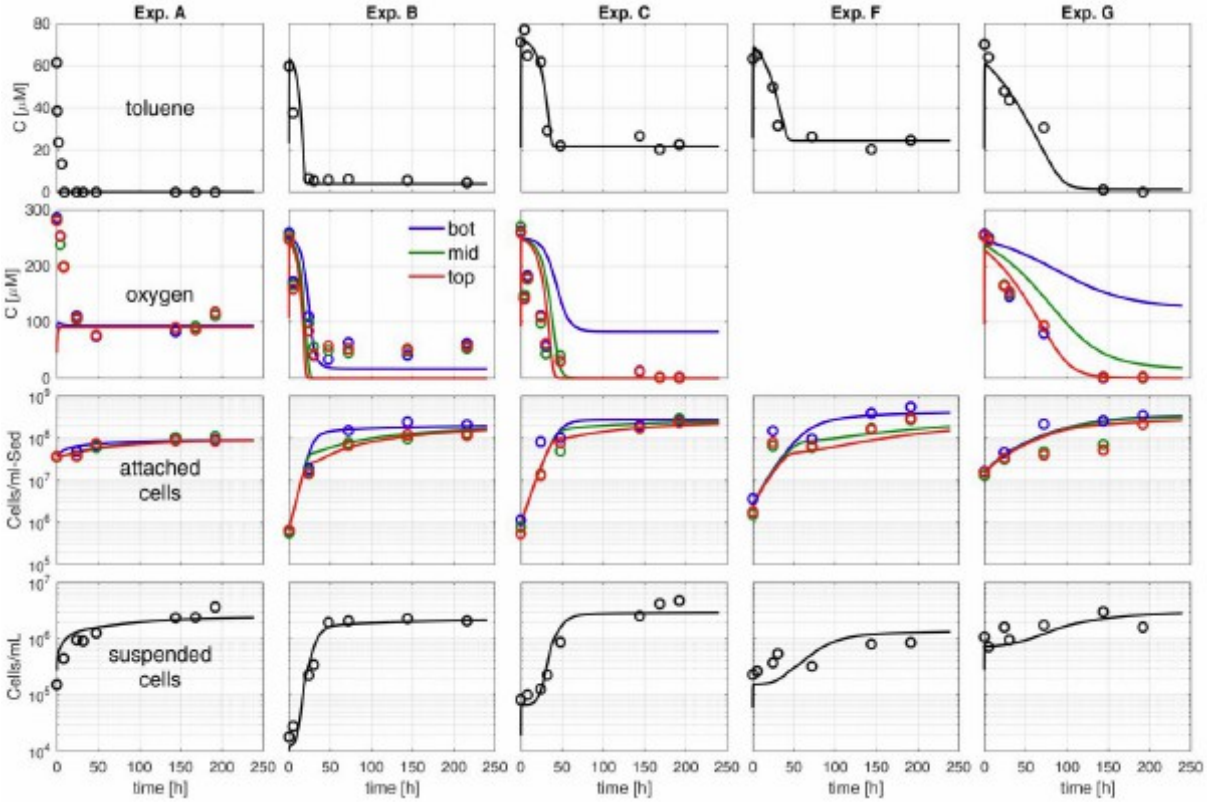

Figure 4.5: Comparison of the experimental data and simulation results for selected column experiments; bot = column bottom (close to inlet), mid = middle part of the column, top = top part of the column (close to outlet).

Because the vast majority of bacteria, and hence biodegradation activity per volume water saturated sediment, was located at the sediment surfaces rather than in the aqueous phase (see Table 4.1), toluene degradation in the columns could be almost exclusively attributed to the attached bacterial populations. Moreover, additional simulations, in which growth of suspended bacteria was accounted for, revealed the same results as simulations in which growth of suspended bacteria was neglected (data not shown).

The concentration profiles of toluene and attached cells could even be reproduced without explicit consideration of mobile cells, if a logistic growth-term of the form $(1-X_{att}/X_{att}^{max})$ was applied to the microbial growth rate, but not to the rates of toluene and oxygen consumption.

While suspended bacteria were found to be unimportant for toluene degradation, between 20% (Exp F) and 93% (Exp E) of the newly grown cells were flushed out of the columns over time (Table 4.1).

The remaining toluene concentration at the outlet of the columns inoculated with *P. putida* F1 increased with the flow rate from Exp B. (1.8 m d^{-1}) to Exp C (3.6 m d^{-1}), and the initial drop in toluene concentration was faster for Exp B than for Exp C. This is also captured by the fitted values of μ_{max} (Table 4.2). While the same value was applicable for Exp B and the batch experiments, a slightly lower one had to be chosen to match the results of Exp. C, which is the experiment with the higher flow velocity.

The fitted maximum specific growth rate μ_{max} of 0.5 d^{-1} in the experiment with the natural microbial community is almost one order of magnitude smaller than the value estimated for the experiment with *P. putida* F1 at the same flow velocity (μ_{max} = 4.5 d^{-1}). Even though the initial abundance of attached cells was about one order of magnitude higher for the experiment with the natural community, as compared to the experiments with *P. putida* F1, the initial drop in toluene degradation was much slower. In this context, it may be worth noting that the simulation was performed under the assumption that all attached cells in the experiment with the natural community were able to readily degrade toluene, which is unlikely for a natural microbial community that consists not only of specific toluene degraders such as *P. putida* F1 (see discussion). Additional simulations, in which we assumed that only a fraction of 1% of the detected number of attached cells was able to readily degrade toluene, however, showed hardly any difference at late times because with the given maximum

specific growth rate μ_{max} the necessary 100-fold increase of biomass at early times only takes 4-7 hours.

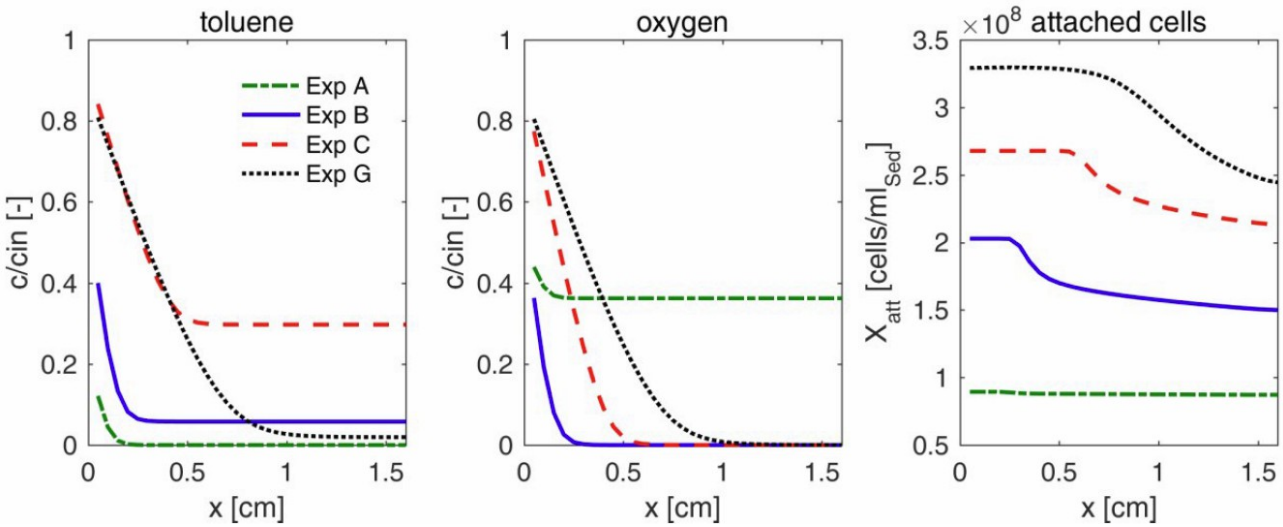

Figure 4.6: Simulated spatial profiles for toluene, oxygen and attached cell concentrations for Exp B-C, and G after steady state has been reached (t = 200h).

The fitted maximum specific growth rate μ_{max} of 2 d^{-1} for the experiment with *A. aromaticum* EbN1 (Exp F) is almost seven times larger than the value obtained from analyzing the batch experiments. Apparently some conditions within the flow-through system provided a better environment for growth of the denitrifying culture than in the batch system, but we don't know the decisive factor.

While the model included attachment of mobile biomass (equation 13), the attachment rate coefficient k_{att} was not a very sensitive parameter. Independent of the flow velocity, all experiments could be reproduced with a value of 50 d^{-1}. Additional model runs, in which k_{att} was set to zero, indicated that the observed increase in the number of attached cells in the middle and top part of the column can be explained to a large extent by microbial growth during the initial phase of the experiment, when oxygen was still available along the entire column.

Figure 4.6 depicts simulated spatial profiles of toluene, oxygen, and attached cells in

the sediment columns for the experiments A-C (*P. putida* F1 with c_{tol}^{in} = 70µM), and experiment G, conducted with the natural microbial community, after steady state has been reached (t = 200h). The model predicts a rapid decrease in toluene concentrations with travel distance, and the complete consumption of oxygen within the first centimeter for all experiments. This matches the fact that no differences in oxygen profiles could be observed between the three locations in the columns.

4.4 Discussion

4.4.1 Batch versus Flow-Through Experiments

Microbial growth observed in batch tests using different initial toluene concentrations exhibited clear first-order degradation kinetics. Model fits of the batch tests showed a maximum specific growth rate μ_{max} of 4.5 d^{-1} for *P. putida* F1, which is somewhat smaller than literature values ranging from 12 to 21 d^{-1} (Abuhamed et al., 2004; Alagappan & Cowan, 2004; Reardon et al., 2000). In the batch experiments with *P. putida* F1 at higher concentrations, we observed that biomass growth continued even after toluene had already completely been consumed (see Fig. 4.2). In these instances the standard Monod growth model could not simultaneously explain the toluene and cell data. Yu et al. (2001) observed a similar pattern and provided evidence that the buildup and further breakdown of 3-methylcatechol, a central intermediate in toluene degradation by *P. putida* F1, was responsible for the observed mismatch. Considering a central intermediate, the chemical nature of which has not been identified in the current experiments, we could fit the batch experiments involving P. putida much better (see solid lines in Fig. 4.2). For *A. aromaticum* EbN1, the denitrifying strain, ten times lower maximum specific growth rates were found with μ_{max} values of 0.3 d^{-1}. Here, literature values range between 2.2 and 2.6 d^{-1} (Evans et al., 1991; Jorgensen et al., 1995).

While the maximum specific growth rates of *P. putida* F1 in the batch and flow-through systems were similar, the mean growth rates differed. In batch systems with initial concentrations of 50µM toluene or more, the mean growth rates were consistently higher than in column experiments with inflow concentrations of 70 µM of toluene. However, in batch systems the bacteria are exposed to a limited mass of toluene, they grow quickly, consume the toluene and stop growing because they run out of substrate. In the flow-through systems, by contrast, the bacteria exhibit a permanent supply of the substrate. They grow until they approach the carrying capacity, when they start to release the new grown cells. That is, the control of biomass growth considerably differs among the two systems.

The picture was different for the denitrifying strain *A. aromaticum* EbN1. Here the growth rates were much higher (factor of 20) in the flow-through experiments. The reason for that is not fully clear. However, there are strong indications from the sediment-column experiments that *A. aromaticum* EbN1 prefers to grow attached to surfaces as seen from the ratio between attached versus suspended cells (Fig. 4.3I) and the very different growth rates of suspended and attached cells in the sediment columns (Fig. S4.10). Without sediments to attach to, *A. aromaticum* EbN1 obviously experiences less favorable growth conditions.

In terms of yield, *P. putida* F1 was more efficient converting toluene carbon into biomass carbon at lower concentrations in the flow-through sediment columns than in the batch systems. At an initial toluene concentration of 10 µM no significant increase in cell numbers could be detected in the batch experiment. As a consequence, at low substrate concentrations, substrate turnover fuels cell maintenance and energy production rather than growth (Egli 2010). At concentrations >100 µM, carbon assimilation efficiencies became pronounced with values between 0.38 and 0.55 in the batch experiments (Fig. S4.1 & S4.2). The latter values are well comparable to the flow-through systems (0.43 to 0.7).

The yield of *A. aromaticum* EbN1 was comparable to *P. putida* in the batch system.

Since there was only one column experiment with *A. aromaticum* EbN1 and we lacked nitrate data for the columns, a direct comparison between batch and flow-through sediment microcosms was not valid.

4.4.2 Column Experiments – Impact of Flow Velocity and Inflow Concentration

4.4.2.1 Toluene Degradation and Bacterial Growth

In all column experiments, the difference between the inlet and outlet concentrations of toluene (ΔTol) initially increased and then reached a steady value, which we denote the maximum degradation efficiency. While steady overall turnover was eventually reached in all column experiments, the time needed to approach the steady value differed between the bacteria involved. *P. putida* F1 generally reached maximum degradation efficiency within 1-2 days, *A. aromaticum* EbN1 needed 3 days, and the natural microbial community was the slowest with a maximum degradation efficiency approached after 6 days. We may explain this by biodegradation being related to the amount of active biomass (specific degrader) and the growth rate being related to the energy gained. In the batch experiments, the denitrifying strain *A. aromaticum* EbN1 had a much smaller maximum specific growth rate μ_{max} than the aerobic strain *P. putida* F1. The difference was smaller in the case of the column experiments. As depicted in Table 4.2 and Fig. S4.10, proliferation of attached *A. aromaticum* EbN1 cells is in the same range as that of *P. putida* F1 (see below). The natural microbial community, by contrast to the specific cultures, consisted of probably thousands of different strains, with only a small fraction readily capable of aerobic toluene degradation and related growth (Bordel et al., 2007; Castillo and Ramos, 2007; Okpokwasili and Nweke, 2005; Vecht et al., 1988).

While the total amount of degraded toluene increased with the flow velocity, toluene removal efficiency, i.e. the percentage of the injected toluene mass which was degraded while being transported through the column, decreased. A similar pattern was observed for the

maximum specific growth rate μ_{max} (see Tables 4.1 and 4.2, Fig. 4.2). An explanation is that the mass-transfer of toluene and oxygen/nitrate from the pore-water to the interior of attached bacteria exerts a stronger control on reactive turnover at higher flow velocities because the characteristic time scale of mass-transfer into the cells is independent of velocity whereas the characteristic time of advection is inversely proportional to velocity. Mass transfer limitations were put forward as explanation for decreased degradation rates in porous media by numerous studies (e.g., Dykaar and Kitanidis, 1996; Simoni et al., 2001; Hesse et al., 2009). At a fixed flow velocity, increasing the toluene concentration in the inflow revealed an increase in total toluene mass degraded per unit time. The toluene removal efficiency per unit volume, on the other hand, declined with increasing concentration in the inflow (see Table 4.1). This is actually expected for inflow concentrations c_{Tol}^{in} that are considerably larger than the half-saturation concentration K_{tol} of toluene:

In this concentration range, the turnover hardly increases with increasing concentration, so that the pseudo first-order coefficient $\frac{1}{Y} \cdot \frac{\mu max}{c_{tol} + K_{tol}}$ decreases.

We estimated the yield by two independent approaches leading to comparable results. In the first approach, we compared the amount of oxygen consumed per toluene degraded, f_{ox}. For the complete mineralization of toluene to carbon dioxide and water, a value of 9 mol_{O2} mol_{Tol}^{-1} is applied, which does not account for cell maintenance, carbon assimilation, and growth. Under growth conditions, however, bacteria oxidize only a part of the substrate to carbon dioxide to gain energy, while they assimilate the other part of the substrate-carbon into new biomass, in which carbon is more reduced than in CO_2 (Rittmann and McCarty, 2001). This reduces the amount of oxygen needed to degrade one unit of toluene under growth conditions. The f_{ox}-values obtained for the different experiments fell in the range of 2.6 to 5.5 μmol_{Tol} μmol_{O2}^{-1}, indicating carbon assimilation efficiencies of approximately 0.4 to 0.7. As can be seen from Figure 4.3D, there is a positive relationship between f_{ox} and flow velocity.

