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Resilience and resistance of a pristine aquifer towards toluene contamination – impact assessment using microbes and elucidation of factors limiting natural attenuation

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Zusammenfassung

Das tägliche Leben von Menschen hängt von der Verfügbarkeit von Grundwasser ab. Grundwasser ist Trinkwasser, Wasser welches für die Bewässerung in der Landwirtschaft gebraucht wird, und wichtiges Lösung- und Kühlmittel in der Industrie. Unglücklicherweise unterliegen unsere Grundwasservorkommen einem zunehmenden Druck durch Übernutzung und Verschmutzung. Verunreinigungen stehen meist in Zusammenhang mit Ablagerungen, Altlasten und dem unbeabsichtigten Entkommen von Schadstoffen in den Untergrund. Eine wichtige Schadstoffklasse ist dabei jene der aromatischen Kohlenwasserstoffe, wichtige Inhaltsstoffe von Ölprodukten. In den letzten Jahren schenkte man dem natürlichen Schadstoffrückhalt (Natural Attenuation), eine wichtige Ökosystemdienstleistung, zunehmend Aufmerksamkeit als kostengünstige Alternative für die Sanierung. Im Vergleich zu dem stetig wachsenden Wissen um das Verhalten und Schicksal von Schadstoffen in Grundwassersystemen, hinkt unser Verständnis für die ökologischen Prinzipien die ‚Natural Attenuation' und der Resistenz und der Resilienz von Ökosystemen zugrunde liegen hinterher. Konsequenterweise, ist ein gezielter Schutz der Ressource Grundwasser, die Voraussagbarkeit für die Veränderungen im Ökosystem bei Belastungen, und ein Management der Ökosystemdienstleistungen sehr eingeschränkt.

Im Rahmen dieser Promotionsarbeit wurde ein umfangreiches Experiment in einem Aquifer-Mesokosmos durchgeführt, welches die Reaktion eines unbelasteten, energie-limitierten Grundwasserleiters auf die organische Verunreinigung mit Toluol (monoaromatische Kohlenwasserstoffverbindung) untersuchte. Hauptaugenmerk wurde auf (1) das im Ökosystem bereits vorhandene spontane mikrobielle Abbaupotential, (2) die kurz- und langfristige Reaktion des Ökosystems bei unterschiedlichen Belastungsszenarien (kontinuierliche Schadstoffquell und katastrophaler Schadstoffeintrag = Spill) und das

Erholungpotential, und (3) die Verwendung von abiotischen und biotischen Parametern zur Ökosystemzustandsbewertung gelegt.

Wie in Kapitel 2 beschrieben ist, konnte die natürliche mikrobielle Gemeinschaft einen kurzen Toluolpuls signifikant in seiner Masse reduzieren. Dies deutet auf ein hohes intrinsisches Potential auch eines energie-limitierten Ökosystems für den Abbau von organischen Schadstoffen hin. Die nachfolgende dauerhafte Belastung des Modellaquifers (Kapitel 3) zeigte ein noch ausgeprägteres Potential für den aerobe Schadstoffabbau, hat die mikrobielle Gemeinschaft Zeit sich durch entsprechende Neuorganisation der Belastungssituation anzupassen. Mittelfristig führte jedoch der aktive aerobe Schadstoffabbau zur vollständigen Zehrung von Sauerstoff, als energetisch günstigster Elektronenakzeptor, was die Abbauleistung des Ökosystems entscheiden habsetzte und den Schadstoffabbau auf die noch sauerstoffversorgten Randbereiche der Kontamination beschränkte. Die Bewertung des Ökosystemzustands mittels ausgewählter physikalisch-chemischer und biologischer Messgrößen zeigte maßgebliche Unterschiede in der funktionellen Antwort (Prozesse und Aktivitäten) und seiner Reaktion hinsichtlich der Zusammensetzung der Gemeinschaften im Verlauf der Verunreinigung und der Rückkehr zum Ausgangszustand. Die im Porenwasser suspendierte mikrobielle Gemeinschaft reagierte viel schneller durch Zunahme der Gesamtzellzahl und Aktivität auf die Toluolbelastung als dies die am Sediment festhaftende Gemeinschaft tat. Die Sedimentgemeinschaft reagierte sehr verzögert hinsichtlich seiner Biomasse, zeigte jedoch eine rasche Veränderung in seiner Zusammensetzung. Diese war gekennzeichnet durch den Verlust sensitiver Arten und das in den Vordergrund treten anderer, die eine Schlüsselrolle für den Abbau innehatten. Im späteren Verlauf des Versuchs, zeigten die Sedimentgemeinschaften noch in ihrer Zusammensetzung und mit ihrer erhöhten Biomasse noch lange eine Störung des Ökosystems an, obwohl sich die ursprünglichen physikalisch-chemischen Bedingungen wieder eingestellt hatten.

Diese Ergebnisse zeigen, dass Grundwasserleiter ein überraschend großes Potential für den unmittelbaren aeroben Schadstoffabbau aufweisen können, jedoch sehr sensibel gegenüber einer kontinuierlichen organischen Belastung, wie etwa der Verunreinigung durch Toluol, reagieren, die zu einer massiven Änderung der Redoxbedingungen und der mikrobiellen Gemeinschaften führt. Das anfangs große Potential zur natürlichen mikrobiellen Schadstoffreduktion ist vor allem durch die für Sedimentsysteme charakteristische eingeschränkte Verteilung und Mischung von Elektronenakzeptor und Schadstoff in seiner Nachhaltigkeit eingeschränkt. Außer Diskussion steht auch, dass für eine Ökosystemzustandsbewertung ein physikalisch-chemisches und auch ein mikrobiologisches Monitoring welches sich nur auf die Wasserphase beschränkt nicht ausreichen. Das Sediment mit seiner mikrobiellen Gemeinschaft stellt ein Langzeitgedächtnis für vorangegangene Störungen.

Abstract

People all over the world depend on groundwater in their daily lives. Groundwater is drinking water, water for irrigation in agriculture, as well as an important solvent and cooling liquid in industry. Unfortunately, groundwater is subjected to an increasing pressure due to contamination by deposition and/or accidental release of multitude amounts of pollutants, including hydrocarbons. In recent years, the ecosystem service of microbially-mediated natural attenuation of contaminants, as a cost-effective clean-up strategy for impacted aquifers, received considerable attention. However, in contrast to the steadily growing knowledge on contaminants´ fate in aquifers, the ecological principles of natural attenuation (NA) and the resistance and resilience of pristine groundwater systems lag behind. Consequently, effective protection of groundwater resources, prediction of pollutant behavior, appropriate decision making and management of ecosystem services is limited. This PhD thesis is based on a mesocosm experiment investigating the response and recovery of a pristine, energy-limited, indoor aquifer to a well controlled toluene contamination scenario using toluene as model pollutant. Particular concern was put on (1) the potential of the intrinsic aquifer microbial community for a spontaneous biodegradation of toluene, (2) the short-term and long-term response of the aquifer to different contamination scenarios (continuous source and spill) and its recovery capacity, as well as (3) the use of abiotic and biotic variables as indicators for ecosystem status and development.

As described in the Chapter 2, a short pulse of toluene injected into the indoor aquifer system was readily reduced in mass by the native microbial community. This fact put emphasize on a very high intrinsic potential, even in pristine and energy-limited groundwater systems, for the aerobic biodegradation of organic pollutants, as demonstrated for toluene. The subsequent treatment of the aquifer with a constant toluene input (Chapter 3) supported

an even higher capacity for the attenuation of toluene, allowing the system to adapt via reorganization of the microbial community and microbial growth. However, depletion of oxygen in the toluene impacted areas slowed down the overall biodegradation, underlining the temporal dimensions of this potential being strongly dependent on the sufficient supply and availability of dissolved oxygen as energetically favorable electron acceptor. The assessment of the groundwater system behavior, following selected abiotic and biotic variables, revealed the differences in functional and compositional response during impact by, and recovery from toluene contamination. Generally, the suspended microbial community reacted much faster to toluene input and its disappearance, in terms of abundance and activity, than the attached members of the community. Sediment communities exhibited a lag phase with respect to total biomass but readily underwent a structural shift in community composition, with sensitive members disappearing and key degrader populations popping up. Later, sediment samples still indicated a disturbed ecological status of the system despite the recovery of the oxic conditions and toluene disappearance.

These results demonstrate, that groundwater systems, despite a surprising potential for spontaneous NA, have a very low resistance to an ongoing toluene stress, which resulted in serious changes of redox conditions and microbial patterns. This underlines their vulnerability and raises the need for an adequate groundwater protection measures. The aquifer`s high biodegradation capacity was shown to be limited by the insufficient transport and mixing processes in porous media, what calls for further understanding and elucidation of factors improving the spread of electron acceptors. Last but not least, a comprehensive assessment of the aquifer`s recovery was only achieved when applying biological indicators, since the fast restoration of the physical-chemical conditions did not reflect the much slower recovery of the sediment communities in the aquifer.

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1. Introduction

1.1 Groundwater - a vulnerable resource

Groundwater is vital to the existence of life on Earth. It is a crucial resource for humanity and the rest of the living world. Globally, groundwater accounts for one third of total freshwater resources, while two thirds are almost exclusively fixed in ice and snow (Danielopol et al., 2003). Worldwide, groundwater represents, where available, the preferred source of drinking water. Around 75% of EU inhabitants depend on groundwater as their main water supply; however groundwater usage in Europe varies between the countries. In Norway usage of groundwater as a drinking water source accounts for only 13%, whereas Denmark, Iceland and Austria depend almost 100% on groundwater supply (EEA, 1999; Danielopol et al., 2003; EEA, 2012). Besides the source of drinking water, society uses it to generate and sustain economic growth and prosperity. Groundwater serves as irrigation water, solvent, cooling liquid and many more. However, there is wider ecological consideration – groundwater plays a crucial role in the hydrologic cycle and thus is a core compound of terrestrial ecosystems, being involved in climate regulation and sustaining groundwater dependent ecosystems. Groundwater reservoirs substantially contribute to the base flow of many rivers and streams. According to U.S. Geological Survey, average groundwater contribution to stream flow is estimated as high as 52 % (Winter et al., 1998).

Groundwater was believed to be an everlasting source of water for human generation, but now the resources are endangered and rapidly diminish in many parts of world. A rapid demographic increase, agricultural practices including irrigation, industry and mining have been the main threats leading to excessive groundwater exploitation and degradation (EEA, 2012). Some of the major anthropogenic groundwater pollutants are: fertilizers, pesticides,

heavy metals, and organic compounds, such as chlorinated solvents and petroleum hydrocarbons, being a “heritage” of maximized agriculture practices, 19th century’s industrial boom or the effect of still today’s practice. The continuous improvement of analytical methods and instrumentation, added a vast array of new compounds used by society to already existing pollutants, including pharmaceuticals, personal care products and food preservation agents reported to occur in drinking water in Germany, UK, Italy, Canada and USA (Lapworth et al., 2012).

According to an assessment of the European Environment Agency (EEA, 2007) heavy metals and mineral oil are the most profound threats to soil, while mineral oil and chlorinated hydrocarbons are commonly found in groundwater. Contamination has been reported at nearly 3 million sites across Europe, which awaits remediation, and if forecasted trends hold true, a 50% increase of polluted sites is expected by 2025. Among mineral oils, petroleum constituents are the most widespread anthropogenic contaminants found in aquifers. Petroleum is a complex mixture of a large numbers of different hydrocarbons, classified as saturated and unsaturated hydrocarbons, cycloalkanes and aromatic hydrocarbons. The last, due to their water solubility and the high toxicity, pose a major threat to our environment. They are divided into three groups: (i) mono-aromatic hydrocarbons, collectively indicated as BTEX (benzene, toluene, ethyl benzene, xylene), (ii) polycyclic aromatic hydrocarbons (PAHs) and (iii) heterocyclic compounds (Nahar et al., 2000; Widdel and Rabus, 2001). As a point source, mainly originating from leaking underground storage tanks, buried petroleum waste and piping, hydrocarbons move through soil and unsaturated sediments and occasionally reach the groundwater table. Light non-aqueous phase liquids (LNAPLs), e.g. BTEX and individual PAHs, float on top of the groundwater table and with time they dissolve into the groundwater forming a contaminant plume. The heavier compounds such as chlorinated solvents, can build dense non-aqueous phase liquids (DNAPLs) sinking

downwards in the saturated zone. The fate of hydrocarbon contamination depends on complex interactions between microorganisms biochemical processes and hydrogeological properties of the aquifer as well as on the nature and on the amount of the contaminant present (Wiedemeier et al., 1999; Christensen et al., 2001).