The faster the toluene is transported through the sediment, the less efficient is the substrate carbon converted into biomass. However, in total more toluene is degraded at higher flow rates and a higher standing stock of bacterial biomass is obtained (Fig. 4.3E). No pronounced differences were obtained for f_{ox} with changing toluene concentrations in the inflow (Fig. 4.3D).

In our second approach, the yield was calculated via the measured cell biomass produced from the toluene degraded. Based on microscopic measurements of cell dimensions (length and width), subsequent conversion into biovolume, and the assumption that carbon makes up 50% of the cell's dry mass, the cell yields were converted into $\mu mol_{cell\text{-}carbon}\ \mu mol_{Tol}^{-1}$. Doing so, and taking into account attached cells, as well as cells suspended in pore-water and continuously flushed out of the sediment columns, we again estimated a range of carbon assimilation efficiencies of 0.4 to 0.7.

The carbon assimilation efficiencies in the batch experiments were found to be around 0.4 to 0.5 and could only be calculated via cell counts since there was a continuous supply of oxygen from the headspace in the batch bottles. Carbon assimilation efficiencies were therefore slightly lower in batch (0.4-0.5) than in the flow-through sediment microcosms (0.4-0.7). This is surprising, since high carbon assimilation efficiencies of > 0.5 are generally reported for batch and chemostat systems (Ho and Payne, 1979; Payne and Wiebe, 1978, and references therein).

Both yield estimates obtained in our experiments are subject to uncertainty. Estimation of cell carbon via cell measurements in the microscope and translation into cell carbon is based on a number of assumptions, such as the carbon content of the dry biomass. Conversely, to obtain f_{ox} values, we used mean oxygen values within the sediment columns ignoring the potential concentration gradients along the three points of measurement (bottom, middle, top; Fig. 4.1). The following additional points may affect the reliability and comparability of the

yield estimates. First, the yield was calculated over the entire duration of the experiments, up to 3 days in the batch experiments and up to 9 days in the column experiments, including the stationary phase in the batch systems and the quasi steady-state phase in the microcosms. Second, in the batch experiments, the cultures received a single donation of substrate at t_0 which then was continuously depleted, while in the flow-through systems substrate was supplied continuously causing a concentration gradient from the column inlet to the outlet. Third, in the batch system, cells generally stop growing when the substrate was depleted. In the flow-through systems, growth decelerated when the maximum cell density was reached, which is termed the carrying capacity in ecology (del Monte-Luna et al. 2004). Finally, if we consider that the substrate is first converted to intermediates which are subsequently utilized for assimilation and mineralization, the overall yield changes during the experiment and can only be deduced by fitting a model that account for a generic intermediate, unless all possible metabolites are monitored. Nevertheless, our experiments provide strong evidence that in flow-through systems cells attached to a solid matrix are more efficiently converting substrate carbon into biomass than suspended ones.

4.4.2.2 Suspended and Attached Cells and Carrying Capacity

Batch systems are closed and optimized systems containing only suspended bacteria, and thus don't resemble the conditions in aquifers. The column systems are a step closer to field conditions as they represent flow-through systems containing both a mobile pore-water phase and an immobile sediment matrix with a solid-to-water ratio close to aquifer conditions (Hofmann et al. 2016). The distribution of cells between these two phases have been reported to depend on several factors including nutrient concentration in the pore-water, flow velocity, surface structure, and nutrient content of the sediment particles (Banfield et al., 1997; Banfield et al., 1999; Bennet et al., 2000; Bennet et al., 2001; Carson et al., 2009; Ehrlich,

1996; Marshall, 1988; Mauck and Roberts, 2007; Rogers and Bennet, 2004; Tuschewitzki et al., 1992; Grösbacher et al., 2016). As a consequence, bacterial growth in flow-through sediment systems can only be monitored if changes in cell numbers are followed in both the attached and suspended populations. Cells that are continuously washed out may be a major contribution to the overall biomass balance. In our column systems, about 98 to 99% of the cells were found attached to the sediment at any time during the experiment; such numbers are also found in most aquifers (Alfreider et al., 1997; Griebler et al., 2002; Lehman et al., 2001a & 2001b).

In all column experiments performed in this study, the amount of attached cells plateaued at a constant level after an initial growth phase, which indicates that the columns had a maximum carrying capacity for attached cells (Zhou et al. 2012). The carrying capacity showed an increasing trend with the amount of toluene degraded in the individual experiments. This suggests that the utilizable mass flux of substrate contributes to the control of the carrying capacity for attached bacteria. While the number of attached cells plateaued at concentrations of 0.9×10^8 to 27×10^8 cells mL_{Sed}^{-1} in the different experiments, 2×10^6 to 4×10^6 cells mL^{-1} were detected in the column outlets under stable experimental conditions. In fact, the ratio of attached to suspended cells was highest at the lowest substrate concentrations and *vice versa*, a pattern that is well known from aquatic sediments including aquifers (Harvey et al, 1984; Bengtsson 1989, Griebler et al. 2001, 2002).

Once the cells had reached the carrying capacity on the sediment, newly formed cells were released into the pore-water where they are transported and occasionally washed out of the columns. Over the course of the entire experiments (max duration of 9 days), this outwash corresponded to about 20-93% of all newly grown cells. An important finding was that growth-facilitated release of attached cells into the mobile phase was very strain-specific. While *P. putida* F1 cells growing on the sediment substantially released cells into the pore-

water, fewer cells were released by the natural community and the fewest by the denitrifying strain *A. aromaticum* EbN1. A significant contribution from budding and detachment cells to the free floating cells in pore-water under growth conditions has been repeatedly observed in earlier studies (e.g., Clement et al., 1997; Murphy et al., 1997; Yolcubal et al., 2002; Jordan et al., 2004) and referred to as cell-division mediated transport by Murphy and Ginn (2000). The finding that the vast majority of cells is attached to the sediment surface suggests that suspended cells have only a minor impact on the overall contaminant degradation, which was confirmed by reactive-transport simulations. In our experiments, the residence time of the pore-water in the columns was rather short (between 6.5 and 38.5 min depending on the flow rate). Still, even if suspended cells do not significantly contribute to contaminant degradation at anywhere within an aquifer, the continuous release of new-grown cells from the sediment to the mobile aqueous phase, which has been observed in our study, increases the ability of bacteria to spread and colonize new sediment surfaces. This may be an important mechanism to establish a high biodegradation potential throughout an aquifer, which would be needed if hydrological fluctuations change the spatial distribution of the contaminants.

Microbes always grow towards the substrate source, even against strong currents. To reduce the effects of steep gradients on the quantification of turnover rates, we have chosen very small and short sediment columns. However, while we observed no significant gradient in cell numbers in most experiments, a distinct gradient in cell density was obvious in experiment B, with the highest cell density in the bottom part of the column near the inlet port for toluene and oxygen. The fact that the oxygen concentrations hardly differed between the bottom, middle, and top parts of the columns in all experiments indicates that microbial activity was indeed mainly restricted to the bottom (inflow) part of the columns, once a stable community of attached bacteria had developed. Interestingly, the continuous release of new cells mainly produced at the column inlet into the pore-water and its subsequent attachment to

sediment particles downgradient, revealed a similar density of attached cells throughout the columns in most of the experiments.

4.4.2.3 Reactive-Transport Modeling

The combined measurement of attached and suspended cells enabled us to develop a quantitative model, which explicitly accounts for the release of new-grown cells from the sediments to the mobile aqueous phase by the dynamic growth-dependent detachment rate $r_{daughter}$. The reactive-transport simulations revealed that suspended cells were irrelevant for toluene degradation in the column experiments. Although it is well known that bacteria transported in porous media are important as seed banks in aquifers (Griebler et al. 2014) and play an important role in partitioning between the mobile aqueous phase and the sediment surface (e.g. Ginn et al., 2002; Tufenkji, 2007; Scheibe et al., 2011; Zhou et al. 2012), the finding that the majority of bacteria in aquifers is attached to the sediment matrix led to the situation that the biomass catalyzing the breakdown of organic contaminants is usually treated as immobile species in reactive-transport models, and the presence of bacteria suspended in the mobile aqueous phase is neglected (e.g., Barry et al., 2002; Schirmer et al., 2000; Prommer et al., 2006, 2009). Moreover, the majority of studies on the transport of microorganisms in porous media was conducted under non-growth conditions and aimed at improving the understanding of the physical processes (e.g. straining and filtration) that govern microbial transport in porous media, which are important for the fate and behavior of pathogens in groundwater. A few studies, investigating the effect of biological processes on microbial transport in porous media, indicated that microbial growth strongly affects the partitioning of bacteria between the aqueous phase and the sediment surface in addition to physical processes (e.g., Clement et al., 1997; Murphy et al., 1997; Yolcubal et al., 2002; Jordan et al., 2004; Eckert et al. 2015). In all of these studies an increase in the number of

suspended bacteria was observed after the addition of a growth substrate to the system.

While the results obtained in this study clearly highlight the flow velocity, the substrate concentration, and the electron-acceptor limitation as different drivers of microbial contaminant degradation in liquid batch and flow-through sediment systems, the data obtained from our lab experiments with single degrader strains should be translated to the field situation with caution. In situ, additional factors like food web interactions, grazing, or competition for resources as well as multiple limitations play an important role within natural microbial communities (Konopka 2000, Griebler & Lueders 2009; Griebler et al. 2014; Meckenstock et al. 2015). Numbers of attached bacteria as high as 10^8 to 10^9 cells mL^{-1} sediment as established in our column experiments are hardly observed in aquifers. Even at comparable high or even higher toluene concentrations, cell densities are typically 1-2 orders of magnitude lower (Winderl et al. 2008; Anneser et al. 2010). In our studies, the tested microbial community, obtained from natural aquifer sediments, degraded toluene at a slower pace than the specialized toluene-degrading strains *P. putida* F1 and *A. aromaticum* EbN1. This is most probably due to the fact that in natural communities, only a small fraction of the community is metabolically active while the majority is in an inactive resting state (Shade et al., 2012). Moreover, only a portion of the active cells in a community might be capable of utilizing petroleum hydrocarbons like toluene, which are toxic at elevated concentrations to many bacterial species (Bordel et al., 2007; Castillo and Ramos, 2007; Herzyk et al. 2013; Okpokwasili and Nweke, 2005; Vecht et al., 1988). Assuming that the toluene-degrading species have a low overall abundance at the beginning of the experiment, the onset of pronounced toluene degradation is delayed until these toluene degrading species reach sufficient cell numbers. We should never expect that degraders under *in situ* conditions reach the carbon assimilation efficiencies and maximum growth rates observed in the lab. Nonetheless, our results are an important step towards a better understanding of ecological

drivers of organic-contaminant biodegradation.

4.5 Conclusions

A recent review by Meckenstock and coworkers (2015) highlights that many common concepts regarding degradation of organic contaminants by microbes in aquifers need considerable revision. One important aspect is that our lab-based knowledge, which is mainly derived from batch experiments, is of limited use in understanding and predicting processes in a natural, heterogeneous, complex, open flow-through sediment system. Our study underlines that degradation and associated growth rates are insufficiently predicted using laboratory batch experiments, which can lead to overestimating anticipated in-situ biodegradation. Also, only flow-through sediment systems allow for an independent assessment of attached and suspended cells. As was found, attached bacteria are responsible for the majority of the observed biodegradation. While attached cells were mainly responsible for toluene degradation, the release of cells into the pore water causes permanent inoculation of the aquifer downstream. In consequence, the ratio of sediment to water is crucial when setting up laboratory experiments representative of field conditions. Finally, mass transfer limitation is an important process controlling toluene biodegradation that cannot be reproduced with laboratory batch experiments. We are convinced that mathematical models that simulate biodegradation and bacterial growth in aquifers will greatly be improved in their accuracy when they are calibrated by data derived from flow-through sediment microcosms and/or directly from field studies at appropriate spatial and temporal resolution.

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5. Dynamics of aerobic toluene degraders in flow-through systems under growth and starvation conditions

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Abstract

The microbially mediated reactions, responsible for field-scale natural attenuation of organic pollutants, are governed by the concurrent presence of a degrading microbial community, suitable energy and carbon sources, electron acceptors as wells as nutrients. The temporal lack of one of these essential components for microbial activity, arising from transient environmental conditions, might potentially impair in-situ biodegradation. This study presents results of one-dimensional (1-D) flow-through column experiments aimed at ascertaining the effects of starvation periods on the aerobic degradation of toluene by *Pseudomonas putida* F1. During the course of the experiments concentrations of attached and mobile bacteria, as well as toluene and oxygen were monitored. Results from a fitted reactive-transport model, along with the observed profiles, show the ability of attached cells to survive starvation periods of up to four months and suggest a highly dynamic exchange between attached and mobile cells under growth conditions and negligible cell detachment under starvation conditions. Upon substrate reinstatement, toluene was readily degraded without a significant lag period, even after a starvation period of 130 days. Our experimental and modeling results strongly suggest that the in-situ biodegradation potential at contaminated field sites is not hampered by periods of starvation.

5.1 Introduction

Natural and/or enhanced bioremediation has been identified as a mildly invasive and cost effective remediation strategy for aquifers contaminated with mono-aromatic BTEX-hydrocarbons. The microbially mediated reactions, responsible for field-scale natural attenuation of organic pollutants, are governed by the concentration distribution and subsequent availability of suitable, energy yielding, electron acceptors[1]. Strong evidence exists that degradation reactions occur where mixing of electron donor and acceptor takes place[2-4].

Besides their intensively documented growth behavior, microbial populations in the subsurface undergo a variety of non-growth processes (i.e. transport, as well as, adaptive behaviors to cope with transient environmental conditions) that have the potential to affect contaminant degradation in the subsurface.

While microorganisms attached to the sediments have been found to comprise the largest portion of microbial biomass in the subsurface[5-7], studies on bioaugmentation and the spread of pathogens have shown that they partition between the sediments and the mobile aqueous phase and that they are transported in groundwater[8-14]. Only a limited number of studies, however, have considered the interdependent effects of microbial growth and transport[9, 15-18]. These studies observed that microbial growth, in addition to physical processes, strongly affects the partitioning of cells between the aqueous phase and the sediment surface. An increase in the number of suspended bacteria was observed after the addition of a growth substrate into the system[15-18]. However, the aforementioned studies did not monitor the response of biomass attached to the sediments throughout the experiments. Harvey et al.[19] observed that this increase in pore-water bacteria did not result in a reduction of attached cells. Similar observations of lower ratios of attached to suspended cells in nutrient rich zones have also been observed in laboratory[20] and field[21] studies.

Microbial populations are known to adapt to fluctuating environmental conditions[22]. In response to fluctuations, organisms may metabolically 'switch' into a so-called anabiotic state, referred to as dormancy[23]. Dormant cells do not exhibit behavioral patterns of living or dead cells, they merely enter a state of lowered metabolic activity in which they do not undergo cell division. Upon reinstatement of favorable conditions, they return to an 'alive' behavioral state[23], that is, the phenotypic shift from dormant to active is reversible[22]. Non-growth functions are maintained by consuming endogenous reserves[24-26].