1.2 Groundwater – a living ecosystem

During the past decades it became more and more recognized that groundwater should not only be seen as a drinking water reservoir, but also protected for its environmental value and considered as ecosystem with individually adapted biocenoses (Danielopol et al., 2003; Hancock et al., 2005). Pristine groundwater systems provide highly specific living conditions and have been considers as "extreme habitats" (Griebler and Lueders, 2009). Due to the absence of light, the biocenoses depend on organic matter once deposited together with the sediments; particulate (POM) and dissolved organic matter (DOM) coming from the surface and/or inorganic chemical energy sources. However, the import of organic substrates to aquifers is limited. The most easily degradable fractions of carbon sources are readily utilized before they reach the saturated zone. In young and shallow groundwater, dissolved organic carbon (DOC) is generally low, ranging between 0.5 to 2 mg L^{-1} (Michalzik et al., 2001; Pabich et al., 2001). Moreover, most of the organic material reaching groundwater is recalcitrant, often only containing degradable amounts of DOC in the range of 10-100 µg L^{-1}. Deep and old groundwater ecosystems have repeatedly been suggested to be free of DOC (Neff and Asner, 2001; Batiot et al., 2003). Since natural non-contaminated aquifers generally lack elevated concentrations of organic carbon, they are characterized by oligotrophy (low productivity) and oligoalimony (low nutrient and carbon conditions). Consequently, the microorganisms – focusing on bacteria mainly - show small cell sizes, a limited morphological diversity (mainly spherical and rod-shaped cells), low densities,

reduced activity and a comparable low diversity (Balkwill and Ghiorse, 1985; Goldscheider et al., 2006; Griebler and Lueders, 2009). Besides the harsh energy-limiting conditions, temperature, pH, water chemistry and flow velocity are comparably constant and predictable in many aquifers. Nevertheless, shallow systems may underlie in dynamics the environmental conditions triggered from the surface. Groundwater recharge, changes in temperature, as well as seasonal land use may cause a ‘seasonality’ of subsurface microbial patterns (Zhou et al., 2012). Shallow oligotrophic aquifers are generally oxic, however, major parts of the subsurface are completely devoid of molecular oxygen (Goldscheider et al., 2006).

The living space in most unconsolidated sand and gravel aquifers is small (µm to mm), and consequently the biocenoses are mainly composed of Eubacteria, Archaea (in the following simply called bacteria), viruses and protozoa (flagellates, ameobae and ciliates). Besides, shallow and oxic aquifers often harbour an invertebrate fauna consisting of crustaceans, nematodes, oligochaetes and mites, among other groups (Madsen and Ghiorse, 1993; Griebler and Mösslacher, 2003). Well adapted to the prevailing nutrient and carbon conditions, heterotrophs are the main inhabitants of the groundwater aquifers, however lithoautotrophs are also present (Ghiorse and Wilson, 1988; Kinkle and Kane, 2000; Alfreider et al., 2009; Kellermann et al., 2012). The total numbers of bacteria in groundwater vary between 10^2 and 10^6 cells mL^{-1} groundwater and 10^4 and 10^8 cells mL^{-1} sediment (Haveman and Pedersen, 2002; Griebler and Lueders, 2009). The distribution of microorganisms in the subsurface is heterogeneous and determined by factors such as the geology, porosity and conductivity, as well as the availability of energy. Interestingly in shallow aquifers, depth is not an important driver of bacterial abundance, as originally suggested. Most microbes in the subsurface are associated with sediment particles and rock surfaces, generally accounting for $\geq 90\%$ of the prokaryotic biomass (Lehman et al., 2001; Griebler et al., 2002; Ahn and Lee, 2003). The attached mode of living is believed to favor survival and growth, especially at low

nutrient and carbon conditions. However a quasi equilibrium exists between attached and suspended cells, strongly influenced by the availability of carbon and nutrients (Bengtsson, 1989). Increased levels of degradable carbon promote the growth of free living bacteria or stimulate the detachment of attached populations resulting in a changed ratio of attached to suspended bacteria (Madsen and Ghiorse, 1993; Alfreider et al., 1997; Griebler et al., 2002). Consequently, often increased numbers of free living bacteria are found in contaminated groundwater (Harvey et al., 1984; Anneser et al., 2010).

Protozoa are generally present at very low numbers in pristine groundwater, with concentrations ranging from 10^0 to 10^2 cells mL^{-1} (Novarino et al., 1997). However, in contaminated sites, their abundance might increase by several orders of magnitude (Zarda et al., 1998). Many protozoa are known to be effective predators, feeding on bacteria or other microorganisms (including other protists), and therefore playing a key role in the microbial food-web with respect to carbon transfer and nutrient cycling (Biagini et al., 1998). Bacterivorous protozoa are affecting microbial communities by size-selective grazing, changing the size structure of prokaryotic population or even community composition (Posch et al., 2001). On the other hand high protozoan abundance is considered as a good indicator of in situ biodegradation since it hints at growing population of degraders (Sinclair et al., 1993; Mattison et al., 2005).

Also viruses contribute an active and abundant component of aquatic microbial communities. Their abundant existence in marine ecosystems has been reported more than 30 years ago, but only recently it became obvious that they can be found anywhere in the environment where life is present (Torrella and Morita, 1979; Wommack and Colwell, 2000; Kyle et al., 2008). The total viral abundance varies along with the prokaryotic productivity and abundance. Typically their concentrations decrease from marine to open oceans, and from surface to deep waters ranging from 10^8 to $< 10^6$ viral-like particles mL^{-1}, on average

one order of magnitude higher than bacterial abundances (Jacquet et al., 2010). Within the last few decades, viruses, previously only viewed as pathogens causing diseases of animals and plants, has been recognized as an important biological compartment in functioning of ecosystems. Viruses as main predators of prokaryotes play a vital role in controlling bacterial populations' density, diversity, size and fitness as well as gene transfer. This virus-mediated mortality has several implications for the environment, including effect in global carbon cycling and re-cycling of nutrients from organic matter (Suttle, 2007; Rohwer et al., 2009).

Given that viruses are responsible for about 10-50% of the total bacterial mortality in surface waters, and up to 100% in the environments where protozoa grazing is of lower importance due to their low abundance, some studies have emphasized the role of grazing in microbe mediated contaminant degradation (Fuhrman, 1999; Kota et al., 1999; Mattison and Harayama, 2001; Head et al., 2006).

1.3 Natural attenuation (NA)

Anthropogenic groundwater pollution has become a worldwide problem diminishing clean water resources in the world. With global human population steadily growing, the demands for clean water will increase in the future. Thus, remediation of contaminated waters remains the only option to ensure sustainable water supply, reduce human health risk and protect the environment. However, at many polluted soil and groundwater sites, it is too expensive and too difficult to successfully conduct remediation. Fortunately, the subsurface is able to attenuate pollutants based on intrinsic and self-sustaining processes. The different biotic and abiotic processes contributing to *in situ* transformation of pollutants are summarized as natural attenuation (NA). It comprises a variety of physical, chemical and biological processes that under suitable conditions will reduce the mobility, toxicity and mass

of contaminants in soil and groundwater without human intervention. These processes include dilution, dispersion, volatilization, precipitation, sorption, ion exchange and most importantly, transformation and degradation (Röling and Verseveld, 2002; Smets and Pritchard, 2003; Scow and Hicks, 2005). However, with exception of degradation mechanisms such as hydrolysis and dehydrohalogenation, abiotic processes do not decrease the contaminant mass, but rather cause dilution. The true decrease of mass and toxicity of contaminants relies on the biodegradation potential of indigenous microorganisms which are able to transform and mineralize a multitude of organic compounds.

Petroleum compounds may undergo biodegradation under oxic or anoxic conditions, and the degree of biological degradation is determined by numerous factors related to contaminant composition, presence of microorganisms, essential nutrients, temperature, concentration of oxygen or alternative electron acceptors such as NO_3^-, Fe(III), Mn(III), SO_4^{2-} (Wiedemeier et al., 1999). In the first instance, the availability of appropriate electron acceptors is key to warrant carbon oxidation. Aerobic microorganisms gain energy by oxidizing organic compounds to carbon dioxide with oxygen serving as the terminal electron acceptor. However, this process is limited in the subsurface since the amount of oxygen dissolved in groundwater is low, and the rate of oxygen supply by advection and diffusion from overlying unsaturated soils is slow. The available dissolved oxygen is quickly consumed. Thus, the contribution of aerobic microorganisms to the overall biodegradation is often moderate (Brune et al., 2000; Bauer et al., 2008). Usually, in heavily contaminated areas of an aquifer, oxygen is only found at the fringes of the contaminated zones. Nevertheless, aerobic transformation processes play a crucial role in early stag plume development, generating oxygen-free areas and initializing anaerobic biodegradation. Anaerobes oxidize organic compounds to carbon dioxide by use of alternative electron acceptors such as nitrate, Fe(III) or sulfate. Once these electron acceptors are depleted,

anaerobic metabolism proceeds by converting organic matter to methane and carbon dioxide. Methane production therefore often dominates close to the source of contaminations where alternative electron acceptors have been depleted. Further down the plume, a succession of anaerobic processes governed by electron acceptor availability was repeatedly reported (Christensen et al., 2000; Lovley, 2001). However, this idealistic pattern is rarely found at contaminated sites (Krumholz, 2000; Anneser et al., 2008). Numerous studies report that the biodegradation of organic pollutants is, in many cases, often elevated at the plume´s fringes where steep chemical gradients and succession of terminal electron accepting processes (TEAPs) are observed. Here electron acceptors mix with contaminants. Experimental results from field and lab studies also show that aerobic and anaerobic degradation was always more efficient in heterogeneous sediments, when compared to homogeneous packing (Cirpka et al., 1999b; Jose et al., 2004; Bauer et al., 2008). This is mainly due to an intensification of mixing of electron donors and acceptors in high conductivity zones.

The behavior of pollutants in the environment is influenced essentially by the nature and amount of the contaminant present, and the interaction among chemical, geochemical and biological factors (Leahy and Corwell, 1990; Bordenave et al., 2007). Regarding the biological factors, the composition of the microbial communities and their metabolic capabilities constitute an important factor. The selective dynamics of the indigenous microbial communities are key drivers determining success and efficiency of biodegradation. The degradation of complex pollutant mixtures such as crude oil and petroleum products requires a combination of different bacterial taxa that, when functioning as a community can degrade a broad spectrum of hydrocarbons that single populations of bacteria could not do. Recent reviews lists 79 bacterial genera distributed over several (sub)phyla (α-, β- and γ-Proteobacteria; Gram positives; Flexibacter-Cytophaga-Bacteroides) that can use hydrocarbons as a sole source of carbon and energy (Röling et al., 2002; Kästner et al., 2006),

and according to Norris et al. (1994), communities containing hydrocarbon degraders are present at 99% of contaminated sites. Without doubt, communities composed of multiple bacterial populations, with different degradative capabilities, sustainably contribute to the overall rate and capacity for measured degradation (Leahy and Corwell, 1990; Haack and Bekins, 2000).

1.4 Resistance and resilience of groundwater systems

The term resilience was brought into ecology by Holling (1973a) and for several decades its diverse meaning and definitions have been extensively discussed. The original definition of resilience is based on the stability concept, however reliance on different assumptions on the presence of either single or multiple equilibria in a system lead to two separate resilience concepts. The concept of *engineering resilience* assumes the existence of a global equilibrium, hence is defined as the ability of a system to absorb disturbance, reorganize while maintaining efficiency of its functions, and return to the conditions from before the disturbance. The speed or time of return is generally used to measure this property (Holling, 1996; Gunderson, 2000). The second definition i.e. the *ecological resilience*, emphasizes conditions far from any steady state conditions, and relies on the assumption of multiple stable states. Therefore, it is defined as the magnitude of disturbance that a system can absorb, before it changes into another regime or stability domain by changing the variables and processes that control its behaviour (Holling, 1973a). This concept has been visually represented by the position of a ball (representing the ecosystem) in a valley (representing stability domain) (Scheffer et al., 2001; Gunderson and Holling, 2002). The deeper is the valley, the higher is the resilience. Thus much stronger disturbances are needed to push the system (ball) into another state (valley) (See Fig.4.10 in the Chapter 4).

The resilience of a system, strongly depends on inner, diverse and overlapping functions within the ecosystem performed by a diverse assemblage of species (Peterson et al., 1998) as well as a wide array of other biotic factors, hence reflects the degree to which a complex system is capable of self-organizing (Folke et al., 2004). Generally, the nature and extent of disturbance, the characteristics of the community and amount of natural variations, functional redundancy, and presence of other stressors determine the reaction to disturbance and recovery of the systems (Folke et al., 2004; Clements and Rohr, 2009). In fact, intense and destructive human activity lead to devastation of most valued aquatic and terrestrial ecosystems. Generally, nature is assumed to respond to graduate changes in a smooth, continuous way, however studies on lakes, wetlands, oceans, coral reefs, forests, semi-arid rangelands have shown abrupt changes of an ecological system to a less productive or less desirable state (Scheffer and Carpenter, 2003; Folke et al., 2004). This is termed as catastrophic shifts, or regime shifts (Scheffer et al., 2001). Pollution, resource overexploitation, land-use change, habitat fragmentations, climate change (Scheffer et al., 1993; Jackson et al., 2001; Folke et al., 2004) entailing further disruptions in both, the biotic components of the ecosystems (e.g. species diversity, flow of matter and energy, food web, ect.) and the abiotic components (habitat structure) are only some implications of human activities that induce lack of resistance and resilience of the ecosystems. In light of these changes and their consequences for human well-being, resistance and resilience of natural systems cannot longer be taken for granted. To assure continuation in delivery of ecosystems goods and services such as drinking water supply, as well as existence of clean groundwater ecosystems, sustainable management actions should focus on maintaining resilience. So far, investigations of critical shifts and implied loss of ecosystems resilience has mainly focused on interpreting historical trends. The development of forecasting tools, however, still remains a scientific challenge (Jackson et al., 2001; Orwin and Wardle, 2004; deYoung et al., 2008;

Thrush et al., 2009) mainly due to lack of long-term studies following exposure-response-recovery trajectories of an ecosystem. In case of aquifers, hardly any study followed contaminant-driven change of pristine systems to impacted stage and tracked back its recovery. Moreover, assessment of groundwater ecosystems status based only on physical-chemical indicators with omission of microbial communities deprives use from extensive understanding of ecosystems processes. To overcome these difficulties, the first step is to understand the processes and drivers that determine ecosystem performance, resistance and resilience, and govern the supply of essential ecosystem services, e.g. purification and storage of drinking water. Further on, it is of crucial importance to understand how the different biotic components of the ecosystem response to disturbance and how they recover with the removal of the stress.

Microbial communities, due to their ubiquity and rapid response to environmental changes are suitable for detection of environmental changes and for risk assessment studies (Goldscheider et al., 2006; Sole et al., 2008). They are characterized by comparably fast growth rates, and responses to low levels of pollutants as well as other physical, chemical, and biotic environmental changes. From detection and effect perspectives, they provide sensitive, meaningful, and quantifiable indications of ecological changes (Paerl et al., 2003). Due to the high complexity of natural microbial communities, identification of all different members of such communities is extremely difficult. However, monitoring the relative abundance of individual key species within their habitats, abundance ratios, and food web structure is of promising and prime importance to identify and understand going on processes.

1.5 Experimental set-up

The indoor groundwater system (see Fig 1.1.) has been designed to mimic a natural aquifer unit at a meso scale, to allow investigations under well controlled conditions, with sampling at a high spatial resolution adapted to the scale of processes and gradients. The system was constructed as a stainless steel box 5 m long, 0.8 m wide and 0.7 m high, filled with quaternary sediment (grain size ≤ 4 mm) from a pristine local gravel pit and directly feed by the local groundwater.

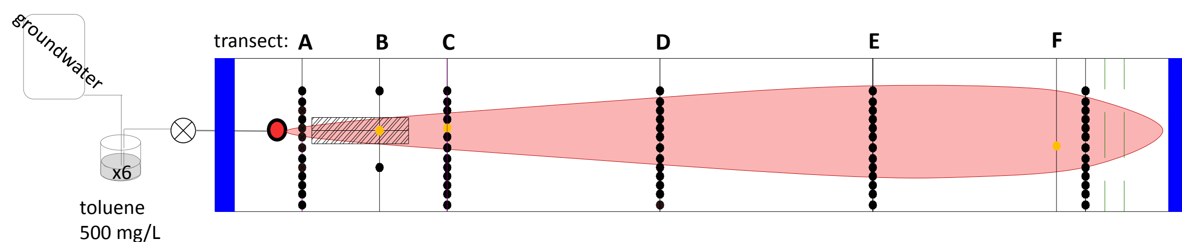

Fig. 1.1 Simplified schematic overview of the indoor groundwater model system with toluene injection set-up, side view, row 2. L=5 m, H=0.7 m, W=0.8 m

The hydraulic conductivity (K_f) was of 5.3 x 10^{-4} m s^{-1} (±10%). The water level was constant with the gradient of (I) 0.0207 in the hydraulic head between the inflow and outflow, and an average water flow rate of 1.6 m day^{-1}. A high conductive lens (12 cm high, 50 cm length, 80 cm width) of homogeneous quartz sand was embedded at the inflow region of the aquifer (transect B) with a measured K_f of 1.11 ± 0.13 x 10^{-3} m s^{-1} to spread the contaminant plume (Huenniger, 2011). For the insulation of the system and its top cover, insulation foam was used. The indoor aquifer was equipped with 134 water sampling ports, forming three (1 to 3) rows and subdividing the system in 6 transect: A; B; C; D; E; F (Fig.1.1 and Fig.2.1). The central row (number 2) contained 13 vertically highly resolved water sampling ports

(with a distance of 4 cm between each other) per transect. The left and right row (1 and 3) had only six vertically distributed sampling ports per transect. Additionally the aquifer was equipped with 20 oxygen sensors installed at 3 transects (A, D, F), 8 temperature sensors located along the water flow path and 84 fluorescence sensors used for an uranine tracer test. Detailed description of the aquifer`s transport parameters and performed tracer tests can be found in the doctoral study of Marko Huenniger (Huenniger, 2011). Monitoring of water flux, inflow and outflow pH, oxygen concentration and temperature was done on daily basis. The aquifer has been in operation for 17 months, before the first sampling campaign (assessment of the pristine stage) was conducted.

1.6 Sampling scheme

In the period of July 2010 to July 2011 five sampling campaigns were conducted. The aim was to follow at a high spatial resolution the physical-chemical and biological changes in the indoor aquifer from initially pristine to highly contaminated conditions and its subsequent recovery. As a consequence, we distinguished five different experimental phases of the experiment: *pristine* (P), *in depth* (*ID) I, ID II, ID III* and recovery phase - *ID IV* from which water and sediment samples were collected at a very high spatial resolution. The *Pristine* sampling campaign was conducted after half a year of aquifer equilibration period to assess the aquifers` physical-chemical status together with features of microbial community. During constant toluene injection, two highly resolved sampling campaigns of water and sediment took place 16 *(ID I)* and 63 *(ID II)* days after start of toluene injection. Additionally one sampling campaign called *basic sampling (BS) I*, where only solute samples were taken, took place 4 days after toluene injection. At the end of continuous toluene injection, the aquifer was exposed to a short pulse of pure toluene, after which the toluene injection was

terminated. The pulse was followed by *ID III* (day 137) sampling campaign, where water and sediment samples were taken at very high spatial resolution to investigate microbial community reaction to pulse injection of pure toluene. 94 days later, the profound sampling campaign *ID IV* took place (day 231 from start of toluene injection) to monitor the recovery of the aquifer and access its resilience potential.

1.7 Aim of the present study

In order to address some of the above mentioned concerns and knowledge gaps, the following research questions have been addressed in this PhD project:

- **What is the intrinsic spontaneous biodegradation potential of a pristine, oligotrophic groundwater aquifer?** This short study focuses on response of native microbial communities to a short toluene pulse in order to assess the intrinsic potential for a spontaneous biodegradation of toluene in pristine, oligotrophic aquifer.
- **How does an oligotrophic, energy limited groundwater aquifer response to continuous toluene contamination and how fast it can recover?** In this chapter, particular concern was put on characterization of a pristine indoor aquifer, its performance and response to toluene contamination with a specific focus on (i) microbial communities' dynamics, (ii) the time needed for the first response and (iii) factors limiting biodegradation. After termination of contaminant supply (iv), recovery of the system was followed and evaluated.
- **Which microbial and abiotic measures reflect the aquifers response to toluene contamination and its recovery? Search for reliable biological indicators.** This chapter addresses the need of ecosystem characterization based on its living components, i.e. microbial communities which are the crucial players of ecosystem

processes and services. A selected set of biotic and abiotic variables determined from toluene impacted areas was compared with non-contaminated areas of the aquifer, and its pristine pre-contamination conditions, to search for sensitive indicators of ecosystem response and recovery.

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2. Intrinsic potential for instantaneous biodegradation of toluene in a pristine, energy-limited aquifer

2.1 Introduction

The terrestrial subsurface harbours one of our most important resources for life, i.e. groundwater. Groundwater is the major source for drinking water in Europe and worldwide and supports a multitude of groundwater dependent ecosystems (EEA, 1999; Danielopol et al., 2003). However, groundwater systems increasingly face severe impacts by contaminants that have been released into the subsurface as a result of deposition (landfills, gaswork plants), leakages of distribution systems and storage tanks (petrol stations), or accidental spills (EEA, 1999; Danielopol et al., 2003; EEA, 2007, 2010).

The subsurface and ecosystems therein are generally considered to be energy poor, due to limited amounts of organic carbon. As a result, microbial cell numbers are usually 1-2 orders of magnitude lower than in surface waters and top soils (Goldscheider et al., 2006; Griebler and Lueders, 2009). Being energy-poor and low in productivity, groundwater systems are expected to display a high vulnerability and thus a low resistance to disturbances, such as the contamination with petroleum hydrocarbons. Yet, an organic contamination is a selective pressure to which diverse microbial communities may react by compositional reorganization with the enrichment of highly specialized degrader populations. Indeed, organically contaminated sites typically harbour microbial biomass significantly above background levels and microbial communities reduced in diversity but with the relative high abundance of microbes involved in contaminant transformation (Franzmann et al., 1996; Haack and Bekins, 2000; Winderl et al., 2008; Anneser et al., 2010; Kostka et al., 2011).

Natural attenuation of organic contaminants by adapted intrinsic microbial communities contributes significantly to pollutant removal (Wiedemeier et al., 1999;

Richnow et al., 2003; Winderl et al., 2008; Berlendis et al., 2010; Yagi et al., 2010). However, since scientific investigations at contaminated sites are delayed by weeks to months or even decades with respect to the time of contamination, our knowledge about the spontaneous biodegradation potential present in aquatic environments and specifically in energy-limited and uncontaminated aquifers is rather poor. Field studies, which in general, come too late and lack an appropriate temporal resolution, may overlook the present indigenous biodegradation potential (Kao and Wang, 2001; Chen et al., 2006; Takahata et al., 2006). In fact, most information on biodegradation is derived from batch experiments and microcosm studies where freshly sampled groundwater or sediment material amended with individual contaminants or contaminant mixtures have been studied (Nielsen and Christensen, 1994; Nielsen et al., 1996; Gieg et al., 1999; Althoff et al., 2001). However, many of these experiments hardly mirror *in situ* conditions, what may be the reason, for the often observed lag phase in contaminant transformation (Arvin et al., 1988; Aamand et al., 1989; Nielsen et al., 1996).

To close the gap between biodegradation of organic pollutants investigated in laboratory studies and *in situ* biodegradation potential, a meso-scale groundwater indoor aquifer was designed. Maintaining the system as close to nature as possible, while still being well controlled, allowed evaluating the spontaneous biodegradation potential of a groundwater system for a selected model contaminant (toluene) at a scale relevant for field applications. A toluene pulse was directed through an initially pristine, oxic, organic carbon and energy poor sandy aquifer. Injection of toluene for 30 h together with deuterated water (D_2O), as conservative tracer, as well as toluene-specific analysis of stable carbon isotope signatures allowed quantification of spontaneous biodegradation after a travel distance of 4.2 meters in less than one week of travel time.

2.2 Material and methods

2.2.1 Experimental set-up and pulse injection of toluene

The study was conducted in an indoor flow-through system constructed as a stainless steel box (indoor aquifer) described in the Introduction. The aquifer has been in operation for 17 months and four months prior to the pulse experiment a sampling campaign was conducted to characterize the pristine stage of the system.