Several studies have investigated the effects of unfavorable growth conditions on biodegradation[23, 25, 27-32]. Observations and modeling results show the resilience of degraders to stress and their ability to resume full metabolic functioning upon reinstatement of favorable conditions. Métris et al.[25] observed a degradation lag phase of a quarter of an hour after a three-day starvation period in a bioreactor system, exposed to changes in inlet concentrations of toluene and xylene. Matínez-Lavanchy et al.[32] observed no significant decline in biomass concentration during a period of oxygen deprivation of 12 h, and observed increased growth of *Pseudomonas putida* and rapid toluene degradation upon the reinjection of an oxygen pulse into their experimental batch system. In batch experiments, Kaprelyants and Kell[31] observed that cells subjected to a starvation period of 75 days readily grew after 16 – 18 h of incubation in a system with a fresh substrate (lactate) containing medium.

In standard reactive-transport models, the degrading biota are assumed to be completely immobile, and their ability to adapt to unfavorable environmental conditions is not considered. The conventional dual-Monod modeling approach solely depends on the instantaneous concentrations of reaction counterparts and does not account for degradation delays due to metabolic adaptations as well as concentration fluctuations[33-35]. A few studies have attempted to incorporate the adaptive dormant behavior that microbial communities exhibit under unfavorable conditions[27-29]. Bradford et al.[36] suggest that there is currently "a need to study and predict microbial transport and survival throughout a range of environmentally relevant conditions in the laboratory scale".

In the present study, we present the results of a meticulously monitored bench-scale one-dimensional (1-D) flow-through system, studying the effects of starvation periods on the degradation of toluene by the aerobic strain *Pseudomonas putida* F1. The build-up of cells in the sediment, the breakthrough of mobile cells at the outflow, as well as the consumption of toluene (electron donor) and oxygen were monitored. The attached degraders were subjected

to starvation periods of up to 130 days in duration. Unlike earlier studies, presented above, the growth of degrading cells and contaminant degradation were monitored under flow-through conditions in natural sediments, a setup closer to natural conditions than well-mixed batch systems. We fitted a reactive-transport model - which takes the aforementioned patterns of microbial behavior into account - to the experimental data in order to quantify the response of the aerobic degraders to phases of toluene starvation of up to four months. The general question addressed by this study is to which extent biodegradation of contaminants is hampered by temporarily unfavorable conditions for the degrading microbial community, as expected at contaminated sites.

5.2 Experimental Section

5.2.1 Experimental Setup

We performed experiments with the aerobic toluene degrader *Pseudomonas putida* F1 in parallel small, cylindrical flow-through columns (length: 1.60 cm; diameter: 1.34 cm). Each column was packed with natural sediment of the middle sand fraction (200 – 630 µm). The measured effective porosity (n_e) in the columns was 30%. Experiments were performed at a seepage velocity of 1.8 m d^{-1}. A multi-channel peristaltic pump (Ismatech, Wertheim, Germany) was used to achieve the desired flow rate. Flow in each of the columns, single-column illustrated in Figure 5.1, was from the bottom to the top.

The columns were inoculated with 10^4 to 10^5 cells mL^{-1}, a bacterial concentration encountered in natural groundwater. A bicarbonate-buffered freshwater medium was injected as mineral medium[37]. After inoculation, toluene was supplied at a concentration of approximately 6 mg L^{-1}. To prevent microbial growth in the inlet tubing, media containing toluene and oxygen (electron acceptor) were injected from separate reservoirs. The two solutions were mixed shortly before the column inlet via T-connectors.

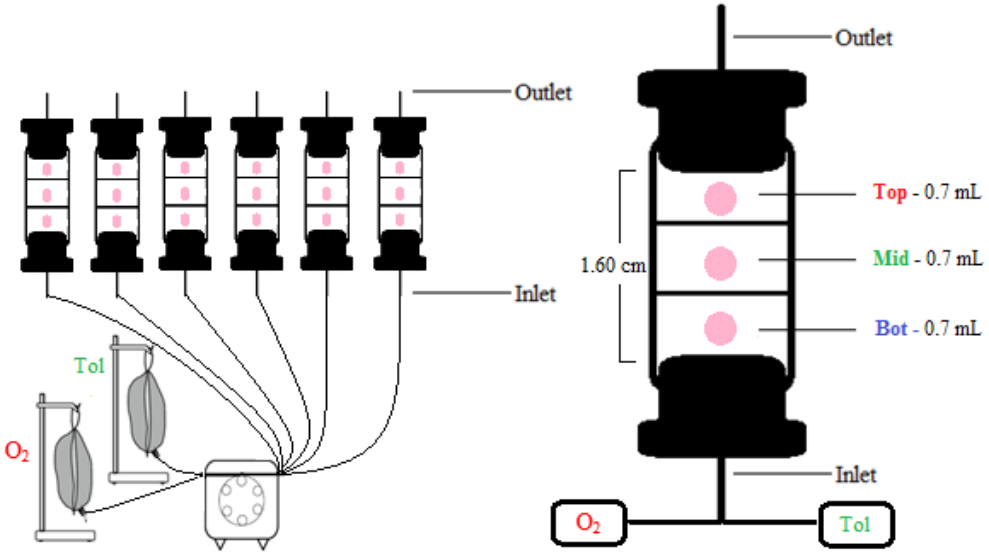

Figure 5.1: Schematic illustration of 1-D glass column used for all flow-through experiments, the columns were each partitioned into three sections of equal dimensions: bottom, middle and top. Each section contained oxygen sensitive strips (pink dots) for the monitoring of O_2 concentrations in the domain.

Water samples were collected at the column inlet and outlet to monitor toluene degradation and transport of bacteria. Toluene concentrations were measured by GC-MS analysis[6], and cell numbers were quantified via flow cytometry[21]. Each experiment was run with a multitude of columns in parallel. At specified time points, column duplicates were dismantled to measure the bacterial abundance, activity (ATP)[38] and mean volume of cells in the sediment. The sediment was partitioned into three layers (bottom, middle, and top), and each sediment layer was separately analyzed for attached cells. Oxygen concentrations in the columns were measured non-invasively via an optode technique[39] (Fibox, PreSens GmbH, Regensburg, Germany) – implementing oxygen-sensitive foils glued to the inner column wall at three different locations (bottom, middle and top).

We used the experimental setup summarized above to run experiments under three different scenarios regarding availability of the growth substrate : (1) an experiment in which the growth substrate, toluene, and the electron acceptor, oxygen, were continuously supplied

(continuous injection); (2) an experiment where the injection of toluene was halted twice for periods of 8 and 21 days, respectively (short-term starvation) and (3) an experiment similar to the second one but with a much longer single toluene-starvation period of 130 days (long-term starvation). During the starvation periods, oxygenated mineral medium was continuously injected into the columns.

5.2.2 Conceptual Model

Experimental results from flow-through experiments (presented herein), involving the aerobic degradation of toluene in porous media by *Pseudomonas putida* F1, showed evidence of microbial transport, attachment, increased cell mobility under growth conditions and the tolerance of attached *P. putida F1* cells towards a four-month starvation period. In order to capture the observed trends, we considered both mobile and immobile microbial cells and incorporated the transport of bacteria in the domain in our model, simulating a scenario in which bacteria are not evenly distributed across all grain surfaces. The model contains rate laws for the attachment of mobile cells to particle surfaces and the detachment of immobile cells into the aqueous phase. This aspect allowed for a more dynamic microbial population. Detachment of cells specifically due to growth was also an important concept incorporated in the model. The ability of bacteria to enter a dormant state in the absence of growth substrate, and resume growth during substrate-rich conditions was introduced via the implementation of an active and an inactive fraction of attached bacteria. The following section presents the specific equations used for each of the simulated scenarios.

5.2.3 Governing Equations

The one-dimensional reactive transport model developed to simulate the column experiments considers three mobile species, namely toluene, oxygen and suspended bacteria, and two immobile

species, namely active attached bacteria and inactive attached bacteria. One-dimensional transport of all mobile species is described by the advection-dispersion-reaction equation:

$$\frac{\partial c}{\partial t} = D \cdot \frac{\partial^2 c}{\partial X^2} - v \cdot \frac{\partial c}{\partial X} + r \tag{1}$$

where c is the aqueous concentration of the relevant mobile species [mg L^{-1}], D is the dispersion coefficient [m^2 d^{-1}], v is the seepage velocity [m d^{-1}], and r represents a reactive source/sink term [mg L^{-1} d^{-1}].

Microbial growth is simulated by dual-Monod kinetics, describing the dependence of microbial growth on the simultaneous presence of toluene (carbon source and electron donor) and oxygen (electron acceptor). The microbial growth rate r_{growth} is then given by:

$$r_{growth} = \mu_{max} \cdot \frac{c_{tol}}{c_{tol} + K_{tol}} \cdot \frac{c_{O2}}{c_{O2} + K_{O2}} \cdot c_{Bio} \tag{2}$$

where μ_{max} [d^{-1}] is the maximum specific growth rate of the bacteria, c_{tol} [mg L^{-1}] is the toluene concentration and c_{O2} [mg L^{-1}] is the oxygen concentration, K_{tol} [mg L^{-1}] and K_{O2} [mg L^{-1}] are the Monod coefficients for toluene and oxygen, respectively, and c_{Bio} is the concentration of biomass. In the model, growth of attached (immobile), c_{Bio}^{im} [cells L_{sed}^{-1}], as well as suspended (mobile) biomass, c_{Bio}^{mob} [cells L^{-1}], is considered.

The experimental results indicated that the number of attached bacteria leveled off at a maximum density, i.e., that there was a maximum carrying capacity of the system for attached bacteria c_{Bio}^{max} [cells L_{sed}^{-1}]. It has been observed that some bacterial strains exhibit less attachment to sediment surfaces when the concentration of cells in the sediment is high, a phenomenon known as blocking[8]. The concept and processes behind “blocking”, resulting in a maximum attachment capacity in sediments, are dealt with in more detail in other works[40-42]. Once c_{Bio}^{max} was reached, in the experiment, attached bacteria continued to grow. All new-grown cells, however, were released to the aqueous phase and finally left the column. This release of new-grown cells from the sediment surface to the mobile aqueous phase, which was observed in

previous studies[13, 14, 40, 41], is also known as cell-division mediated transport[34] and was implemented in the model by the dynamic, growth-depended detachment rate, $r_{daughter}$ [cells L_{sed}^{-1} d^{-1}]:

$$r_{daughter} = r_{growth}^{\mathrm{im}} \cdot \left(\frac{c_{Bio}^{\mathrm{im}}}{c_{Bio}^{max}} \right) \tag{3}$$

in which r_{growth}^{im} [cells L_{sed}^{-1} d^{-1}] is the growth rate of the attached bacteria. The expression considers all attached cells whether they are actively degrading or dormant – more details on these distinctions below.

In order to account for adhesion of suspended bacteria to particle surfaces, as well as the possible mobilization of attached bacteria due to non-growth processes, first-order rate expressions for attachment r_{att} [cells L^{-1} d^{-1}] and detachment r_{det} [cells L_{sed}^{-1} d^{-1}] were incorporated into the model:

$$r_{att} = k_{att} \cdot c_{Bio}^{mob} \cdot \left(1 - \frac{c_{Bio}^{\mathrm{im}}}{c_{Bio}^{max}} \right) \tag{4}$$

$$r_{det} = k_{det} \cdot c_{Bio}^{\mathrm{im}} \tag{5}$$

in which k_{att} [d^{-1}] and k_{det} [d^{-1}] are the first-order rate coefficients for attachment and detachment, respectively. The term $\left(1 - c_{Bio}^{\mathrm{im}} / c_{Bio}^{max}\right)$ was introduced to account for the carrying capacity of the system for attached cells[42]. When the density of attached cells approaches c_{Bio}^{max}, the attachment rate approaches zero.

In order to implement microbial dormancy, we consider active, $c_{Bio}^{\mathrm{im}\,ac}$, and inactive, $c_{Bio}^{\mathrm{im/in}}$, attached bacteria. Deactivation of active attached bacteria under unfavorable conditions, i.e., in the absence of toluene as a carbon and energy source, and reactivation of inactive bacteria, once conditions become favorable for microbial growth again, are implemented as pseudo first-order kinetic processes[27] in the model:

$$r_{deac} = (1 - \theta) \cdot k_{deac} \cdot c_{Bio}^{\mathrm{im}/ac} \tag{6}$$

$$r_{reac} = \theta \cdot k_{reac} \cdot c_{Bio}^{\mathrm{im/in}} \tag{7}$$

where k_{deac} [d^{-1}] and k_{reac} [d^{-1}] are the first-order coefficients for deactivation and reactivation, respectively, and θ [-] is a switch function describing the transition between the active and inactive state of attached bacteria. The switch function is modified from Stolpovsky et al.[27]:

$$\theta = \frac{1}{\exp\cdot\left(\frac{c_{tol}^{thresh} - c_{tol}}{0.1\cdot c_{tol}^{thresh}}\right) + 1} \tag{8}$$

in which c_{tol}^{tresh} [mg L^{-1}] is the threshold toluene concentration for microbial growth. The switch-function θ determines whether conditions are favorable or unfavorable for bacterial growth. It can take on values between 0, denoting maximally unfavorable conditions, and 1, marking optimal conditions.

Combining all expressions gives rise to a model system in which microbes grow when both substrate and electron acceptor are present. Their growth may result in detachment which implies the presence of a mobile population of biomass. Like toluene and oxygen, the mobile biomass undergoes transport but it can adhere to particle surfaces along its flow path. Both attached and suspended bacteria are able to grow, consuming toluene and oxygen. However, due to the short mean residence time, growth of the mobile bacteria was found to be insignificant. The governing equations are summarized as follows:

$$\frac{dc_{tol}}{dt} = D\cdot\frac{d^2 c_{tol}}{dx^2} - v\cdot\frac{dc_{tol}}{dx} - \frac{1}{n_e}\cdot r_{growth}^{im/ac}\cdot\frac{1}{Y} - r_{growth}^{mob}\cdot\frac{1}{Y} \tag{9}$$

$$\frac{dc_{ox}}{dt} = D\cdot\frac{d^2 c_{ox}}{dx^2} - v\cdot\frac{dc_{ox}}{dx} - \frac{1}{n_e}\cdot r_{growth}^{im/ac}\cdot\frac{1}{Y} - f_{ox}\cdot r_{growth}^{mob}\cdot\frac{1}{Y} \tag{10}$$

$$\frac{dc_{Bio}^{im/ac}}{dt} = r_{growth}^{im/ac} - r_{daughter} + n_e\cdot r_{att} - r_{det} - r_{deac} + r_{reac} \tag{11}$$

$$\frac{dc_{Bio}^{mob}}{dt} = D\cdot\frac{d^2 c_{Bio}^{mob}}{dx^2} - v\cdot\frac{dc_{Bio}^{mob}}{dx} + r_{growth}^{mob} + \frac{1}{n_e}\cdot r_{daughter} - r_{att} + \frac{1}{n_e}\cdot r_{det} \tag{12}$$

$$\frac{dc_{Bio}^{\mathrm{im/in}}}{dt} = r_{deac} - r_{reac} \tag{13}$$

The rates of change of both toluene and oxygen concentrations depend on their consumption by the bacteria, which is proportional to microbial growth. The yield coefficient Y [cells mg_{sub}^{-1}] describes the amount of cells produced per mass of toluene degraded in the growth reactions and the stoichiometric coefficient f_{ox} [mg_{ox}/mg_{tol}] relates the mass of oxygen consumed to the mass of toluene degraded. The effective porosity n_e is used as a conversion factor for unit agreement between the rates depending on the concentrations of attached bacteria and those of mobile bacteria.