Four serum bottles were filled with 30 mL of pure toluene phase and 210 mL D_2O. The total injected mass of toluene and deuterium was 0.486 g and 204 g, respectively. The bottles were tightly sealed with viton stoppers, avoiding any headspace and connected to each other in line using steel capillaries and fluran tubings (Ismatec, Wertheim, Germany). The capillaries were placed to the bottom of the bottles to avoid transfer of the floating toluene phase. To ensure that the injection solution was maximally saturated with toluene, bottles were placed on a magnetic stirrer during the time of injection. The first bottle of the injection set-up was connected to a D_2O reservoir, and the last bottle was connected to a peristaltic pump feeding the toluene saturated D_2O into 16 injection ports made of stainless steel capillaries. These ports were connected with a horizontally oriented, perforated steel tube allowing infiltration of the contaminant solution and tracer across the entire width of the model aquifer, generating a contaminant plume. The injection rate for the contaminant and D_2O solution was q= 0.78 L day^{-1} and lasted for 30 hours.

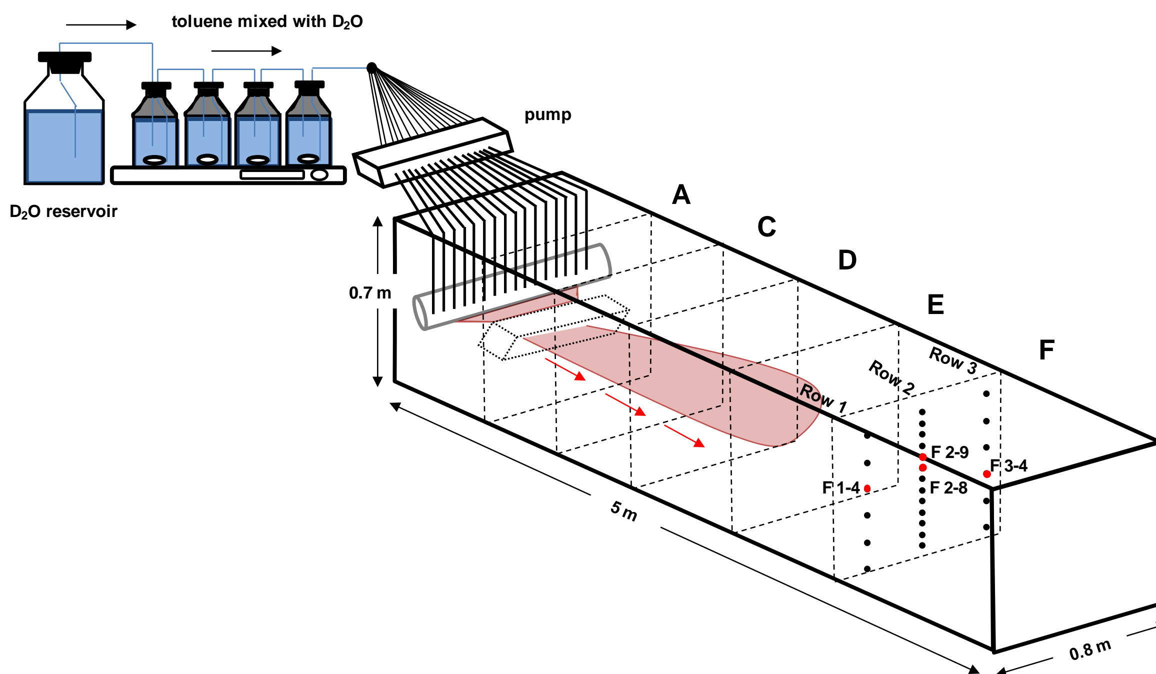

Fig. 2.1 Contaminant injection set-up and schematic overview of the indoor aquifer with 5 transects (A, C, D, E, F) and sampling ports in different depths.

2.2.2 Sampling

Water samples were repeatedly collected at 4 ports in transect F, 4.2 m downstream from the injection port. Two ports, F2-8 and F2-9, were located in the central longitudinal transect (middle row) at a depth of 0.36 and 0.32 m below surface (bs), respectively. Ports F1-4 and F3-4 were located in the longitudinal transects 1 and 3, left and right from the middle row 2, at a depth 0.30 m bs (Fig.2.1). Water was sampled continuously into glass syringes (50 mL) by means of a multichannel syringe pump. The pumping rate was 0.5 mL min^{-1}. The collected volume of 50 mL was subsequently divided into: (i) aliquots for toluene carbon isotope analysis, stored without headspace in 40 mL vials; (ii) aliquots for toluene concentration measurements (2 x 3.5 mL), transferred to a 10 mL headspace GC vials; (iii) aliquots for water isotopes analysis (1 mL) stored in 1.5 mL Supelco vials. Sample aliquots

for toluene analysis were fixed with NaOH (100 mM). Additionally, samples for toluene concentration measurements were spiked with an aqueous ethylbenzene (0.01 mM) solution as internal standard.

2.2.3 Deuterium analysis

Water samples were analyzed for D_2O by pyrolysis in a reactor loaded with "glassy carbon" and Ni-coated carbon (EuroVector) at 1480°C. Separation of CO and H_2 was done in a molecular sieve column at 95°C. Both gases were transferred to an isotope ratio mass spectrometer (IRMS) in a continuous He stream using a ConFlowIII system (Thermo Finnigan). δ2H measurements were conducted with a IRMS (Thermo Finnigan MAT 253) with relation to VSMOW (Vienna Standard Mean Ocean Water), where δ2H is expressed as:

$$\delta^2 H = \frac{(\frac{^2H}{^1H})sample - (\frac{^2H}{^1H})standard}{\frac{^2H}{^1H}_{standard}} x\ 1000\ [‰] \quad (1)$$

2.2.4 Toluene and oxygen measurements

Toluene concentrations were determined via headspace analysis on a Trace DSQ GC-MS instrument (Thermo Electro, Germany) equipped with a Combi PAL autosampler (CTC Analytics, Swizerland) as described in Anneser et al (2008). Separation was done in a DB5 capillary column (J&W Scientific, USA) with helium used as the carrier gas. The detection limit for toluene was 10 μg L^{-1}.

Dissolved oxygen concentrations were measured *in situ* via an optode-array technique (FIBOX3, PreSens GmbH, Regensburg, Germany). Light conducting silica fibers with a spot of oxygen sensitive polymer foil glued to the tip were buried at different depths in the water saturated sand.

2.2.5 Compound Specifics Isotope Analysis

The carbon stable isotope composition ($\delta^{13}C$) of toluene was measured using a Velocity XPT purge and trap (P&T) sample concentrator with an AQUATek 70 liquid autosampler (Teledyne Tekmar) coupled to a gas chromatograph – combustion – isotope ratio mass spectrometer (GC-C-IRMS). The GC-C-IRMS system was composed of a TRACE GC Ultra gas chromatograph (GC) (Thermo Fisher Scientific, Milan, Italy) connected to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) (Thermo Fisher Scientific, Bremen, Germany) via a FinninganTM GC Combustion III interface (C). An Optic 3 temperature programmable injector with LN2-cryofocusing option (ATAS GL International) was attached to the GC. The analytical column used, was a DB-5 (30 m x 0.25 mm; 1 µm film; J&W Scientific, Folsom, CA).
The $\delta^{13}C$ values are reported in permille [‰] relative to Vienna PeeDee Belemnite (VPDB):

$$\delta^{13}C = \frac{(\frac{^{13}C}{^{12}C})_{sample} - (\frac{^{13}C}{^{12}C})_{standard}}{\frac{^{13}C}{^{12}C}_{standard}} \; x \; 1000 \; [‰] \qquad (2)$$

CO_2 reference gas was calibrated to V-PDB and was introduced at the beginning and the end of each run. The $\delta^{13}C$ of toluene were determined for samples collected at the port F2-8. The

resulting concentrations of ^{13}C and ^{12}C were calculated based on the following equations: (Kopinke et al., 2005).

$$C^{13C} = C_{total} \times \frac{1}{1+\frac{1}{(1+\frac{\delta^{13}C_{sample}}{1000})\times R_{standard}}} \quad (3)$$

$$C^{12C} = C_{total} - C^{13C} \quad (4)$$

The C^{12C} and C^{13C} stands for concentration of ^{12}C and ^{13}C whereas C_{total} is the total concentration of carbon; $R_{standard}$ is the isotope ratio of the reference carbon source.

The mass balances of both isotopes ^{13}C and ^{12}C and their relative recoveries (RR) were further calculated by applying Equation 11. The stable carbon isotope signature of toluene passing through sampling port F2-8 was calculated according to the following equation:

$$\delta^{13}C_{F2-8} = \frac{\frac{C_{initial}^{13C}\times RR^{13C}}{C_{initial}^{12C}\times RR^{12C}} - R_{standard}}{R_{standard}} \; x\, 1000 \quad [‰] \quad (5)$$

2.2.6 Mathematical Modeling

In general, the transport of solutes in a natural medium should be described by a three-dimensional (3-D) dispersion equation in which dispersion has a tensor form (Bear, 1961;

Scheidegger, 1961). However, the 3-D transport equation may be simplified to a 2-D model in the case of: (i) horizontal line-injection, performed on the infinity long line, spread in the y-direction, and (ii) assumed homogeneous granular porous medium, where the movement of the solute is considered along the x-axis parallel to the flow lines. Such simplified 2-D equation, coupled with the instantaneous equilibrium reaction model and exponential degradation, reads as follows:

$$D_L \frac{\partial^2 C}{\partial x^2} + D_T \frac{\partial^2 C}{\partial z^2} - v \frac{\partial C}{\partial x} = \frac{\partial C}{\partial t} + p \frac{\partial C_s}{\partial t} + \lambda C \qquad (6)$$

with

$$C_s = k_3 C \qquad (7)$$

$$p = \rho(1-n)/n \qquad (8)$$

where v is the mean water flow velocity; D_L, and D_T are the longitudinal and the vertical transversal dispersion coefficients, respectively [L^2T^{-1}]; t is the time variable [T]; C is the solute concentration in water [ML^{-3}]; Cs is the concentration of solute adsorbed by aquifer material due to an instantaneous equilibrium reaction with a linear adsorption isotherm [M/M]; k_3 is the distribution coefficient for the instantaneous equilibrium sorption with a linear adsorption isotherm [L^3M^{-1}], p is the factor needed to express C_s in the same units as C in Equation 6 [ML^{-3}] with n being the effective porosity of the granular medium, and ρ being the density of the aquifer material [ML^{-3}]; λ is the degradation constant [T^{-1}], which represents an irreversible first order reaction, and accounts for biodegradation in the liquid

phase. For substances undergoing biodegradation, the value of λ depends on conditions existing in the investigated system; here λ was assumed to be constant.

In modeling pollutants which undergo an instantaneous equilibrium sorption (Equation 7), the so-called retardation factor (R_3) is often used instead of k_3. R_3 is defined as follows (Maloszewski et al., 2003):

$$R_3 = 1 + pk_3 \tag{9}$$

In the case of non-reactive and non-degradable substances (k_3=0; λ=0), Equation 6 reduces to:

$$D_L \frac{\partial^2 C}{\partial x^2} + D_T \frac{\partial^2 C}{\partial z^2} - v \frac{\partial C}{\partial x} = \frac{\partial C}{\partial t} \tag{10}$$

and describes the transport of a "so-called" ideal tracer.

The analytical solution of Equation 1 or 5, C(x, z, t), in the case of a pulse injection, as applied in the present study, was provided by (Carnahan and Remer, 1984).

The relative recovery (RR) of a tracer mass observed in the cross-section having the surface (F) perpendicular to the flow direction (x) after sufficiently long time (t=∞) can be estimated as follows (Leibundgut et al., 2009):

$$RR(\infty) = Fvn \int_0^{\infty} C(x,z,t')dt'/M \tag{11}$$

where n is the effective porosity of the saturated zone and M is the mass of tracer injected.

2.3 Results

2.3.1 Comparison of toluene and D_2O concentration data

Modeling of the deuterium break-through curves, by using the solution of Equation 10, provided values for water flow velocity (v), as well as for both dispersivities, longitudinal and transversal ($\alpha_L = D_L/v$ and $\alpha_T = D_T/v$). The water velocities found in different observation ports varied between 1.58 m d^{-1} at F3-4 (z = 0.30 m bs) to 1.77 m d^{-1} at F1-4 (z = 0.30 m bs). Modeling results based on the deuterium tracer (D_2O) are summarized in Table 2.1. In the central longitudinal row of the indoor aquifer variations in water velocity were smaller, i.e. v = 1.61 m d^{-1} and v = 1.67 m d^{-1}at F2-8 (z = 0.36 m bs) and F2-9 (z = 0.32 m bs), respectively. The longitudinal dispersivity α_L = 2.18 cm was similar for all ports. The calculated mean water velocity v = 1.65 m d^{-1} was taken for further modeling of the transversal-vertical dispersivity as well as for calculating the mean porosity of the porous material from the observed volumetric flow rate of water through the system. The transversal vertical dispersivity was found to be α_T = 0.035 cm while the calculated effective porosity was equal to n = 0.30.

Under consideration of those transport parameters, the solution of Equation 5 was used to model the toluene concentration curves. Calibration of the model with measured concentrations of toluene was then performed with two fitting parameters: R_3 (Equation 9) and λ. The results of the modeled toluene concentrations as observed in all ports are summarized in Table 2.2, while the curves with best fit are shown in Fig. 2.2 – 2.5.

Tab. 2.1 Transport parameters resulting from modeling of deuterium concentrations observed at different ports (depths) at the cross-section (transect) F at a flow distance of x = 4.2 m from the inlet. ME stands for model efficiency as defined by (Hornberger et al., 1992); ME=100% means an ideal fit.

Port	z [m bs]	T [hrs]	v [m d^{-1}]	α_L [cm]	ME [%]
F2-8	0.36	62.5	1.61	2.18	99.0
F2-9	0.32	60.2	1.67	2.18	99.0
F1-4	0.30	57.0	1.77	2.18	97.5
F3-4	0.30	64.0	1.58	2.18	99.5

Tab. 2.2 Reactive parameters of modeled toluene concentrations for the different ports (depths) at cross-section (transect) F at a flow distance x = 4.2m. ME stands for model efficiency as defined by (Hornberger et al., 1992); ME=100% means ideal fit.

Port	z [m bs]	R_3 [-]	λ [hr^{-1}]	ME [%]
F2-8	0.36	1.26	0.156	98.0
F2-9	0.32	1.27	0.190	98.0
F1-4	0.30	1.26	0.190	92.0
F3-4	0.30	1.25	0.014	95.0

Toluene followed an instantaneous equilibrium sorption with a retardation factor being equal to R_3= 1.26 ± 0.01. The first-order degradation rate (λ) found for toluene varied between 0.156 and 0.190 d^{-1} excluding the results for port F 3-4, where the degradation rate was more than one order of magnitude lower (0.014 d^{-1}). Such low degradation rate would mean that in that part of the system the toluene followed only an instantaneous equilibrium reaction

practically without any degradation. Taking into account the other three ports, the mean degradation rate was found to be λ=0.178 d^{-1}. This corresponds to a mean half-life time constant $T_{1/2}$ (= ln2/λ) of 3.87 days.

Based on transport and reaction parameters (Table 2.1 and 2.2), the relative recovery of deuterium and toluene was calculated according to Equation 11. For the cross-section area (F) being at a flow distance of x = 4.2 m, the relative recoveries of deuterium and toluene were 100% and 61%, respectively. Thus, along the flow distance of 4.2 m and within 150 hours, about 40% of the toluene injected was instantaneously and irreversibly eliminated from the liquid phase via biodegradation.

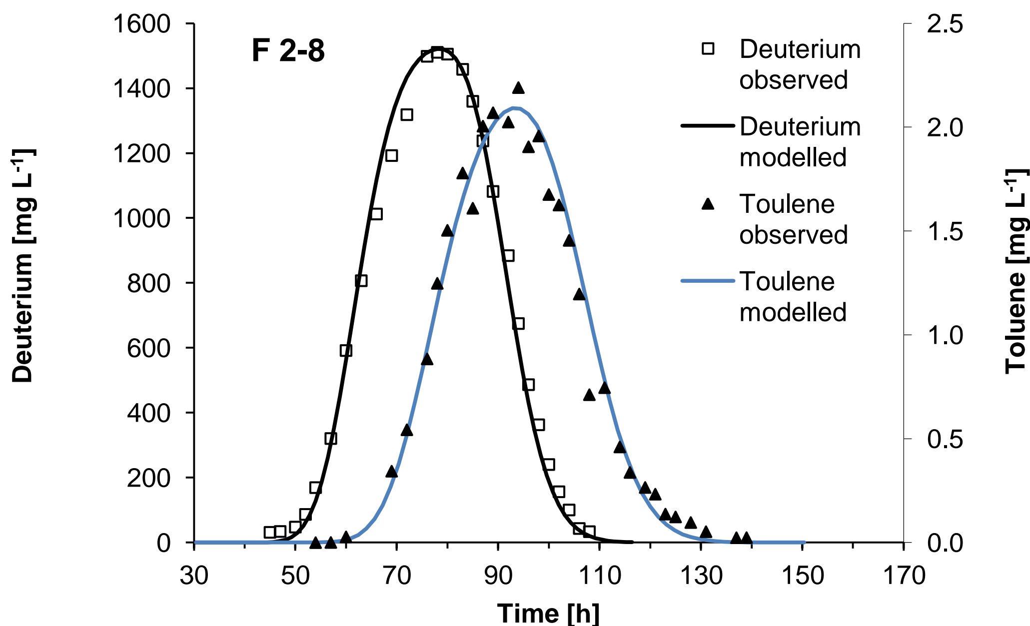

Fig. 2.2 Port F 2-8 (x=4.2 m, z=0.36 m bs); measured (squares, triangles) and modelled (black and blue line) break through concentration curves of deuterium and toluene, respectively.

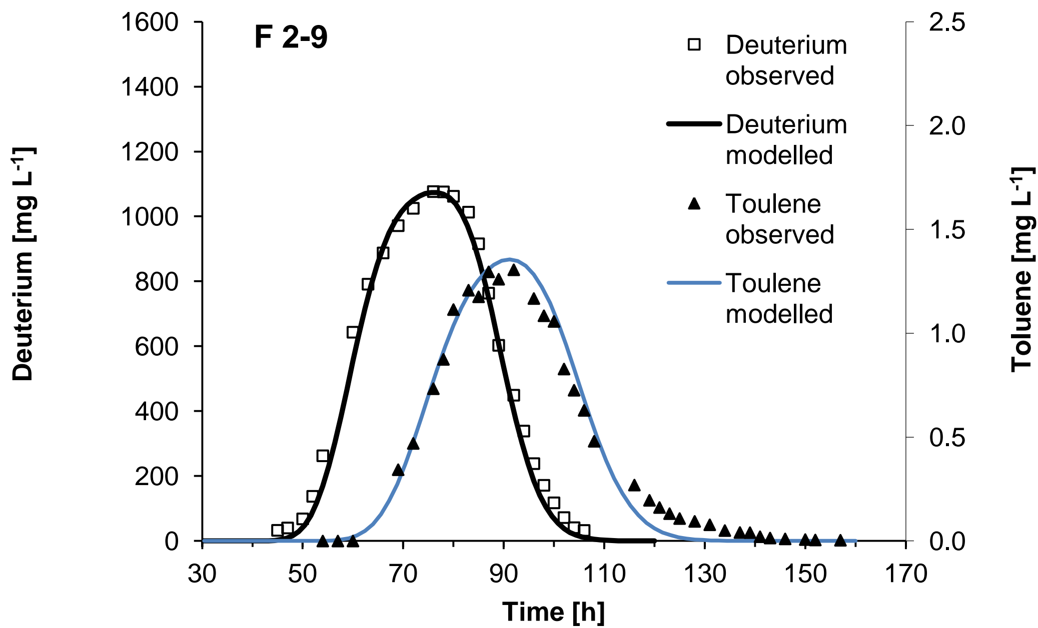

Fig. 2.3 Port F 2-9 (x=4.2 m, z=0.32 m bs); measured (squares, triangles) and modelled (black and blue line) break through concentration curves of deuterium and toluene, respectively.

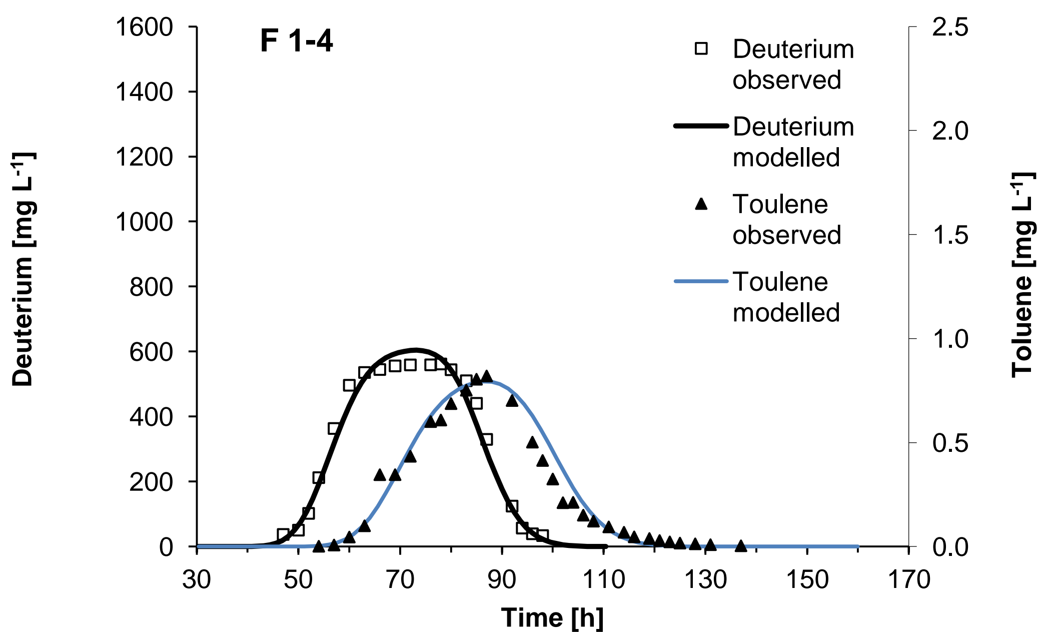

Fig. 2.4 Port F 1-4 (x=4.2 m, z=0.30 m bs); measured (squares, triangles) and modelled (black and blue line) break through concentration curves of deuterium and toluene, respectively.

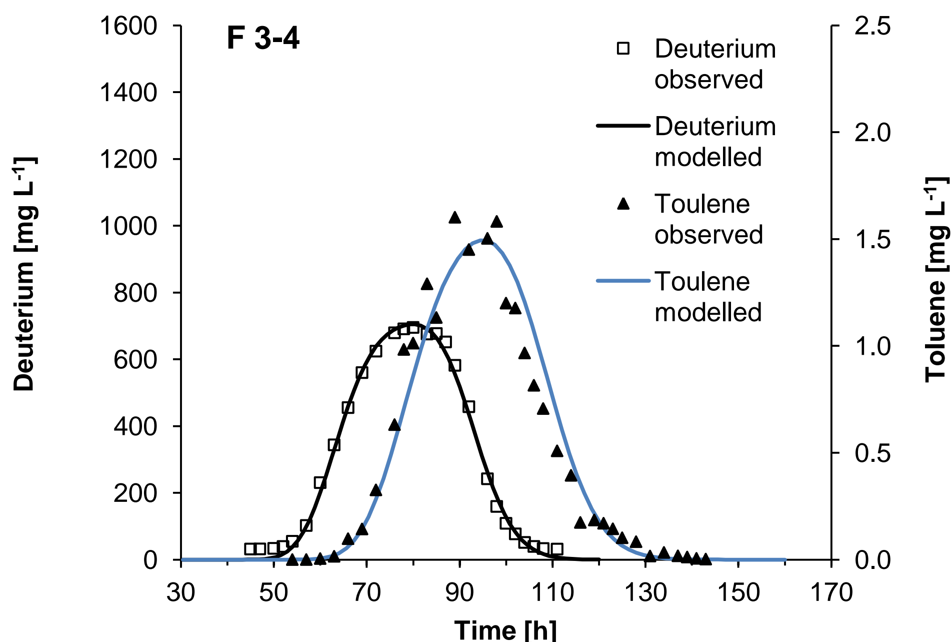

Fig. 2.5 Port F 3-4 (x=4.2 m, z=0.30 m bs): measured (squares, triangles) and modelled (line, bold line) break through concentration curves of deuterium and toluene, respectively.