5.2.4 Numerical Methods

The coupled system of five nonlinear partial differential equations was discretized in space by the cell-centered Finite Volume method (Δx = 0.1 mm), applying upwind differentiation of the advective term. The global implicit approach was adopted for the coupling of transport and reaction terms. The resulting system of nonlinear algebraic equations was linearized by the Newton-Raphson method. The code is written in MATLAB.

5.3 Results & Discussion

5.3.1 Experimental Results

Figure 5.2 depicts the experimental data along with the corresponding simulation results. The figure includes the results for the continuous injection experiment as well as the short- and long-term starvation experiments.

In the flow-through experiment with continuous toluene injection, steady-state conditions were reached after 1.5 to 2 days of experiment run-time – data points are illustrated in the left column of Figure 5.2. The marked drop in toluene concentrations is strongly mirrored by the

oxygen consumption in the system. The temporal evolution of dissolved oxygen concentrations measured in the bottom, middle and top part of the column are almost identical, indicating that oxygen consumption, and hence microbial activity, was restricted to the bottom (inflow) section of the column. The bacterial growth curves for the attached cells follow the expected exponential growth trends, leveling out at a maximum of about 2×10^8 cells per mL of sediment (mL_{sed}) for the bottom section of the domain and about 1×10^8 cells mL_{sed}^{-1} for the middle and top sections. Once the maximum number of attached cells was reached, the amount of washed out cells was constant throughout the remainder of the experiment. The continuous wash-out of about 2×10^6 cells mL^{-1} indicated that attached cells, even though their density in the sediment stayed constant, were still replicating, releasing the daughter cells to the mobile aqueous phase.

Over the course of the experiment, 76 % of the new-grown cells were flushed out of the column. To estimate the distribution of bacteria between water and sediment, we compared the amount of cells attached to the sediment in the top part of the column (close to the outlet) and the amount of cells in the outflowing water. Even though the majority of new-grown cells were flushed out of the column over time, the vast majority of cells per volume of porous media (99%) was found to be attached to the sediment surface.

The data points in the second and third columns of Figure 5.2 show the results of the short--term (middle column) and long-term starvation experiment (right column), respectively. In the short-term starvation experiment, the oxygen concentration in the column outlet rapidly drops to effectively zero during the periods of toluene injection and initially rebounds to about 8 mg L^{-1} when toluene injection is stopped, but decreases to a level of about 6 mg L^{-1} for the remainder of the starvation periods. The maximum toluene concentrations detected after the resumption of toluene injection at the end of the two starvation phases was less than half of the injected concentration, and the concentration values declined to the level observed before

the onset of starvation within hours. This indicates that the attached bacteria were able to degrade toluene right away, even after the absence of toluene for a period of three weeks during the second starvation phase.

The initial growth phase in the short-term starvation experiment (from day 1 to 4) resulted in the highest numbers of attached cells in the three experiments, with cell densities of about 1×10^{9} cells mL_{sed}^{-1} in the bottom (inflow section) of the domain. While the number of attached cells decreased to a level similar to the one observed in the other two experiments during the first starvation phase, the number of attached cells stayed constant during the second starvation phase. The breakthrough of cells in the aqueous phase showed evidence of periodic variations in conjunction with injection switches. The number of out-washed cells was highest during growth periods, reaching cell concentrations in the outflowing water similar to the ones detected in the continuous-injection experiment, and precipitously declined by around one order of magnitude during the starvation periods, followed by a swift increase to the previous level upon reinstatement of substrate injection.

The right column of Figure 5.2 shows the results for the long-term starvation experiment with a single starvation phase of four months. Trends are similar to those of the short-term starvation experiment. The number of attached cells increased by a factor of 15 during the first three days and stayed constant during the 130-day starvation period in the bottom (inflow) part of the column, while it decreased by approximately 30 and 70% in the mid and top part, respectively. The maximum observed density of attached cells was approximately 7.8×10^{8} cells mL_{sed}^{-1} and thus about two times larger than in the continuous-injection experiment. Similar to the short-term starvation experiment, we observed a constant out-wash of about 2×10^{5} cells mL^{-1} during the starvation phase. The detection of similar cell densities at the column inlet, however, indicated that these cells originated from the injection media. The bottom right sub-plot of Figure 5.2 includes measurements of cells in the inlet, indicated by

blue diamonds. Markedly, the number of cells in the inlet is almost identical to the number of cells in the outlet during long-term starvation. These results indicate that no substantial number of attached *P. putida* F1 cells was lost due to detachment or biomass decay within four months of toluene starvation and that the attached cells were able to regain their full toluene-degradation potential within less than a quarter of a day after toluene injection was resumed on day 133.

In addition to the measurement of cell numbers, Figure 5.3 shows ATP concentrations and the bio-volume of the attached cells recorded during the four month starvation period. The ATP measurements suggest a reduction in cell activity by 70 to 90% after the injection of toluene was ceased, and an increase in cell activity after toluene injection was resumed on day 133. The cell volume decreased by a factor of four during the four months of toluene starvation. We hypothesize that the reduction in cell volume is at least partially associated with a loss of biomass, which may be utilized to gain energy for cell maintenance. While the oxygen measurements suggest a slight, but consistent, oxygen consumption throughout the two starvation phases of the short-term starvation experiment, the detected oxygen consumption during the long-term starvation experiment was not consistent.

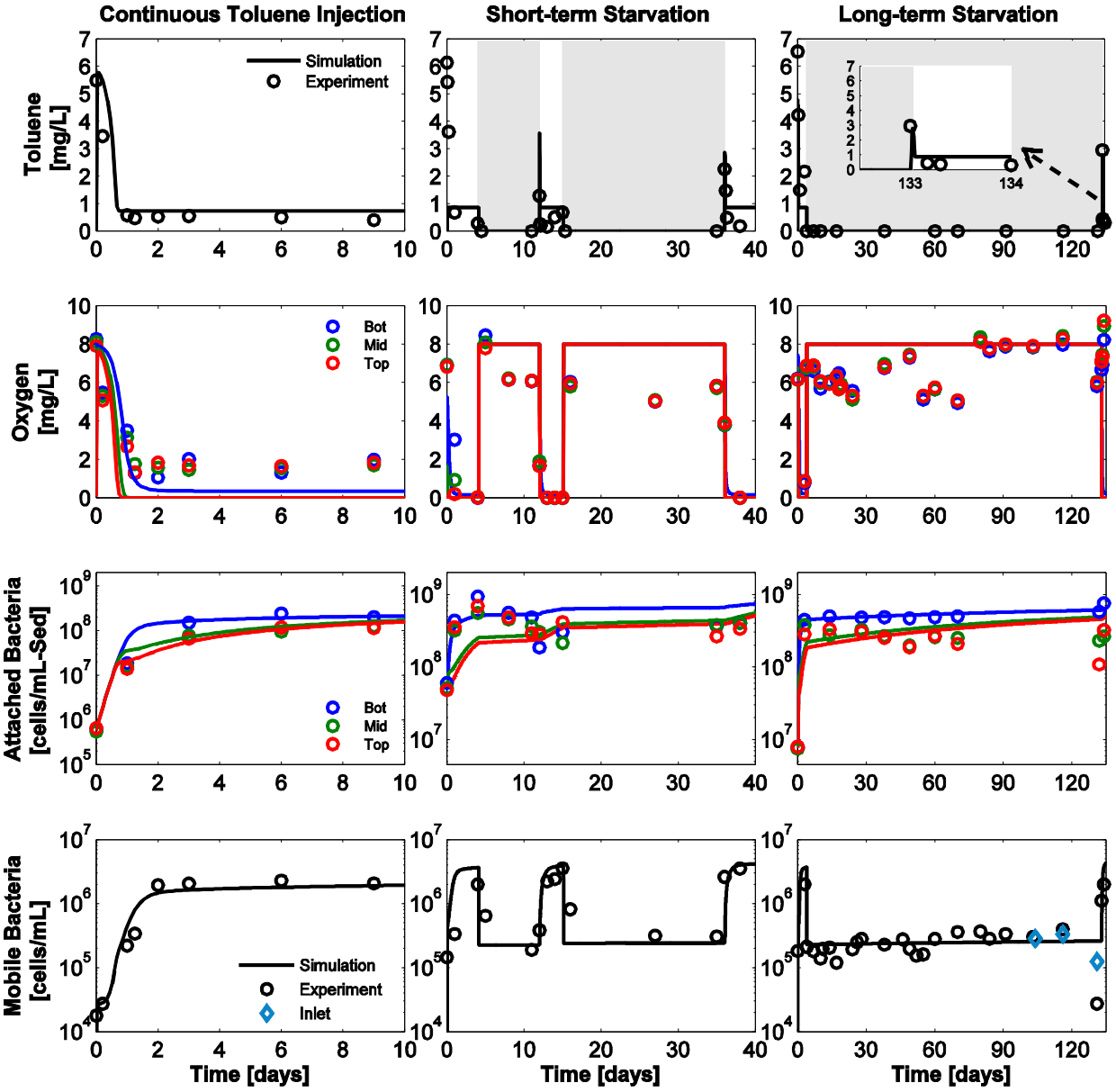

Figure 5.2: Experimental and simulation results for all three experimental setups (from left to right): continuous toluene injections, short-term (8 days and 21 days) starvation and long-term (130 days) starvation. The grey rectangles in the toluene plots in the upper row indicate the toluene-starvation periods.

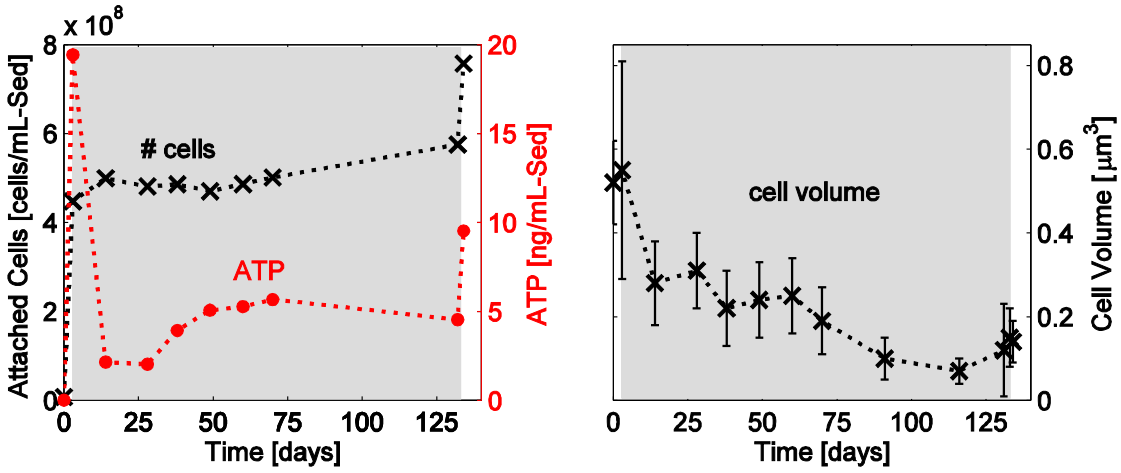

Figure 5.3: Concentrations of attached cells and ATP in the bottom (inflow) part of the column for the long-term starvation experiment (left subplot) and the temporal change of the cell bio-volume of attached cells (right subplot). The area shaded in grey indicates the toluene-starvation periods of 130 days.

5.3.2 Simulation Results

Figure 5.2 contains a comparison of the experimental data to the simulation results of all three scenarios. The parameters of the reactive-transport model, applied to simulate the column experiments, are summarized in Table 5.3. A discussion and justification of individual parameter values is given in the supporting information S1. The parameters in bold are fitted, whereas all other parameters were determined prior to the reactive-transport simulations in batch and column experiments, or were taken from the literature. Except for slight variations in the yield and the observed maximum amount of attached bacteria, the three experiments could be adequately simulated using the same parameters. This effectively yielded a single model that satisfactorily explained all experiments.

Table 5.3: Model parameters which best simulated the growth curves and concentration profiles observed in the experimental data. Parameters which were fitted to the data of the column experiments are given in bold. All remaining parameters were determined from batch and column experiments prior to the reactive-transport simulations or were taken from the literature.

Parameters		Continuous Injection	Short-term Starvation	Long-term Starvation	
μ_{max}	[d^{-1}]	4.5	4.5	4.5	batch
Y	[cells mg_{tol}^{-1}]	3.9×10^{8}	8.5×10^{8}	8.5×10^{8}	column[a]
K_{Tol}	[mg L^{-1}]	0.1	0.1	0.1	batch
K_{O_2}[43]	[mg L^{-1}]	0.32	0.32	0.32	literature
f_{O_2}	[mg_{ox} mg_{tol}^{-1}]	1.5	1.5	1.5	column[b]
C_{Bio}^{max}	[cells mL-PM^{-1}]	2.5×10^{8}	9.4×10^{8}	7.6×10^{8}	column[c]
$\boldsymbol{C_{Tol}^{thresh}}$	**[mg L^{-1}]**	**-**	**1×10^{-3}**	**1×10^{-3}**	**fitted**
$\boldsymbol{k_{att}}$	**[d^{-1}]**	**50**	**50**	**50**	**fitted**
$\boldsymbol{k_{det}}$	**[d^{-1}]**	**0**	**0**	**0**	**fitted**
$\boldsymbol{k_{deac}}$	**[d^{-1}]**	**-**	**2**	**2**	**fitted**
$\boldsymbol{k_{reac}}$	**[d^{-1}]**	**-**	**2**	**2**	**fitted**

[a] mass balance (new-grown cells/toluene degraded); [b] mass balance (oxygen consumed/toluene degraded); [c] max. observed attached cell density

The simulation of the continuous-injection experiment yielded results in which the initial consumption of oxygen and toluene resulted in growth curves that closely fit measured attached cell concentrations during the initial growth phase. The parameterization of cell-division mediated transport, which simulated the expulsion of new-grown (daughter) cells from the sediment surface to the mobile aqueous phase - once the carrying capacity of the system for attached cells was reached - resulted in a simulated breakthrough of bacteria at the outflow similar to the one observed. The measured oxygen concentrations plateaued at 1-2 mg L^{-1} under stable conditions, while toluene could still be detected at the outflow. Oxygen profiles were not accurately fitted to the measured profiles after the initial growth phase. Because the reaction is highly energy yielding, it seems unlikely that oxygen values would all stabilize at such high levels. Since the simulated oxygen profiles during the dynamic toluene injection experiments follow similar trends as the data, the measured values for the continuous injection

experiment were treated with caution and the lack of a proper fit was attributed to a measurement bias.

Results for the short-term starvation (middle column in Figure 5.2) simulation yielded oxygen profiles that plateaued at values higher (about 2 mg L^{-1}) than the observed ones. This might indicate that other pathways of oxygen consumption were at play in the columns. A possible explanation for this finding could be endogenous respiration of *P. putida* F1, the oxidation of cell reserves in the absence of external substrates[26, 34]. The consumption of oxygen in the absence of external energy sources has been observed in previous flow-through experiments[18, 44]. The results of the oxygen measurements in the long-term starvation experiment were not as conclusive as for the short-term starvation experiments. While most of the measurements before day 70 of the experiment where about 2 mg L^{-1} lower than expected, the measured concentrations for later times were similar to the inflow concentration.

While the model reproduced the concentrations of attached cells well for the continuous injection experiment, experimental and simulation results showed some discrepancies for the two starvation experiments. The model was not able to accurately reproduce the increased initial growth spurt and subsequent decrease of cells attached to the sediment during the first starvation phase in the short-term starvation experiment. It was also not able to model the concentration of attached cells in the middle and bottom part of the column towards the end of the long-term starvation experiment.