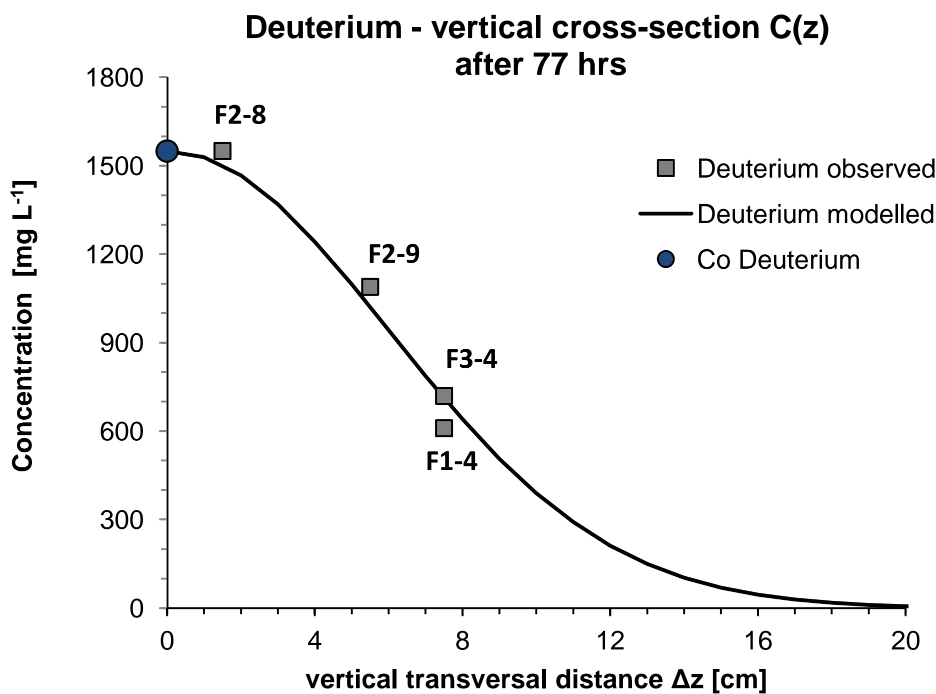

Fig. 2.6 Vertical distribution of deuterium concentrations C(z) measured (squares) and modelled (line) in the flow distance x=4.2m for the time of observation t = 77 hrs after starting of the injection. The blue cycle point stands for calculated Co of deuterium leaving the horizontal mixing well.

2.3.2 Toluene carbon stable isotope composition

Independent of the total mass balance of toluene and D_2O, carbon stable isotope signatures were used to calculate toluene ^{13}C and ^{12}C concentrations. Based on these data, a relative recovery of toluene ^{13}C and ^{12}C (Equation 11) at port F2-8 was found to be 60.5 %, showing the overall enrichment of the heavy isotopes by 0.57 ‰.

2.4 Discussion

The enormous potential of oxic aquifers, and thus aerobic populations of bacteria, to degrade a wide array of organic compounds has been well established and reported in the literature (Gibson and Subramanian, 1984; Aelion et al., 1987; Barker et al., 1987; Aelion and Bradley, 1991; Cerniglia, 1992; Dobbins et al., 1992; Gibson and Parales, 2000; Alfreider and Vogt, 2007) and the role of aerobic biodegradation as a fast and effective process in the initial removal of a contaminant bulk and at the later stage, the formation of an anaerobic plume, has been underlined repetitively (Wiedemeier et al., 1999; Kao and Wang, 2001). However, in many cases investigations of the biodegradation potential of a given oxic aquifer, rely on laboratory experiments with groundwater and/or sediment from that particular site. Since the physical, geochemical and hydrological constrains for the transport and biodegradation of contaminants in field situations are complex and very different from small scale lab settings, very often results from the lab cannot be easily extrapolated to the

field. Moreover, due to sampling schemes with a temporal resolution of days or weeks and an inappropriate spatial resolution (Anneser et al., 2008; Anneser et al., 2010) one may fail to uncover the instantaneous *in situ* biodegradation potential present.

The 30h pulse of a total of 486 mg toluene injected into the pristine, energy limited, indoor aquifer – in fact the natural DOC concentration was always < 1 mg L^{-1} and total bacterial cells numbers accounted for $3 \pm 1.25 \times 10^4$ cells mL^{-1} and $1.28 \pm 0.40 \times 10^6$ cells cm^{-3} in groundwater and attached to sediment particles - has resulted in an instantaneous degradation of 40 % of contaminant mass along a transport distance of 4.20 m and a mean travelling time of 150 hours. Toluene and deuterium break-through curves followed at four selected sampling points close to the outlet of our indoor aquifer system (transect F) indicated toluene reduction to be almost exclusively attributed to biodegradation. Taking into account, that toluene is a compound that also occurs naturally (Hornafius et al., 1999; Head et al., 2003), we may assume that there are always microbes present in indigenous microbial communities, capable of aerobic toluene degradation. However, with respect to the moderate number of isolates of toluene degrading aerobic bacteria, as well as the lacking selective force for toluene oxidation before a contamination occurs the first time, these organisms should account for only very low relative abundances within microbial communities in pristine aquifers. Moreover, mean bacterial growth rates in oligotrophic aquifers are considerably low, with community doubling times typically in the range of days to months or even years, rather than hours (Griebler and Mösslacher, 2003; Wilhartitz et al., 2009). Thus, the 30 hour toluene pulse was most probably not long enough, to give sufficient time for indigenous communities to react, in terms of activation of dormant cells, and substantial growth of specific degraders, but was attributed to an instantaneous response of the aquifer´s native microbial community. As estimated from incorporation of [^{3}H]-leucine into bacterial proteins (Kirchman, 1993) and bacterial abundance data from a foregoing and later

experiment (data not shown), the bacterial carbon production as determined in groundwater from pristine aquifer and after 16 days of constant toluene injection yielded generation times in the range of years. Microbial grow rates estimated form sediment samples were considerably higher compared to groundwater. However, even 16 days long, continuous exposure to toluene did not bring any significant changes in the community generation time. The average generation time of attached bacterial community prior to contamination was 159 ± 74 days and decreased only slightly to 63 ± 44 days upon 16 days of a continuous toluene input, showing no observable increase of the bacterial biomass in pore water and sediment. Mineralization of 40% of toluene mass within that short time is therefore fully attributed to toluene degraders that must have been present in the pristine system and responded immediately once the contaminant appeared. The overall enrichment of $\delta^{13}C$ in the residual toluene at transect F clearly and independently confirms microbial degradation taking place. Some examples from other aquatic ecosystems showed the presence of hydrocarbon-degrading populations of bacteria prior to contamination, providing the backbone for a fast intrinsic biodegradation potential (Delille and Delille, 2000; Margesin et al., 2003; Smets and Pritchard, 2003; Scow and Hicks, 2005; Hazen et al., 2010; Kostka et al., 2011). Thus, with respect to the pulse experiment, we conclude that the pronounced degradation of toluene relies almost exclusively on the instantaneous reaction of aerobic indigenous bacteria already present and active in the indoor aquifer. We might however speculate that in the aquifer facing continuous flow of contaminants, insufficient replenishment of oxygen, as energetically favorable electron acceptor, into the toluene contaminated areas, will result in a continuous decline of this potential with time, turning the contaminated zones from oxic to anoxic. Therefore the high potential for instantaneous aerobic degradation of toluene, or a comparable monoaromatic contaminant, might become electron acceptor-limited as soon as the contamination is not a pulse but continues for days and weeks.

The calculated first-order toluene degradation rate constant (day^{-1}) was estimated as high as λ=0.178 day^{-1}. It is comparable with rates reported for aerobic toluene degradation from *in-situ* mesocosms installed in the oxic, sandy groundwater aquifer (Nielsen et al., 1996), where degradation rates of toluene varied between 0.1 - 0.4 day^{-1}. Much higher toluene degradation rates (λ= 12.5 – 54.8 day^{-1}) were found in sediment column experiments performed by (Angley et al., 1992). A field study of Kao and Wang (2001) conducted in an oxic, porous aquifer contaminated with toluene reports up to 2 orders of magnitude lower degradation rate constant (λ= 0.0043 day^{-1}) compared to our findings. The huge variations in degradation rate constants among different studies might of course be due to the very individual natural attenuation potentials of the systems and material investigated. However, we suggest the different experimental conditions (size of experiment, temperature, (initial) concentration of pollutants) and used approaches (sampling intervals, chemical analysis) substantially contributed to this discrepancy. Our experiment, carried in the field-scale relevant system, import additional information on toluene degradation rate that might better reflect a real field scenario.

This study reports on the presence of a surprising high potential for instantaneous, aerobic degradation of toluene, present in the indigenous microbial community of a pristine, energy-limited indoor aquifer. The toluene virgin groundwater ecosystem was able to cope with a short pulse of toluene, mineralizing 40% of a 486 mg toluene mass within the travel distance of only a few meters and the time frame of less than a week.

2.5 References

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3. Response and recovery of a pristine groundwater ecosystem impacted by toluene contamination – a meso scale indoor aquifer experiment

3.1 Introduction

The widespread problem of groundwater pollution by organic contamination has become an important concern. According to recent estimates, approximately 3 million sites across Europe are seriously polluted. Nearly 250000 sites are scheduled for clean-up, while for the remaining ones investigations continue to establish whether remediation is required. Mineral oil products and chlorinated compounds are the biggest threats for groundwater resources (EEA, 2007). Transport, distribution and biodegradation of organic contaminants in porous aquifers have been studied extensively in the past decades at the lab scale as well as in the field (Stapleton and Sayler, 1998; Christensen et al., 2000; Thornton et al., 2001; Anneser et al., 2008; Bauer et al., 2008). Studies focused on different aspects of microbial community activity and composition, as well as on environmental factors, e.g. sediment heterogeneity (Bauer et al., 2009) and transverse dispersion (Prommer et al., 2002; Cirpka and Valocchi, 2007) influencing degradation processes. Huge variations in degradation rates, extent and completeness of biodegradation from one site to another indicate that extensive knowledge of the subsurface as a microbial habitat is indispensable, especially in terms of where the biodegradation potential comes from and how it is activated in pristine systems.

The behaviour of pollutants in aquifers is influenced by the nature and the amount of the contaminant and the interplay of chemical, hydrogeological and biological factors (Leahy and Corwell, 1990; Galvão et al., 2005). The functional capabilities of indigenous microbial community constitute other important driving force for a systems` natural attenuation (NA). While our understanding of the fate of specific contaminants in aquifers is steadily growing,

the knowledge on the ecosystems` reaction to the contamination remains still poor. Groundwater ecosystems are expected to be extremely vulnerable to contamination. Their highly specific environmental conditions (e.g. relatively secluded, dark, scarce in energy), low productivity of their communities, simple trophic networks, and the proposed narrow ecological amplitude of most groundwater organisms (Balkwill and Ghiorse, 1985; Danielopol et al., 2000; Gibert and Deharveng, 2002; Griebler and Lueders, 2009), lead to extended times of recovery from disturbance. Consequently, a profound understanding of ecosystems` performance under pristine and contaminated conditions, with a special focus on microbial communities as resistance and resilience trajectories of groundwater systems, is of great importance and still a major challenge.

Detailed information on how and in what time frame pristine groundwater ecosystems respond to contamination with petroleum hydrocarbons is yet limited. The same holds true for their recovery. Reasons are the very few studies conducted to assess the impact of petroleum contamination on pristine ecosystems (Evans et al., 2004; Kaufmann et al., 2004; Bordenave et al., 2007), and even more important, the lack of pre-disturbance data which allow to properly assess the ecosystem resilience. Although there is a pile of laboratory and field studies on NA of aromatic hydrocarbons, none have comprehensively addressed the succession of geochemical conditions and indigenous microbial community patterns from pristine conditions through the phase of contaminant exposure to a quasi steady state of the system and back to predisturbance status.

In this study, we present a comprehensive dataset of spatially and temporally connected biochemistry and microbiology of water and sediment samples from an indoor sandy groundwater system. Changes in the microbial community composition along with its functional patterns (eg. abundance, activity, diversity) were followed upon exposure of the initially pristine system to constant input as well as an accidental spill of toluene. Moreover,

the systems recovery upon the switch off of the contaminant source was evaluated. Natural attenuation of toluene is analyzed in terms of dominating redox processes and in perspective of possible limitations.

3.2 Material and methods

3.2.1 Experimental design

The experiment has been conducted in the indoor aquifer which in detail is described in the Introduction of this thesis. The results presented in this chapter cover following sampling campaigns: P (pristine, before contamination), day 4, 16, 63 (contaminated conditions, constant toluene injection), 137 (contaminated conditions, pulse injection of toluene phase) and day 231(recovery). Water data originates from the highly resolved sampling ports of row 2, collected at transect A, C and F. Sediment liners were obtained along the row 2 at transect A, C and F.

3.2.2 Toluene injection

Water fully saturated with toluene (500 ± 30 mg L^{-1}) and amended with 250 mg L^{-1} of bromide, as an internal tracer, was injected into the aquifer over a period of 133 days with a volumetric flow rate of 0.78 L day^{-1}, which corresponds to a contaminant mass of 390 ± 23.4 mg day^{-1}. At a later stage of the experiment, directly following the continuous injection, a 6h pulse of 200 mL of pure toluene was injected to the aquifer.

The toluene was injected via 16 stainless steel capillaries into a horizontally placed perforated tube located in the inflow area of the system, allowing distribution of the contaminant across

the entire width of the model aquifer generating a contaminant plume (see the Introduction, Fig.1.1).

3.2.3 Sampling conditions

To prevent contaminant sorption, volatilization and eventual re-oxidation of reduced components, Fluran tubes (Ismatec, Wertheim, Germany) and stainless steel capillaries submerged to the bottom of the narrow-neck, sterile, carbon free bottles were used. Peristaltic pumps with a pump rate of 0.5 mL min^{-1} were used for sampling. Water samples were always collected starting from the furthest downstream transect (F) and moving upstream, sampling simultaneously all chosen ports at 3 rows within one transect. Detailed depth profiles of certain variables derived from water samples are presented only from row 2 due to its high resolved water ports location. The collected water was processed immediately for the analysis of a number of biotic and abiotic parameters. Water samples for microbial community analysis were collected in 1 L 230°C baked Schott bottles, sealed on the top and kept on ice during sampling time. In total approximately 1150 mL of water was collected from one sampling port and used for different assays as described below. Sediment sampling was always conducted at the end of the water sampling campaign. During each sampling campaign at minimum three sediment cores, with diameter of 4 cm and length of approx. 60 cm, were taken by direct push coring technique using a manual geoprobe device (Eijkelkamp Agrisearch Equipment, Netherlands). Each sediment liner was divided in max. 12 pieces and each unit was sub-sampled immediately for a selected set of parameters or frozen in -80 °C for subsequent DNA extraction and further analysis of the microbial community composition. The boreholes were refilled with wet sediment of the same batch that had been used for packing the indoor aquifer.

3.2.4 Physical-chemical analysis

The water samples for determination of the major anions and cations was filtered through a 0.45 µm filter and analysed by ion chromatography on a Dionex DC-100 device (Dionex, Idstein, Germany). For toluene concentration analysis, a volume of 3 mL was transferred into 10 mL GC-MS vials and amended with NaOH (0.1 M final concentration) to terminate microbial activity. Vials were tightly closed with polytetrafluoroethylene (PTFE) caps. If not analyzed immediately, samples were stored at -20 °C. Toluene concentration was determined by GC-MS headspace analysis (Thermo Electron, Dreieich, Germany). The dissolved organic carbon was measured using high temperature combustion with infrared detection of CO_2 (Shimadzu TOC-5000). Dissolved oxygen concentration was measured in situ via oxygen optodes connected to a FIBOX3 Fiber-optic oxygen meter (PreSens GmbH, Regensburg, Germany). Temperature measurements were performed using 8 digital semiconductor temperature sensors connected to a data logger from which the temperature could be read out immediately. The oxygen, pH and temperature measurements in the inflow and outflow were performed using field sensors (WTW, Weilheim, Germany).

3.2.5 Determination of bacterial cell numbers

For the enumeration of bacterial cell numbers 1 mL of water and 0.5 mL of sediment aliquots were fixed with glutardialdehyde (final concentration 2.5 %) and kept at 4 °C until further processing. Total cell counts were quantified in a flow cytometer (LSR II, Becton Dickinson, Heidelberg, Germany) equipped with a 488 nm and 633 nm laser. Sample preparation, flow cytometric analysis and instrument settings were as described by Hammes et al. (2008) and Bayer et al. (submitted). Briefly, 1 mL of water sample was mixed with 250 µL of PBS buffer containing commercially purchased beads serving as an internal standard

(TrueCount Tubes, Becton-Dickinson) and stained with SYBR Green I at a ratio of 1:10 000. Attached cells had to be separated beforehand and abiotic particles had to be removed via density gradient centrifugation following the protocol of Lindahl and Bakken (1995). Subsequently, 100 µL of supernatant from the sediment was mixed with PBS buffer and stained with SYBR Green I. Samples were always measured in duplicate and the BD FACSDiva Software Package (Becton Dickinson) was used for data analysis. Calculation of total bacterial cell numbers were performed as described in Nebe-von-Caron (2000).

3.2.6 Determination of living biomass

The concentration of adenosine tri-phosphate (ATP) was used to assess the viable microbial biomass in water and sediment samples. Cellular ATP was measured using the BacTiter-Glo Microbial Viability Assay reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA) following in principle the protocol of (Hammes et al., 2010), with slight modifications. In short, preheated (38 °C) 1 mL aliquots of water samples and 50 µL of reagent was combined in sterile 2 mL reaction tubes (Greiner Bio-One). After 20 sec of incubation at the same temperature, luminescence was measured. The same procedure was repeated for 0.1 µm filtered (Millex®-GP, Milipore filter unit) samples, thus allowing the determination of total and cellular ATP. Handling of sediment samples was as follows: 200 µL of sediment sample aliquots containing additionally 50 µL of ATP-free water were warmed for 10 min at 38 °C. Thereafter, 100 µL of preheated reagent and 900 µL of ATP-free preheated water were transferred to the samples. The mixture was incubated and shaken for 2 min on a thermoshaker (Eppendorf) at 38 °C, 500 rpm. Subsequently, samples were shortly spun down (14000 rpm, 10 sec.) and the collected supernatant (1mL) was transferred to a separate sterile reaction tube and measured.

Data were collected as relative light units (RLU) and which were converted to ATP [M] by means of a calibration curve with known ATP concentrations (Roche, Mannheim, Germany).

3.2.7 Measurement of microbial activity

The [^{3}H]-leucine incorporation method modified as originally described by Chin-Leo and Kirchman (1988) was used to determine bacterial carbon production. For each water sample, three aliquots of 25 mL each and one formaldehyde-killed control were incubated with [^{3}H]-leucine at a saturating concentration (10 nM, final concentration) for 14h at *in situ* temperatures in the darkness. Incubation was stopped with the addition of formaldehyde (final concentration 3.7%). Anoxic water samples were filled into the vials without leaving the headspace. To extract and collect the macromolecular fractions (RNA, DNA and proteins), samples were placed on ice for 15 min and trichloroacetic acid was added (final concentration 5%). Following extraction, samples were filtered through 0.2 µm cellulose nitrate filters (Whatman, Germany). The filters were rinsed twice with 5 mL of ice cold 5% TCA and Mili-Q water, before placed in scintillation vials, dried and dissolved in 1 mL of ethylacetate. Subsequently, scintillation cocktail was added (Ultima Gold XR, Perkin Elmer) and samples were counted with a liquid scintillation counter Tri-Carb 2900TR (Packard BioScience). For each sediment sample, three replicates and one trichloroacetic acid-fixed control were prepared, 0.5 mL sediment, in 2 mL vials (Biozym) containing 700 µL of sterile Mili-Q water. Samples were incubated with a mixture of [^{3}H]-labeled and non labeled leucine (concentration 500 nM, at ratio of 1:1) reaching a final concentration of 100 nM in the samples. Incubation was conducted over 6h at *in situ* temperature in the dark and was terminated by adding of TCA (final concentration 5%) to the replicates. Samples were kept at +4°C until further processing. Washing and extraction of proteins was done by two series of

centrifugation (14000 g, 10 min) and Mili-Q water rinsing steps. Proteins were extracted by adding 900 µL of alkaline extractant and 1h incubation on a thermoshaker at 99 °C and 1000 rpm. Afterwards samples were centrifuged (10 min, 14000 rpm) and 100 µL of supernatant was placed into the vial, and radioassayed in scintillation counter. Quenching curve, linearity of substrate turnover and substrate saturation concentration had been determined before.

3.2.8 DNA extraction and T-RFLP

DNA was extracted in triplicates from each sediment aliquots of liner A, C and F. A modified protocol from Gabor et al. (2003), Lueders et al. (2004) and Winderl et al. (2008) was implemented. Due to the low microbial abundance in groundwater ecosystems, DNA extraction had to be as yield-optimized as possible. Therefore, cell disruption was performed using a combination of an enzymatic (Lysozyme and Proteinase K), a mechanical (bead beating with 0.1 and 0.7 mm Zircona/Silicia beads) and a chemical (phenol-chloroform) method. DNA was extracted over two days, with a precipitation step overnight, afterwards eluted in 20-30 µl EB buffer and stored at -20° C. For terminal restriction fragment length polymorphism (T-RFLP), PCR amplification of bacterial 16S rRNA gene was conducted with bacterial primers Ba27f-FAM and 907r, and MspI enzyme used for digestion as described in (Pilloni et al., 2011). Purified (Min Elute, Qiagen), restricted and desalted (DyeEx, Qiagen) fragments were subjected to capillary electrophoresis on a 3730 ABI DNA Analyzer (Applied Biosystems). Primary evaluation of electropherograms was performed using Gene Mapper 5.1 software (Applied Biosystems). Further analyses were done with free access software T-REX (available under http://trex.biohpc.org/) as reported by Winderl et al. (2008). Diversity indices, e.g. Shannon-Wiener index (*H*') were calculated using the PAST software (http://folk.uio.no/ohammer/past/index.html). Pareto-Lorenz distribution curves

were plotted from the cumulative relative abundance of each T-RF and cumulative relative proportion of the number of T-RFs for each sample. To access community evenness, the Gini coefficient (G) ranging from 0 to 1 was calculated geometrically by estimating the area under the Lorenz curves. The higher the Gini coefficient is the more uneven the community is. The number of T-RFs was used to evaluate species richness.

3.2.9 Statistical analysis

To explore the relationship between microbial community variables in different experimental conditions, One Way Analysis of Variance (ANOVA) was applied if the dataset was normally distributed and showed equal variance between the groups. In all other cases a Kruskal-Wallis ANOVA was used. Normal distribution was tested using the Shapiro-Wilk test. A p-value of 0.05 was set as significance threshold. Correlations among measured variables were explored by a Pearson product correlation or by a Spearman rank order correlation matrix according to the results of the normality test. All statistical analyses were performed using the statistic package in SigmaPlot 12.0 for Windows. Bacterial community patterns were explored by means of correspondence analysis using free software PAST.

3.2.10 Conservative transport model

The conservative transport model was carried out to simulate the transversal vertical distribution of toluene $C(z)/C_o$ for different transects using the parameters summarized in the Tab.3.S1 assuming continuous injection of toluene through the section (mixing pipe) with vertical width of 0.06 m. For theoretical calculations the migration of toluene was assumed to be conservative, following only advection and dispersion processes.

3.3 Results

3.3.1 Characterization of pristine starting conditions

Prior to the contamination, physical – chemical parameters as well as microbial patterns of the indoor aquifer exhibited features typical for pristine groundwater ecosystem. The experimental system was characterized by oxic conditions (7.27 - 8.93 mg L^{-1}), low concentrations of dissolved organic carbon (DOC) (0.69 – 0.78 mg L^{-1}), low sediment total organic matter (TOM) content (0. 25 ± 0.1 mg g^{-1} dw), and low concentrations of nitrate (5.27 ± 0.93 mg L^{-1}) and sulfate (7.34 ± 1.11 mg L^{-1}). The oligotrophic nature of the system was further underlined by low cell numbers, small cell size and reduced microbial activities.

Designing the aquifer as close to nature as feasible, (e.g. implementation of a highly conductive quartz sand lens and use of unsieved sediment as the matrix), while still being controllable, led to an aimed sediment heterogeneity reflected further in natural variations of microbial patterns. Total number of cells (TNC) in the groundwater varied between the transects ranging from 2.08 ± 1.01 x 10^4 cells mL^{-1} at transect A to 4.38 ± 0.54 x 10^4 cells mL^{-1} further downgradient, at transect F. ATP, referring to living and active biomass, was in the range of ATP concentrations found in drinking water (Lautenschlager et al., 2010) and ranged between 4.62 ± 3.11 pM at transect F to 12.80 ± 5.82 pM at transect A. Measurements of ^{3}H-Leu incorporation in suspended bacterial cells revealed exceptionally low carbon production rates ranging from 2.66 to 13.6 ngC L^{-1} h^{-1} resulting in doubling times between 3.21 x10^3 and 8.74 x10^3 days.

The pristine sediment was characterized by a significantly higher abundance of attached bacteria, higher ATP concentrations and bacterial carbon production (BCP) rates. The abundance of attached bacteria was comparable for the different transect. The mean density of attached bacterial cells in the aquifer was 1.28 ± 0.40 x 10^6 cells mL^{-1} of sediment,

on average 2 orders of magnitude higher compared to the water samples. Mean heterotrophic bacterial carbon production was 7.90 ± 3.08 x 10^{-3} ngC mL^{-1} h^{-1}. Cellular ATP concentrations of attached bacteria were 6 to 16 folds higher when compared to ATP concentration in pore water, with an average value of 73.82 ± 5.8 pM.