The simulations of both the long-term (right column in Figure 5.2) and short-term starvation experiments were able to capture the abrupt drop in cell breakthrough during toluene-starvation conditions, pointing out the importance of considering peak cell detachment under growth conditions, which arises from the continuous replication of attached cells and the release of new-grown cells to the aqueous phase once the carrying capacity of the system for attached cells is reached. Cell detachment during the toluene-starvation phases was found to be

negligible and the concentration of cells in the outflow during these periods was similar to the concentrations in the inflow.

During the periods of toluene-starvation the bacteria enter a resting (inactive) state in which they survive by consuming cell reserves, this causes cells to decrease in size and also leads to a reallocation of resources which induces a change in cell elemental composition[24]. It is usually the case that biomass exposed to a new substrate needs to first initiate enzyme production and metabolic pathways and may need to repair cell damage, from the time of dormancy, before substrate consumption can begin, which is also known as metabolic lag[30]. The first-order activation rate coefficient determines the speed at which the bacteria are able to switch between their active and inactive states. The fitted activation coefficient of 2 d^{-1} accurately modeled the awakening period evident from the close fit of the peaks in toluene concentration at the outlet upon reinjection.

The overall model fit and ability to reproduce the experimental system is good and its performance is encouraging. The array of variables considered and measured depicts a complex system. Discrepancies between model fit and experimental results are likely due to the transient nature of natural processes and the fact that certain conditions can shift an organisms' response to its environment. Such variability can lead to unexpected patterns, such as the initial growth spurt in the short-term starvation experiment – not captured by the simulation. Information on processes governing transience of model parameters was, however, not available therefore a simplified approach was implemented.

5.3.3 Implications for in situ Biodegradation Processes

In column experiments performed with the aerobic toluene degrader *P. putida* F1, we found that cells attached to the sediment matrix survived a toluene-starvation period of four months. There was no significant loss in numbers of attached cells and the attached cells regained their

full biodegradation potential within a quarter of a day after toluene reinjection into the system. Furthermore, the model-based analysis of the experimental data showed the highly dynamic nature of microbial detachment. While detachment of *P. putida F1* cells from the sediments was found to be negligible under toluene-starvation conditions, most of the new-grown cells were released to the mobile water phase under growth conditions.

Our experimental and modeling results strongly suggest that in-situ biodegradation at contaminated field sites is not hampered by periods of unfavorable conditions. The temporal lack of electron donors or acceptors, which might arise from changes in contaminant plume position or variations in groundwater flow, will most likely not lead to a breakdown of the degradation capability of the microbial community if it has been established by exposure to the contaminants in the past, at least not under aerobic conditions.

So far, experiments were performed for toluene degradation under aerobic conditions. At many contaminated field sites, however, oxygen is only present at trace amounts, and contaminant degradation is governed by anaerobic processes[45, 46]. Towards this end, it is not clear if the results from the aerobic system can be directly transferred to biodegradation under anaerobic (e.g., sulfate reducing) conditions. Anaerobic conditions are energetically considerably less favorable than aerobic biodegradation - microbial growth and hence contaminant degradation rates are much smaller[47]. Similar arguments may hold for aerobic degradation of less energy-rich contaminants.

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6. General Discussion

While the key contaminant degraders and redox gradients at the site in Düsseldorf, Flingern have been identified in previous studies already (Anneser et al., 2008, 2010, Winderl et al., 2008, Pilloni et al., 2011, 2012, Larentis et al., 2013), it was so far unknown which factors are mainly responsible for shaping the microbial community composition in organically contaminated aquifers. In this thesis we evaluated for the first time individual selective forces, like the degree of contamination, sediment properties and water chemistry. Our results indicated that the organic contamination in highly polluted areas superimposed all other factors, indicated by the community composition on different minerals, i.e. mica and translucent quartz. In areas strongly contaminated with BTEX or PAHs, the community composition on different types of minerals showed greater similarities than in uncontaminated areas. T-RFLP analysis revealed that OTUs characteristic of certain BTEX degrading strains, which were previously identified by Winderl et al., 2008, Anneser et al., 2010, Pilloni et al., 2011, 2012 and Larentis et al., 2013, were only present in areas with high BTEX contamination, while lesser contaminated and uncontaminated areas exhibited different OTUs which were absent in contaminated areas. Thus we conclude that the petroleum hydrocarbon contamination shaped the bacterial community by functioning as a carbon and energy source for the degraders while being toxic to others (Sikkema et al., 1995, Isken & de Bont, 1998).

Since there is no possibility to study microbial growth and degradation behavior directly in the field, and also because field conditions are very complex due to a multitude of factors being involved, a simpler approach is needed to study the processes of interest in the lab. Batch cultures have long been a method of choice, but since these systems aren't very suitable for studying processes in aquifers, it is advisable to design experiments based on small- and medium-scale flow-through microcosms. Two very prominent examples of such setups are

sediment-filled column systems and 2-dimensional flow-through microcosms, both of which resemble the conditions in the field much better than batch systems as they take a variety of factors into account such as: a solid matrix for cell attachment, flow velocity, and advective transport of electron donors and acceptors, nutrients, and microorganisms. Unfortunately and unlike the 2-dimensional flow-through microcosms, the column system setup applied in this thesis cannot take transverse dispersion into account due to the columns being one-dimensional only. On the other hand, many parallel run columns allow for repeated analysis of the sediment during an experiment by sacrificing replicate columns.

Our study revealed the following main differences between perfectly mixed and enclosed batch systems and one-dimensional flow-through column systems: while the maximum specific growth rates of *Pseudomonas putida* F1 were comparable in both systems, substrate depletion stopped growth in batch systems. In the sediment-filled columns, on the other hand, cells grow attached until reaching the carrying capacity on the sediment, and after that they won't cease growth but instead release newly grown cells into the sediment pore water. Bacteria seem to have different preferences in growing attached or suspended. It seems that the denitrifying strain *Aromatoleum aromaticum* EbN1 prefers to grow attached to the sediment, as it has exhibited a higher growth rate in the column setup than in batch. Both strains showed no measurable growth in the batch systems with the lowest toluene concentration, indicating that the substrate carbon was used for energy production and maintenance instead (Egli, 2010). In the column systems *P. putida* F1 was fastest in reaching its maximum specific growth rate, followed by *A. aromaticum* EbN1, and finally by the natural community, likely because only a small fraction of this community consisted of aerobic toluene degraders (Bordel et al., 2007; Castillo and Ramos, 2007; Herzyk et al. 2013; Okpokwasili and Nweke, 2005; Vecht et al., 1988), and also because only a small portion of the community is metabolically active in aquifers (Shade et al., 2012). Total toluene mass

degraded increased with both increasing flow velocity and inflow concentration. However, both toluene removal efficiency and maximum specific growth rate decreased with increasing flow rate, hinting at mass transfer limitations due to the time of advection being inversely proportional to the flow velocity. Fitting to this, the toluene removal efficiency declined as well with increasing toluene concentration in the inflow. We attribute this to the fact that when the substrate concentration exceeds the half-saturation constant of K_{Tol} considerably, substrate turnover will increase only marginally due to an overabundance of the substrate, so the removal efficiency in percent decreases. Yield was estimated via two distinct approaches, one was via the f_{ox}-value (oxygen consumed vs toluene degraded), and the other was via the newly formed cells produced by toluene degradation. It was found that our two methods of yield estimation were comparable, yielding carbon assimilation efficiencies of 0.4-0.7. Strong evidence was found that attached cells are better at converting carbon into biomass than suspended cells. 98-99% of cells were found to be attached to the sediment at any time, which seems to be the norm in aquifers according to other studies (Alfreider et al., 1997; Griebler et al., 2002; Lehman et al., 2001a & 2001b). However, a considerable number of cells were washed out over the course of the experiments, referred to as cell-division mediated transport (Murphy and Ginn, 2000). Our results show that growth of attached cells plateaued after reaching the system's carrying capacity (Zhou et al. 2012), and newly grown cells were then released into the medium. The highest ratio of attached to suspended cells was found in the experiment with the lowest substrate concentration, a finding that has also been reported in other studies (Harvey et al., 1984; Bengtsson 1989, Griebler et al., 2001, 2002). The highest number of suspended cells was produced in the experiments with *P. putida* F1, the lowest number was released by *A. aromaticum* EbN1, which might be an indication that the cells released into the pore water after reaching the carrying capacity resembles the growth rate. Likewise, strains which do not release any cells into the pore water don't grow anymore. Due

to short residence times, suspended cells were only of lesser importance to biodegradation in our experiments, but they could be very important in aquifers owing to their colonization of new sediment surfaces and distribution of degrading strains over a wide area, thus increasing the biodegradation potential within the aquifer. The oxygen profile indicated that degradation activity was restricted to the inflow part of the column, and those newly grown cells produced at the inlet attached to the sediment downgradient, leading to a relatively even cell density throughout the columns. In models, the biomass responsible for degradation is usually treated as immobile while suspended cells are neglected (e.g., Barry et al., 2002; Schirmer et al., 2000; Prommer et al., 2006, 2009). Other researchers (Meckenstock et al, 2015) also came to that same conclusion that data obtained from batch experiments is unfit for predicting degradation processes in aquifers, as mass transfer limitations cannot be reproduced in batch, and degradation and growth rates in batch tend to be considerably higher than in the field. The new findings of our study are about how strongly certain factors that cannot be studied in batch systems influence bacterial growth and degradation behavior. The results clearly indicate that flow velocity, substrate concentration and electron acceptor limitation are key drivers of contaminant degradation. One has to be aware, however, that in the field there is still a multitude of additional factors at play which were not accounted for in our study, like food-web interactions, grazing, or competition for resources (Konopka, 2000; Griebler & Lueders, 2009; Griebler et al., 2014; Meckenstock et al., 2015). Also, cell concentrations in aquifers tend to be in the range of 10^6 to 10^7 and are therefore 1-2 orders of magnitude lower than those found in our column experiments (Winderl et al., 2008; Anneser et al., 2010). Nevertheless, we recommend the implementation of data obtained from flow-through sediment microcosms into models predicting natural attenuation in order to improve their reliability and accuracy.

The final novel discovery of this thesis is the response and adaptation of *Pseudomonas putida* F1 to short-term and long-term starvation conditions. In two experiments, cells of *P. putida* F1 were subjected to starvation periods of several weeks up to four months, respectively, and in both cases a significant fraction of the starving cells was readily able to degrade toluene when reapplied within only hours. During periods of growth highest cell numbers were found in the outflow and these declined by one order of magnitude during starvation conditions. However, the number of attached cells remained fairly constant during starvation periods, indicating that no significant loss of cells due to death and detachment occurred during starvation periods. Cells' volume and activity, however, declined considerably during the starvation periods, as the results of ATP measurement and microscopic analysis of the cells' size showed. ATP values monitored, indicated a reduction in cellular activity of about 70-90%, which is supported by the cells' reduction in volume to about a quarter of their initial size. Both of these measurements indicate that the cells are shrinking due to loss of water. It seems that still small amounts of oxygen were consumed during starvation periods, which was also observed in other studies (Murphy et al., 1997, Eckert et al., 2015) and may be explained as oxidation of internal cell reserves (Mason et al., 1986, Murphy & Ginn, 2000). After reapplying toluene, the cells were readily able to reach their pre-starvation degradation level within hours. Natural attenuation will therefore most likely not be hindered by changes in groundwater flow or contaminant plume position, as the degradation potential of microbial communities, at least under aerobic conditions, was shown to remain largely conserved despite the lack of an electron donor for months. In most contaminated aquifers, however, anaerobic conditions are most prevalent (Lovley, 2001; Wiedermeier et al,. 1999), and we don't know for sure if our findings from aerobic starvation experiments can be translated to anaerobic contaminant degradation especially considering that anaerobic processes yield less energy than aerobic

processes and thus lead to lower growth and degradation rates for anaerobic degraders (Rittmann & McCarty, 2012). However, similar behavioral patterns seem likely.

7. Conclusions and Outlook

This thesis provides several new insights and details about microbial communities in aquifers contaminated with petroleum hydrocarbons. The fact that microbial community composition is driven by sediment mineralogy as well as hydrocarbon contamination highlights the importance of microbial phylogeny for biodegradation despite various environmental parameters still awaiting further research. Future studies should therefore pay more attention to biotic factors like protozoan grazing and viral lysis (Šimek et al. 2003, Kent et al. 2007, Salcher et al. 2005, Wey et al. 2008), or to the continuous exchange of cells between the sediment and the porewater to evaluate temporal dynamics of microbial communities (Zhou et al. 2012, Hug et al. 2015). It should also be kept in mind that such highly specialized degrader communities as found in contaminated aquifers influence adjacent ecosystems like soils and surface waters by providing certain microbial species and ecosystem functions (Hug et al. 2015). One main focus of our study was to emphasize and elaborate on the differences between perfectly mixed, enclosed batch systems and heterogeneous open flow-through systems. The results of our work suggest that growth rates and degradation rates obtained from batch experiments aren't very suitable for predicting actual conditions in the field.

As stated in Meckenstock et al. 2015, the differences between enclosed and perfectly mixed laboratory batch bottles and complex, heterogeneous open flow-through aquifers are just too big to compare these two systems accurately. Especially growth rates and degradation rates usually tend to be much higher in batch cultures due to an overabundance of substrate and nutrients and a lack of competition. Also, since batch bottles aren't flow-through systems, they're unsuitable for studying mass transfer limitations. Another important factor is the impossibility to assess attached and suspended cells independently in batch bottles, and considering that degradation was mainly performed by attached cells while suspended cells

harbor the potential for sediment colonization further downstream this differentiation is vital for estimating an aquifer contaminant's long-term fate. Overall we can conclude that models simulating growth and biodegradation within contaminated aquifers benefit tremendously from data derived from flow-through sediment microcosms rather than batch experiments, provided that spatial and temporal resolutions are chosen appropriately.

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9. Authorship Clarifications

Chapter 2: "Organic contamination versus mineral properties: competing selective forces shaping bacterial community assembly in aquifer sediments"

The concept for the experiment was devised by Dr. Christian Griebler. The sampling was performed by Carolin Spicher, Anne Bayer, Giovanni Pilloni, and Christian Griebler. Subsequent laboratory analyses were performed mainly by Carolin Spicher, whereas sediment sorting, DNA extrcaction and T-RFLP analysis were performed by both the PhD candidate and Carolin Spicher. Clemens Karwautz and Hagen Scherb gave the PhD candidate an introduction into statistical evaluation, and together the three of them performed the Principal Component Analysis. Data evaluation was performed by Christian Griebler, Carolin Spicher and the PhD Candidate. The PhD candidate wrote the manuscript, under the supervision from and guidance by Christian Griebler, and made corrections accordingly.

Chapter 3: "Mini Sediment Columns and Two-Dimensional Sediment Flow-Through Microcosms: Versatile Experimental Systems for Studying Biodegradation of Organic Contaminants in Groundwater Ecosystems"

The part about the one-dimensional sediment column system was written by the PhD candidate, the other part about the two-dimensional flow-through microcosm was written by Roland Hofmann. Christian Griebler supervised the writing of both parts.

Chapter 4: "Source concentration versus mass flux – drivers of biodegradation and microbial growth in groundwater model systems"

The concept for the experiment was devised by Dr. Christian Griebler and Dr. Olaf Cirpka. The PhD candidate performed all laboratory experiments for both systems and their related

analyses. Dominik Eckert and Olaf Cirpka developed the model that was fed with the data from the experiments with the one-dimensional sediment-filled column system. Data evaluation was done by Christian Griebler and the PhD candidate. The part of the manuscript concerning the laboratory experiments, as well as the abstract, the introduction and the conclusions were written by the PhD candidate, under guidance by Christian Griebler, and the part concerning the model was written by Dominik Eckert and Olaf Cirpka.

Chapter 5: "Dynamics of aerobic toluene degraders in flow-through systems under growth and starvation conditions"

The concept for the experiment was devised by Dr. Christian Griebler. The PhD candidate performed all laboratory experiments. Adrian Mellage, Dominik Eckert and Olaf Cirpka developed the mathematical model. The part of the manuscript concerning the laboratory experiments was written by the PhD candidate under the supervision of Christian Griebler, while the part concerning the model was written by Adrian Mellage, Dominik Eckert and Olaf Cirpka.

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First and foremost, my biggest thanks go to my parents Angelika and Rudolf Grösbacher, for they have not only financed my entire study and my flat in Munich, but have also provided me support all the time.

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My thanks also go to Sigrid Kaschuba, for she has shown me around at the Helmholtz Center Munich and helped me with several administrative and organizational matters during the first few days after I arrived here.

I'd also like to thank several colleagues who have helped me at one or several points of my work, among them being Anne Bayer for her help with the anaerobic culture medium preparation and for showing me how to cultivate *Aromatoleum aromaticum* strain EbN1 during the early stages of my PhD, Michael Granitiotis for introducing me to the fluorescence microscope, and Giovanni Pilloni for showing me how to culture *Pseudomonas putida* strains. Gabriele Barthel for ebiss, Zhou Yuxiang for DAPI filtration unit, Maria Avramov for headspace vials and crimping, Agnieszka Herzyk for Ultracentrifuge and FACS introduction.

11. Curriculum Vitae

Professional experience:

Sep. 2015 - ongoing:	Quality management representative at the Landeskontrollverband Tirol, Prüfstelle Labor Rotholz. Tasks: Bacterial and chemical analysis of drinking water samples, raw milk analysis (flow cytometry, ELISA, PCR), quality management, supervision of compliance according to EN ISO 17020 and 17025
05.10.2011-04.10.2014:	PhD-Student at the Helmholtz Zentrum München (Institute of Groundwater Ecology, Group Microbial Ecology). Tasks: Flow Cytometry, GC-MS-Analysis, HPLC-Analysis, PCR, T-RFLP Analysis, PCR, Bradford Protein Assay, ATP-Test, DOC-Measurement, gel elctrophoresis, manufacturing of growth media, cultivation of bacterial cultures, light microscopy, fluorescence microscopy.
01.08.2009-31.03.2010:	Research assistant & collaborator on the project „Microcolumbus“ at the University of Salzburg (Faculty of natural science Salzburg, Department of molecular biology, division of microbiology). Tasks: Biochemical and molecular biological analysis of halobacteria, manufacturing of growth media, cultivation of bacterial cultures, irradiation with UV, antibody assays, staining of bacteria, fluorescence microscopy.
July 2004:	Laboratory trainee at the Hospital Schwarzach-St.Veit. Tasks: acceptation and distribution of samples, plating and cultivation of samples, analysis of blood and urine.

Educational training:

05.10.2011-04.10.2014:	Promotion in Biology at the Technical University of Munich and the Helmholtz Zentrum München (Institute of Groundwater Ecology, Group Microbial Ecology).
Oct. 2002-May 2010:	Baccalaureate Genetics at the University of natural science in Salzburg, Master's program Genetics/Biotechnology at the University of natural science in Salzburg
Sep. 1994 – July 2002:	Higher general education at the private academic high school St. Rupert

Scientific Conferences:

11.-12. July 2013:	Participation on international science congress in Leipzig, Germany Young Scientists Meeting, Water Science Alliance
11.-14. Oct. 2009:	Poster presentation on an international science congress in Brussels, Belgium. 9th European Workshop on Astrobiology EANA'09

Publications:

Grösbacher M, Eckert D, Cirpka OA, Griebler C (2018) Contaminant concentration versus flow velocity - Drivers of biodegradation and microbial growth in groundwater model systems. *Biodegradation* **29:** 211-232 https://doi.org/10.1007/s10532-018-9824-2.

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12. Supporting Information

12.1 Organic contamination versus mineral properties – Competing selective forces shaping bacterial community assembly in sediments

Supporting Information

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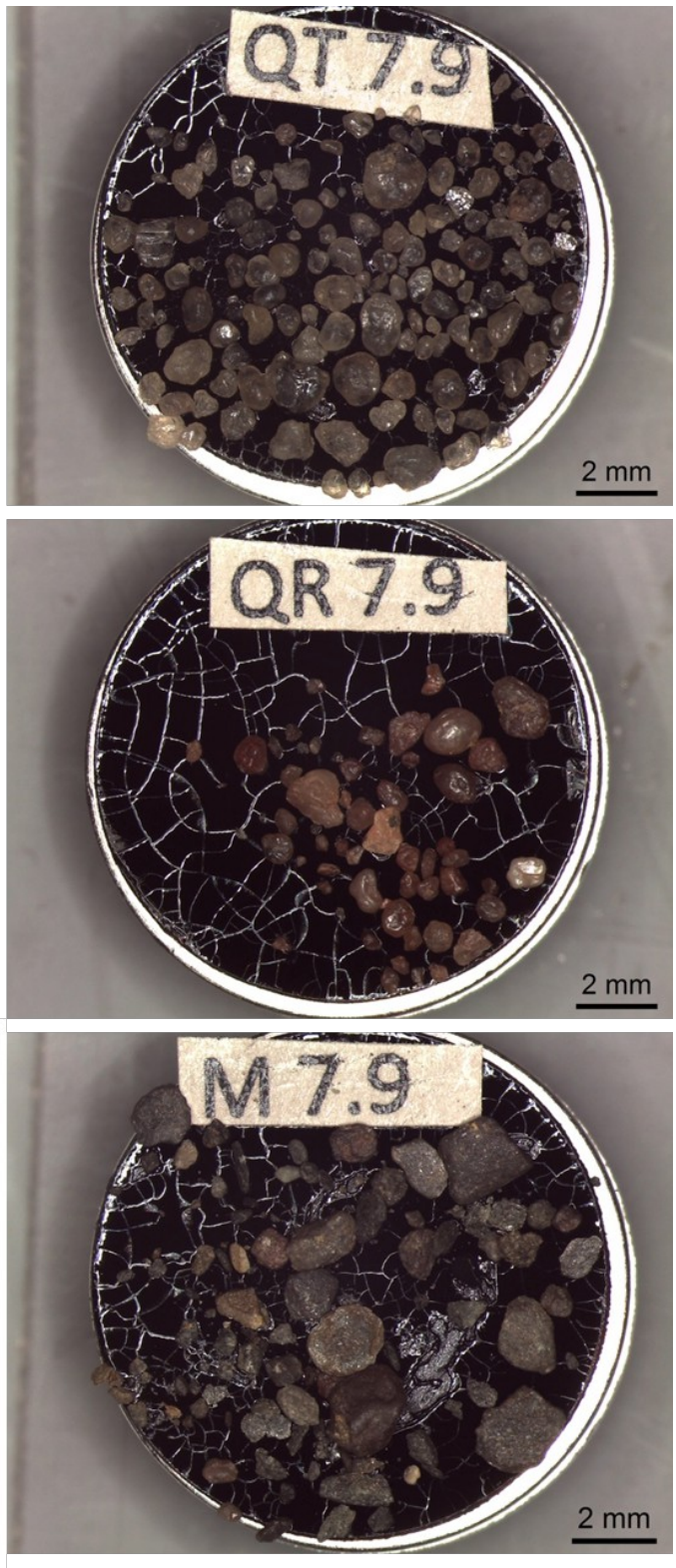

Figure S2.1: Sorted grains from Zone 2 dedicated to SEM-EDX analysis. QT = translucent quartz; QR = remaining sediment fraction mainly composed of 'coated quartz'; M = mica.

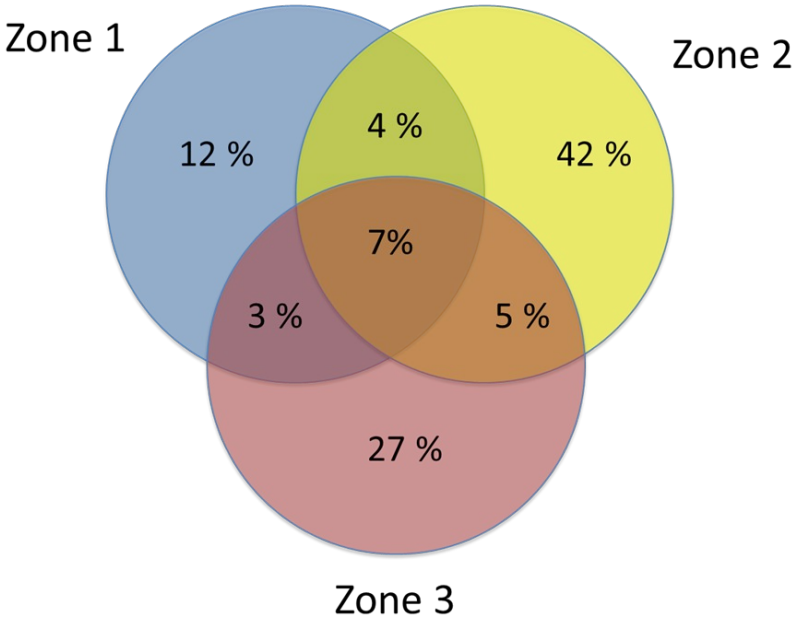

Figure S2.2: Venn diagram with the percentage of operational taxonomic units (TRFs) shared by the individual zones of the aquifer investigated.

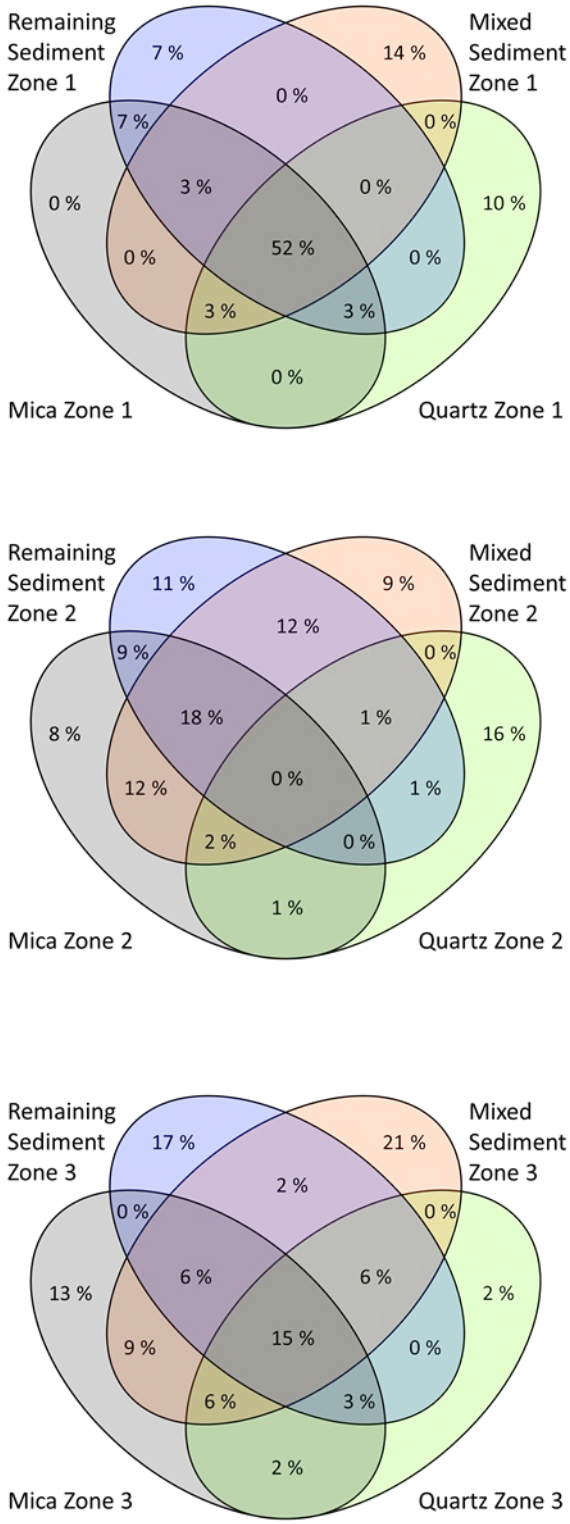

Figure S2.3: Venn diagrams with the percentage of operational taxonomic units (TRFs) shared by the individual mineral fractions selected in the three zones of the aquifer investigated.

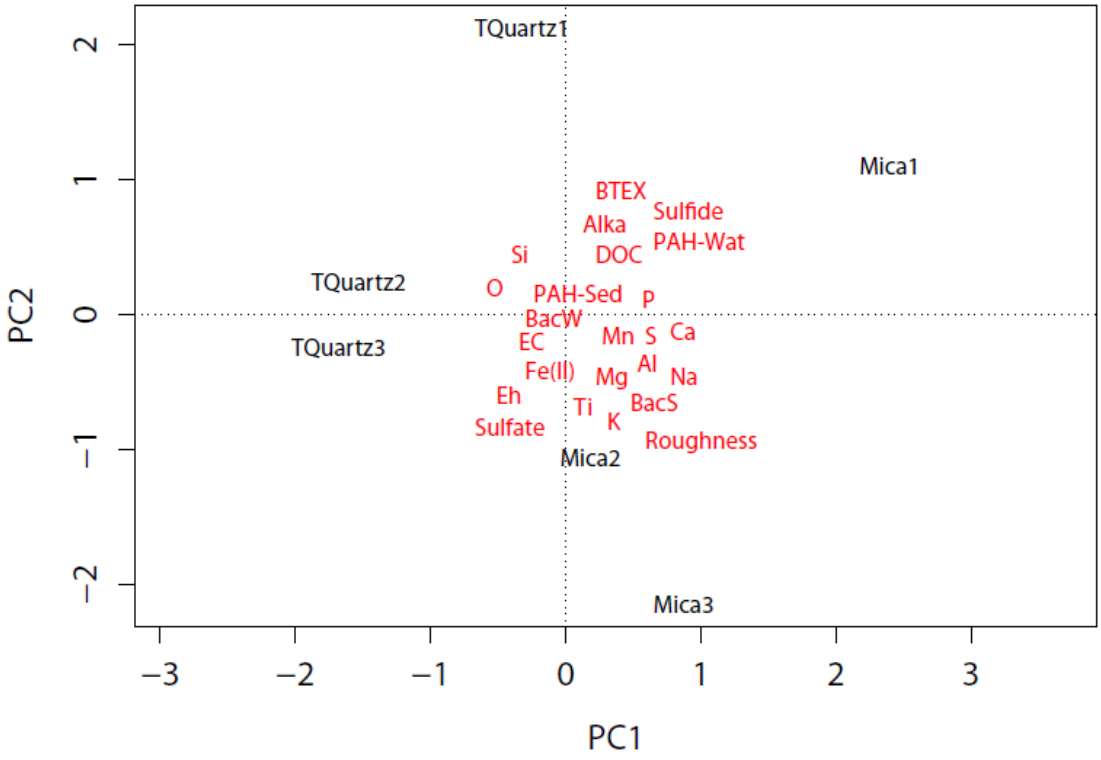

Figure S2.4: PCA plot that separates the individual samples (different zones and the mineral fractions translucent quartz and mica) according to the influence of environmental variables

12.2 Contaminant Concentration versus Flow Velocity – Drivers of Biodegradation and Microbial Growth in Groundwater Model Systems

Supplementary Information

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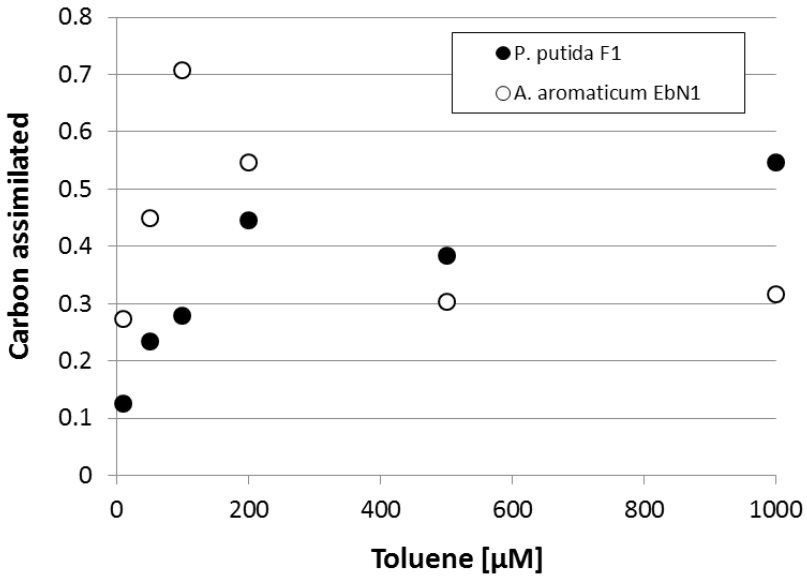

Figure S4.1: Substrate carbon converted into biomass carbon in batch experiments with the aerobic strain *P. putida* F1 and the nitrate reducing strain *A. aromaticum* EbN1 at varying initial concentrations of toluene. Toluene was the sole carbon and energy source.

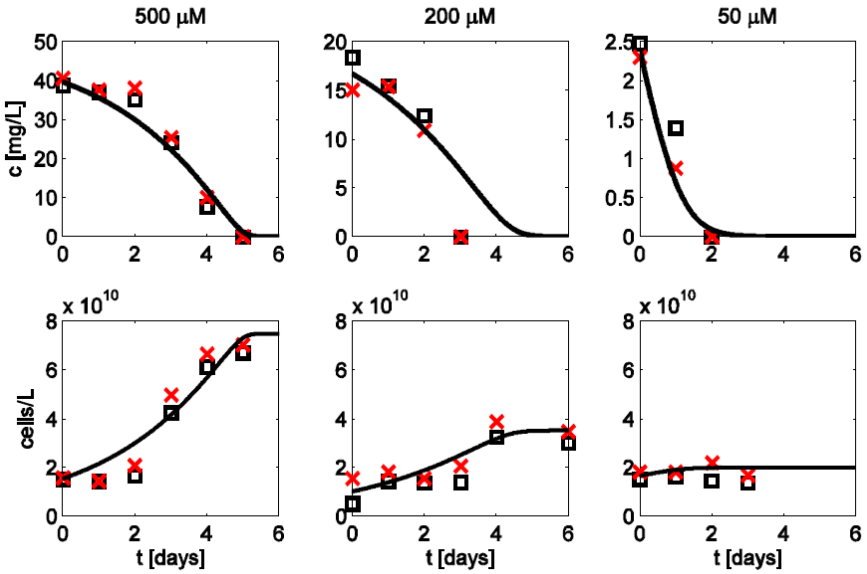

Figure S4.2: Measured and simulated toluene concentrations and bacterial growth of *A. aromaticum* EbN1 over the course of batch experiments with different substrate starting concentrations.

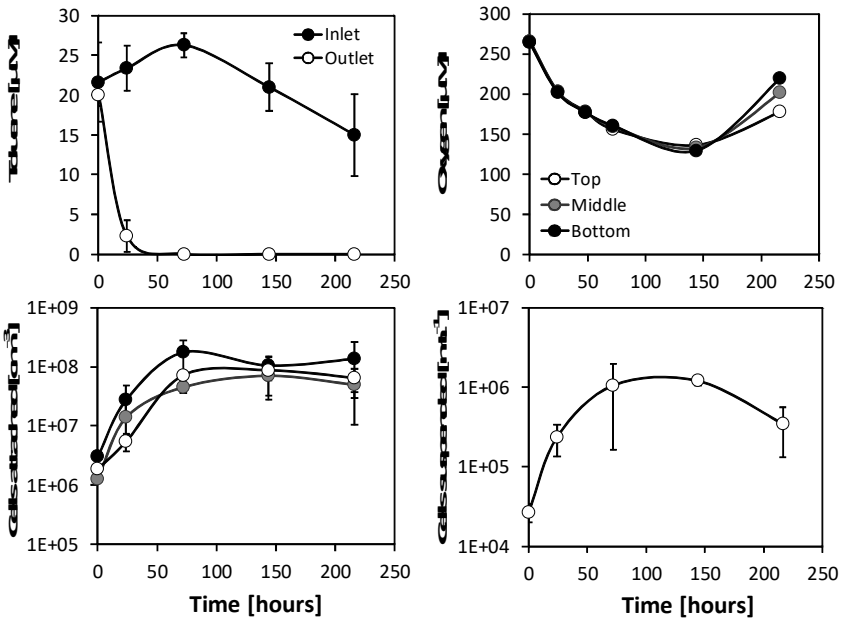

Figure S4.3: Aerobic toluene degradation and growth of *P. putida* F1 in mini-sediment columns flow-through experiments at a continuous source concentration of 30 µM and a flow velocity of 1.8 m d^{-1} (Exp. D). Values are means of triplicate measurements ± SD.

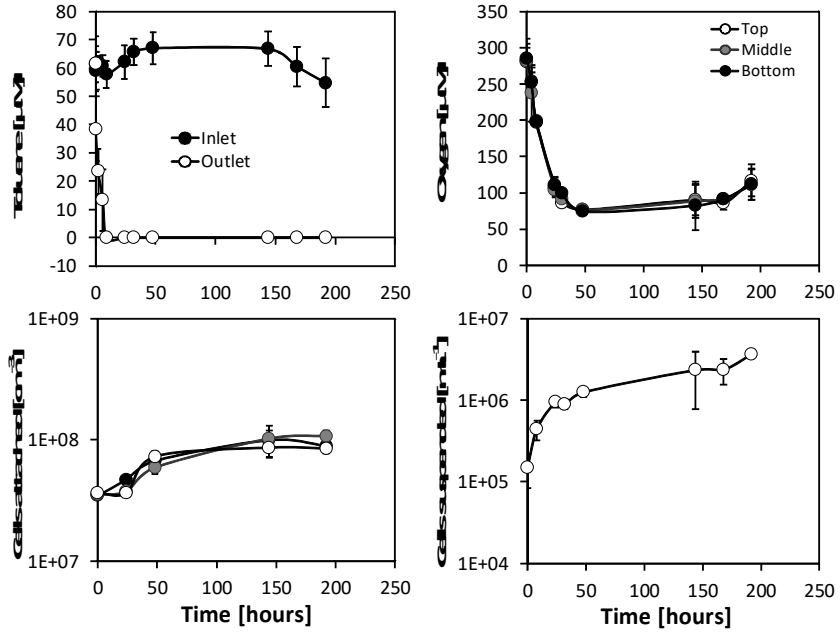

Figure S4.4: Aerobic toluene degradation and growth of *P. putida* F1 in mini-sediment columns flow-through experiments at a continuous source concentration of 70 µM and a flow velocity of 0.6 m d^{-1} (Exp. A). Values are means of triplicate measurements ± SD.

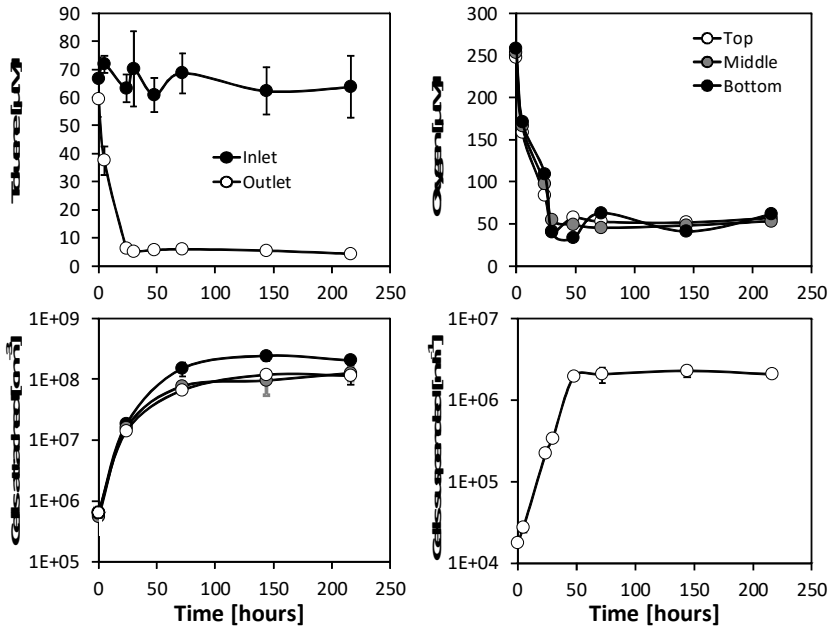

Figure S4.5: Aerobic toluene degradation and growth of *P. putida* F1 at a continuous source concentration of 70 µM and a flow velocity of 1.8 m d^{-1} (Exp. B). Values are means of triplicate measurements ± SD.

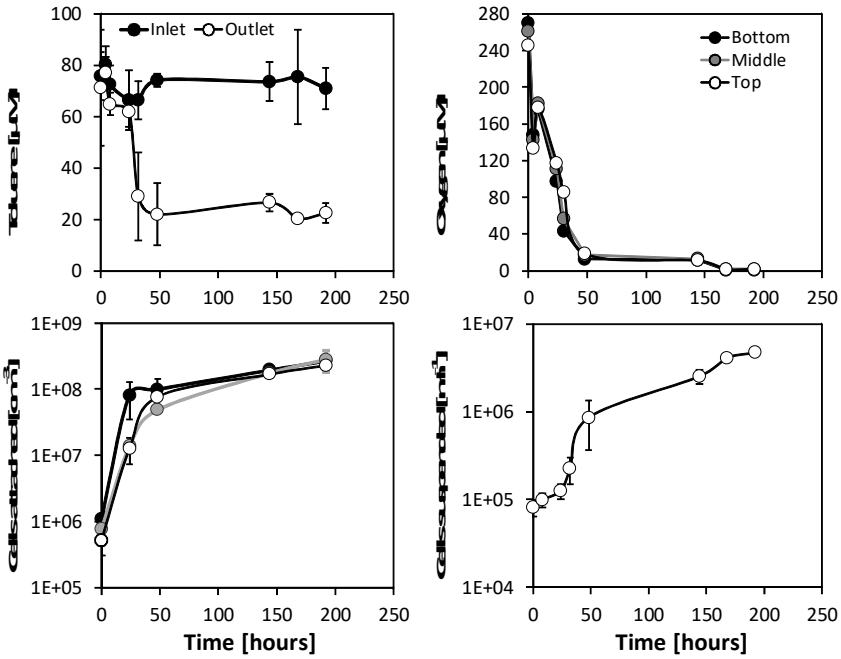

Figure S4.6: Aerobic toluene degradation and growth of *P. putida* F1 at a continuous source concentration of 70 µM and a flow velocity of 3.6 m d^{-1} (Exp. C).Values are means of triplicate measurements ± SD.

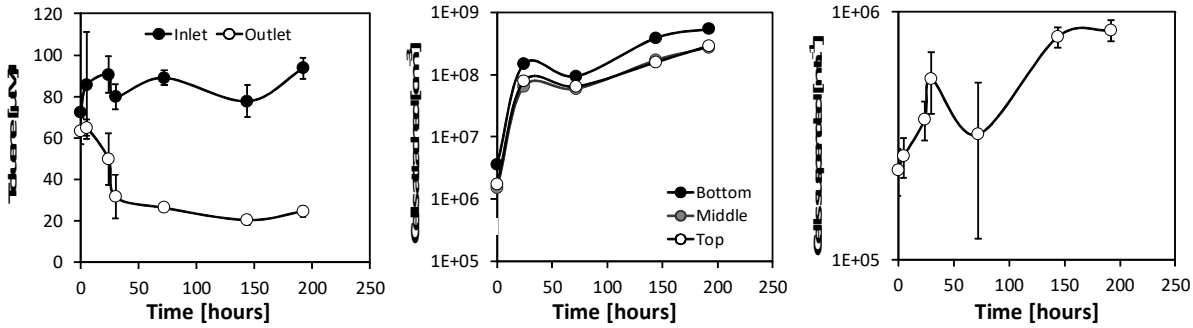

Figure S4.7: Toluene degradation and growth of *A. aromaticum* EbN1 under nitrate reducing conditions at a continuous source concentration of 70 µM and a flow velocity of 1.8 m d^{-1}. Values are means of triplicate measurements ± SD.

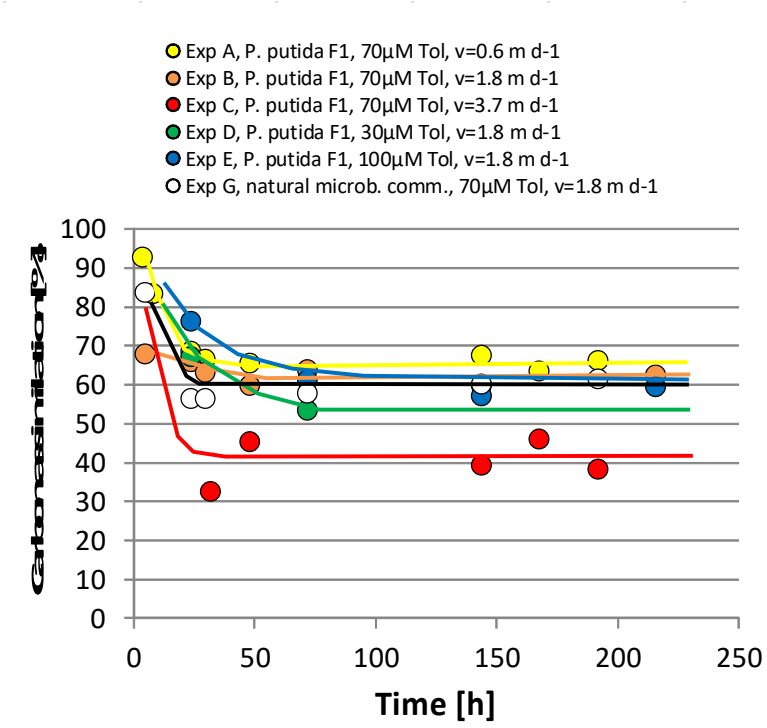

Figure S4.8: Carbon assimilation efficiency calculated from f_{ox} during different stages of the aerobic flow-through sediment column experiments with P. putida F1 and the natural aquifer microbial community.

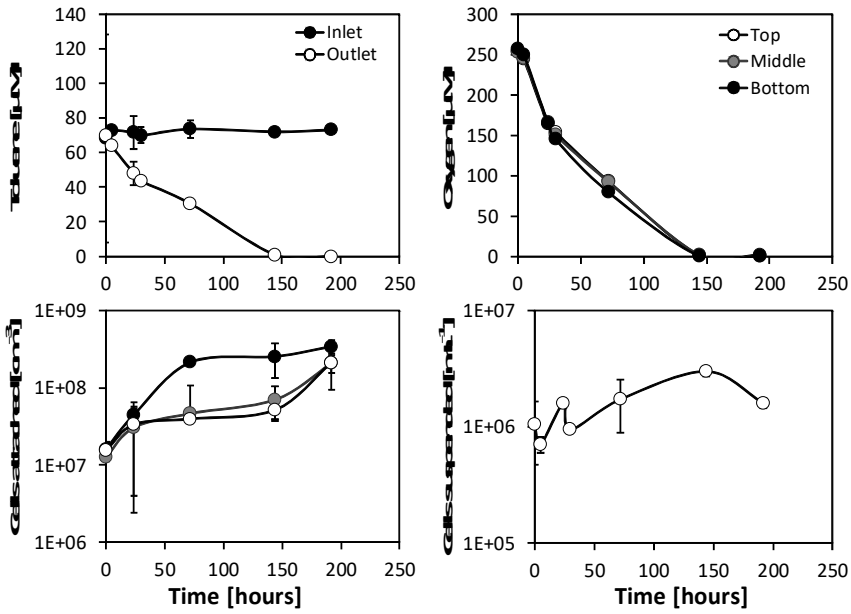

Figure S4.9: Aerobic toluene degradation and growth of a natural aquifer microbial community at a continuous inflow concentration of 70 µM and a flow velocity of 1.8 m d^{-1}. Values are means of triplicate measurements ± one standard deviation.

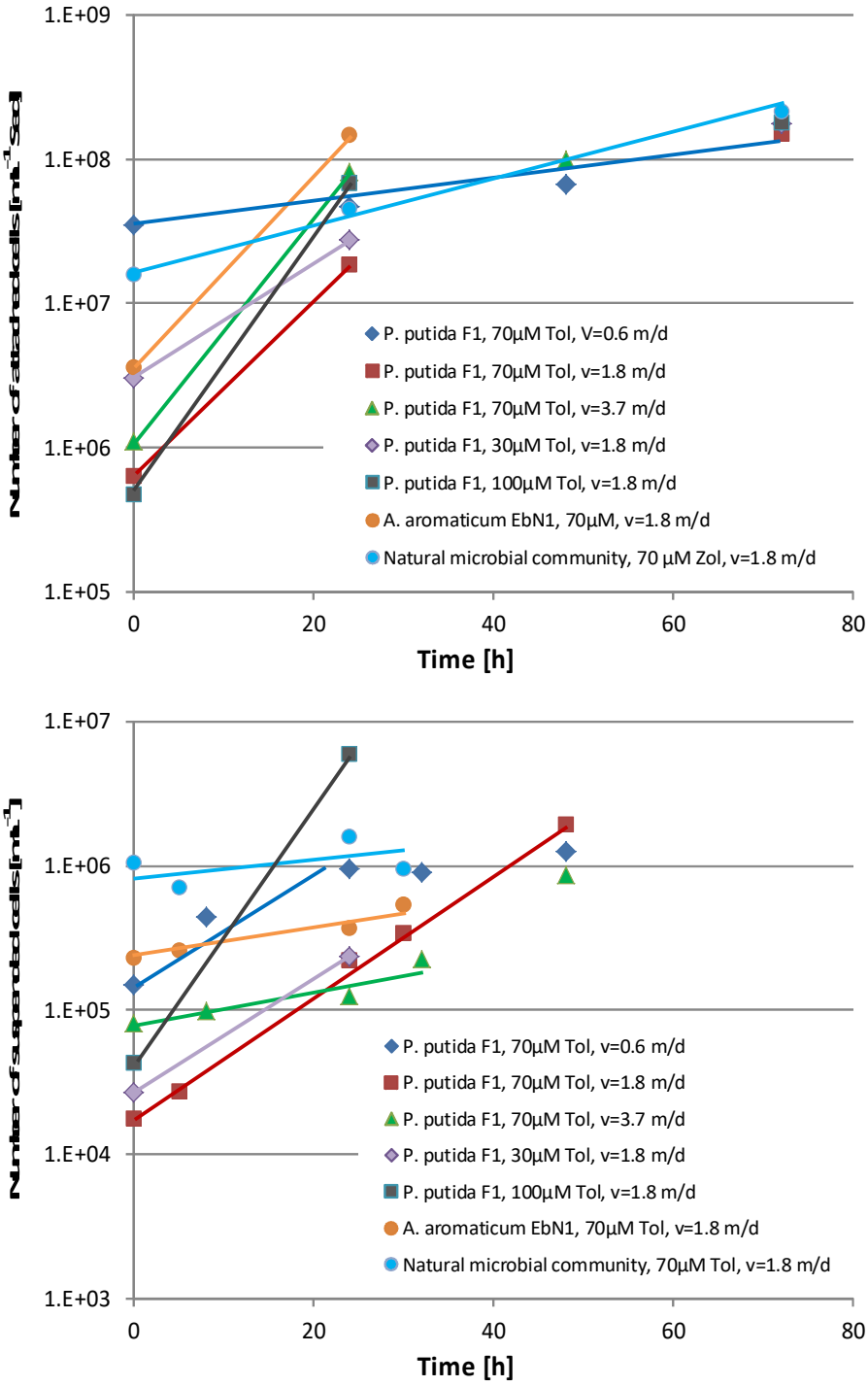

Figure S4.10: Comparison between the early stage growth patterns of attached and suspended bacterial populations during the different sediment column experiments.

Appendix

A. Bacterial strains and growth medium

Pseudomonas putida strain F1

The aerobic bacterium used was Pseudomonas putida strain F1, belonging to the class of the γ-Proteobacteria. It is a gram-negative, aerobic, motile bacterium (Chapelle, 2000; Madigan et al., 2003) with an average cell size of 1.5*1 µm. The optimum growth conditions were found to be at 25-30°C at a pH of 7.2-7.4.

Aromatoleum aromaticum strain EbN1

The anaerobic bacterium used was the denitrifyer Aromatoleum aromaticum strain EbN1, a gram-negative, facultatively anaerobic γ-Proteobacterium (Trautwein et al., 2008; Zink and Rabus, 2010) whose cells are rod-shaped, motile and have a size of about 0.6-0.8*1.5-2.5 µm. The optimum growth conditions are at 26-31°C and at a pH of 7.1-7.4 (Rabus and Widdel, 1994).

The growth medium

Both strains use almost the WSM medium (Widdel Sweet-Medium) for growth, the only difference being that *Aromatoleum aromaticum* strain EbN1 needs anoxic conditions and a source of nitrate (50 mL of 1M $NaNO_3$ L^{-1} medium). 1L of medium contained the following: 1g NaCl, 0.4g $MgCl_2*6H_2O$, 0.2g KH_2PO_4, 0.25g NH_4Cl, 0.5g KCl, 0.15g $CaCl_2$ x $2H_2O$, 0.2% trace element solution SL-10 (Widdel et al., 1983), 0.2% selenite-tungsten solution (Tschech and Pfennig, 1984), 0.1% 7 vitamin solution (Widdel and Bak, 1992). The medium was autoclaved at 121°C for 30 min, and gas exchange was performed using N_2/CO_2 gas in an 80:20 ratio. Once the medium has cooled to room temperature, 30 mL of 1M $NaHCO_3$ buffer and 0.5 mL of 1M Na_2SO_4 were added. Afterwards, the pH was adjusted to around 7.2 to 7.4. The medium was then filled into several 120 mL serum bottles, approximately 50-70 mL per bottle. The bottles were closed with viton stoppers, and the medium was stored in the dark at

room temperature. When preparing an anaerobic medium, the bottles were exposed to N_2/CO_2 gas for a couple minutes after filling them with medium, to remove any remaining oxygen.

B. Experiment with a natural community and the alternative substrate α-Ketoglutarate

Another column experiment with the same natural community was performed using α-Ketoglutarate as a substrate to compare the growth and degradation potential of the same natural community between two different carbon sources. The concentration of α-Ketoglutarate was constantly measured using the α-Ketoglutarate Colorimetric/Fluorimetric Assay Kit from BioVision. The natural community degraded α-Ketoglutarate much faster than toluene. Two days after the start of the experiment, no α-Ketoglutarate was measurable in the outflow anymore (data not shown). Contrary to the experiment with toluene as the sole carbon source, degradation started instantly along with immediate and fast bacterial growth, most likely because α-Ketoglutarate is not toxic and a highly sought-after substrate for many bacterial species.

12.3 Dynamics of aerobic toluene degraders in flow-through systems under growth and starvation conditions

Supporting Information

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S1. Model Parameters

The maximum specific growth rate, μ_{max}, as well as the half-saturation concentrations of toluene, K_{Tol} were determined from batch experiments by Grösbacher et al.[1]. The half-saturation concentrations of oxygen K_{O_2} was obtained from literature[2]. Simulations using these parameters fit the datasets of the continuous-injection experiment as well as the short- and long-term starvation experiments well and were able to reproduce microbial growth in the column experiments with a transport velocity of 1.8 m d^{-1}.

The carrying capacity, quantifying the maximum amount of attached bacteria, C_{Bio}^{max}, was set to the maximum concentration of attached cells measured in the respective experimental run. It was necessary to include this site-saturation behavior in order to accurately simulate the maximum amount of attached cells in the system and the expulsion of cells into the mobile phase under substrate-rich conditions. This agrees well with results of Ding[3] who stated that the inclusion of an "attachment-site capacity" term is important when modeling the distribution of attached microbes under growth conditions. The magnitude of the observed maximum capacity used in these experimental simulations falls within the range of values presented in Ding[3] and references cited therein.

The rate coefficient of cell attachment, k_{att}, which was not a very sensitive parameter, was fitted to a constant value of 50 d^{-1} for all three experiments. Detachment under non-growth conditions was negligible, and consequently the detachment rate coefficient k_{det} could be set to zero.

In order to match the base-level cell outwash observed in the experimental measurements, a constant base inflow of cells needed to be considered. Without considering addition of cells from an external source, during toluene-free periods, cell numbers at the outflow would drop to zero due to the lack of new growth. A continuous baseline injection of background cells was confirmed by the three separate measurements of cell counts in the inlet during the long-

term starvation trial, plotted in Figure 2 of the main article. The cell concentration in the inlet was found to be consistently above 1×10^5 cells mL^{-1} at all sampling time-points. The source of this background contamination of cells is unknown.

The growth yield (Y) of *P. putida* F1 in the column experiments was estimated based on the ratio of newly-grown cells and degraded toluene mass during the course of the individual experiments. The estimated yield values ranged between 13 and 29% (that is, 3.9×10^8 - 8.5×10^8 cells mg_{tol}^{-1}) of the value obtained from the batch experiments conducted by Grösbacher et al.[1] (Y = 2.9×10^9 cells mg_{tol}^{-1}). The considerably lower yield coefficients in the column experiments, as compared to the well-mixed batch-system, suggests that the efficiency with which *P. putida* F1 utilizes toluene for growth is decreased under flow-through conditions. The discrepancy in microbial growth yield between batch and flow-through experiments is discussed in more detail by Grösbacher et al.[1].

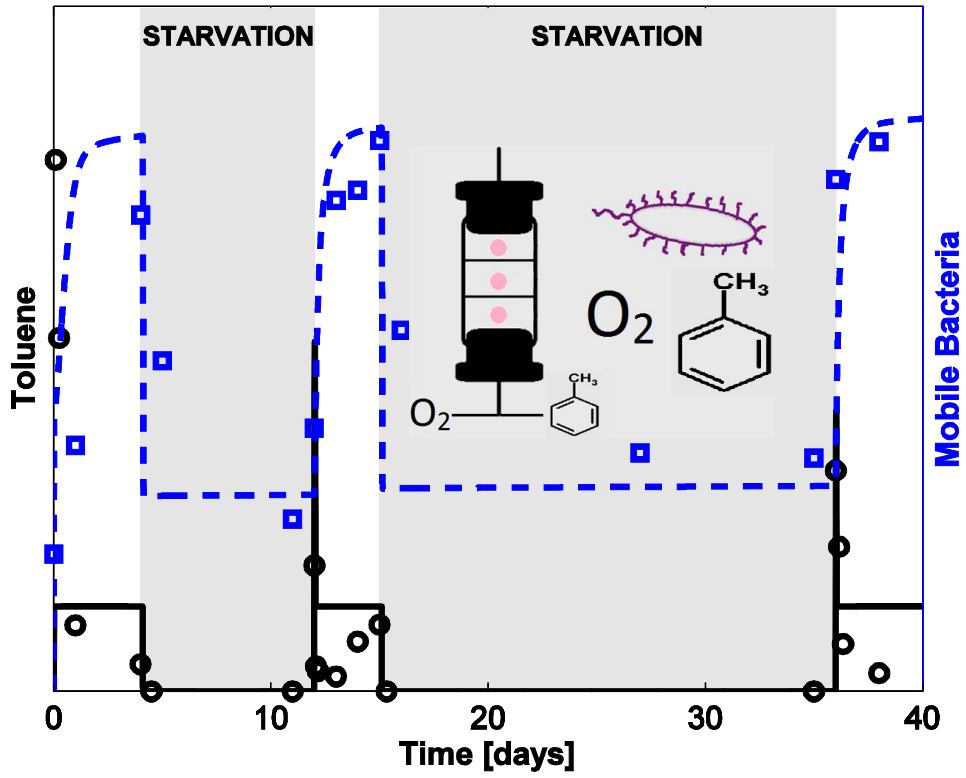
The toluene threshold concentration for microbial activity C_{Tol}^{thresh} of 1 µg L^{-1} was merely chosen in order to facilitate a switch in conditions. The actual value of the threshold concentration was not significant, because the change between substrate-poor to substrate-rich conditions in the column experiments was quite abrupt.

The coefficients for deactivation of attached bacteria during phases of toluene (substrate) – starvation, k_{deac}, and reactivation after the resumption of toluene injection, k_{reac}, were both fitted to a value of 2 d^{-1}, which captured the speed with which cells entered and exited the dormant phase. Stolpovsky et al.[4] fitted separate reactivation and deactivation rate coefficients for experimental batch data involving *P. putida* F1, namely 24 d^{-1} and 72 d^{-1} for reactivation and deactivation respectively. The quicker response to changing conditions observed by Stolpovsky et al.[4] might be due to the fact that the starvation period was only 12 h long in a well-mixed bioreactor system, as well as the fact that microbes were deprived of oxygen and not toluene[5].

Except for slight variations in the yield and the observed maximum amount of attached bacteria, the three experiments could be adequately simulated using the same parameters. This effectively yielded a single model that satisfactorily explained all experimental scenarios.

It may be noteworthy that in order to jointly fit the toluene and oxygen concentration profiles, the stoichiometric coefficient for oxygen consumption f_{O_2} needed to be changed to about half of the value expected for the complete mineralization of toluene to carbon dioxide and water of 3.1 mg_{ox} mg_{tol}^{-1}. While the same pattern was observed in a previous large-scale flow-through experiment[6], we are currently not able to explain this observation. For a detailed discussion of this issue we refer the reader to Grösbacher et al.[1].

GRAPHIC – TOC/Abstract Art



SYNOPSIS

This study focused on the degradation dynamics of microbial degraders when subjected to starvation conditions in porous flow-through systems. The response of a toluene degrading community of *Pseudomonas putida* F1 - subjected to periods of no substrate availability in 1-D bench-scale column systems - was monitored. The concentrations of both suspended cells at the outlet and cells attached to the sediment within the column domains were monitored, along with the concentrations of electron acceptor (O_2) and donor (toluene), in order to relate their consumption to growth. Measurements and simulation results show that degrading microbes remain in their majority attached to the sediment, after an initial growth stage, during extended starvation periods of up to four months. Contaminant (toluene) degradation was resumed without any significant delay upon its re-injection into the system, after each starvation period. Our results suggest that extended periods of starvation have no effect on the degradation efficiency of microbial populations in contaminated systems.

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