T-RFLP fingerprinting of the pristine sediment samples revealed a quite diverse and even bacterial community. The inferred Shannon-Wiener index used as a measure of diversity was in the range of H' = 2.0 - 3.5 and evenness calculated from Gini Coefficient was G = 0.62 ± 0.06.

3.3.2 The continuous toluene source

Toluene was injected at a concentration of 500 ± 30 mg L^{-1} into the horizontal mixing well at an injection height of 0.35 m initializing a thin contaminant plume expanding horizontally over the whole width of the indoor aquifer system. As calculated, a constant point source contamination of 25 mg L^{-1} of toluene was leaving the horizontal mixing well. Vertical spreading of toluene was restricted to a narrow (approx. 0.06 m wide) zone at transect A. After passing the highly conductive lens, the plume exhibited an approximate vertical thickness of 0.10 m and 0.20 m at transect C and F respectively. The vertical position of the plume core was subjected to some fluctuations by ± 4 cm with transect and experiment time most probably due to the hydraulic gradient present and repeated disturbances by sediment sampling activities. A computed steady state transversal vertical spreading of toluene at different transects is shown in the Fig.3.S2.

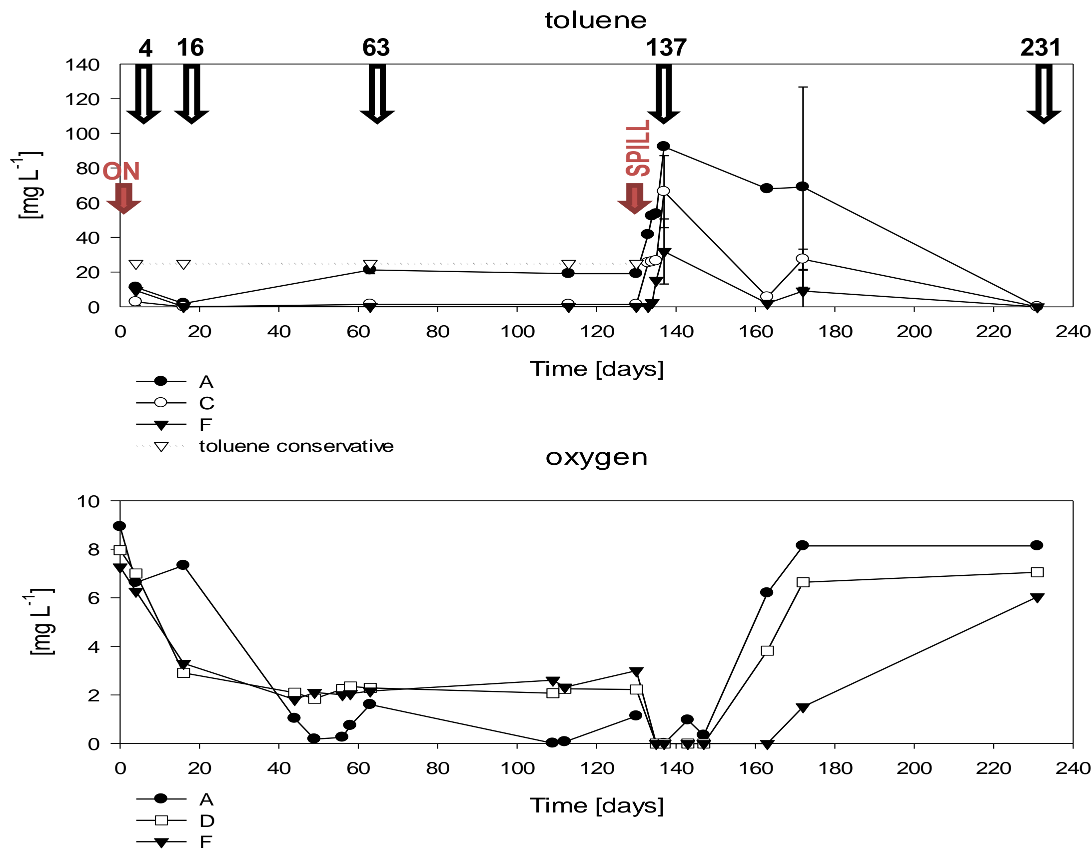

Fig. 3.1 Spatio-temporal dynamics of toluene (transect A, C, F) and oxygen (transect A, D, F) in the indoor aquifer during contaminant exposure and recovery phase. Dotted line shows conservative, modelled toluene concentration.

3.3.3 System response to the continuous toluene source – Day 16

At the early stage, the introduction of toluene to the oligotrophic, pristine aquifer did not significantly alter the total number of attached and suspended cells, however, it led to a dramatic reorganization of the bacterial communities inside the toluene plume. Moreover, 16 days after switching on the continuous toluene source, the toluene concentration at transect A was 1.85 ± 0.06 mg L^{-1}, which equal to an overall decrease of toluene mass by 95% (Tab.3.S2). Further downgradient, at transects C and F, concentrations were already close or below the detection limit (10 ng L^{-1}) pointing at a complete degradation of toluene. The disappearance of toluene went along with a decline in oxygen concentrations in the aquifer,

indicating aerobic mineralization. At transect A the dissolved oxygen dropped to 7.33 mg L^{-1}, whereas concentration at transect D and F were significantly lower with 2.91 mg L^{-1} and 3.30 mg L^{-1}, respectively (Fig.3.1). The still high oxygen concentration at transect A was unexpected, however, might be explained by fluctuations of the very narrow toluene plume which could led to a temporal appearance of the oxygen sensor outside of the plume core. No significant decrease of nitrate and sulfate concentrations in the toluene plume compared to the ambient water occurred at this stage of the experiment.

Sediment bacterial community fingerprinting data revealed a strong decrease in the Shannon diversity inside the toluene plume, especially pronounced close to the source of the plume. At transect A the average microbial diversity outside the toluene plume was H`= 3.14 ± 0.25 and dropped to $H$`= 2.34 ± 0.45 in the contaminated area. Here, the Shannon diversity was found to be negatively correlated to the toluene concentration (r = -0.902, P = 0.005). Downgradient the bacterial diversity varied from H`= 3.12 ± 0.48 outside the plume to $H$`= 2.68 ± 0.37 inside, at transect C and H`= 3.35 ± 0.33 outside and $H$`= 2.87 ± 0.50 inside at transect F, respectively. Shifts in the bacterial community composition in the toluene impacted areas went along with changes in community evenness, again most pronounced at transect A. Exemplarily, Pareto–Lorenz distribution curves for each sediment sample from transect A are shown in Fig.3.S1. The Gini coefficient revealed a significantly more uneven microbial community in the toluene plume, G = 0.75 ± 0.09, than in non-impacted areas of the aquifer, G = 0.65 ± 0.03 (ANOVA, Q = 2.38, p = 0.017). The significant changes in community composition and the high values of Gini coefficient in the toluene plume hint at the disappearance of sensitive species and the establishment of specialized degrader populations. The low bacterial richness (determined from the number of T-RFs) supports those findings. Bacterial richness ranged from 30 to 113 T-RFs in individual samples, always lowest inside the toluene plume.

Bacterial community profiles from transect A, C and F showed the establishment of individual T-RFs, (eg. 490 bp, 488 bp, 437 bp, and 428 bp) found almost exclusively inside the toluene plume (Fig.3.3). In contrast, the T-RFs 139 bp, 147 bp, 159 bp and 180 bp appeared to be present under non-contaminated conditions as well as in the contaminated areas, however, at a much lower relative abundance. Other bacterial lineages seemed to completely disappear while facing contamination, such as the T-RF 491 bp suggesting its intolerance against toluene (toxic effect). Among T-RFs found in the toluene plume, the T-RF 490 bp was the most dominant one, with a relative abundance of 24%, 30% and 23% at the transect A, C and F, respectively, and not detected before the contamination. At transect A the new ecological niche was shared with the T-RFs 428 bp, 437 bp and 488 bp accounting for a 19%, 8% and 7% of the total community in the plume. Those T-RFs were less abundant at transects further downgradient. To provide taxonomic and phylogenetic information of the microorganisms involved in the early response to toluene contamination, selected samples will be subjected to sequencing.

ATP, a proxy for the active microbial biomass, pointed at higher proportion of active cells within the toluene plume compared to the non-contaminated areas. The difference in ATP was especially pronounced at transect A. Here, the ATP concentration of attached cells was 8 folds higher compared to the pristine conditions with an average of 0.61 ± 0.35 nM (ANOVA, $Q = 5.04$, $p = 0.05$). No obvious change of the ATP concentration could be observed in the water fraction.

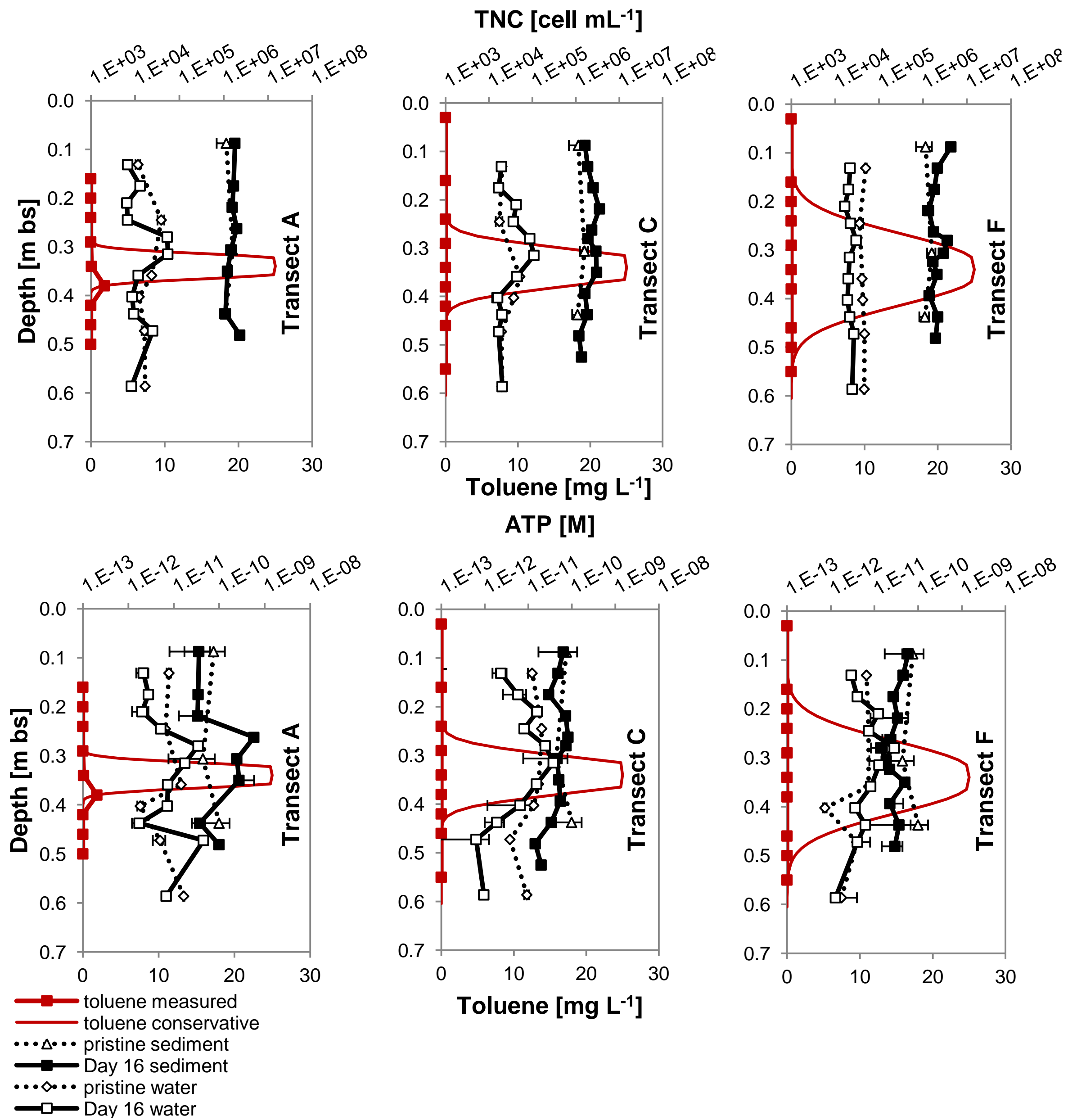

Fig. 3.2 Depth profiles of toluene (measured and modelled concentration for conservative transport), total bacterial cell numbers (TNC) and ATP concentration of attached and suspended cells at transect A, C and F after 16 days of constant toluene injection.

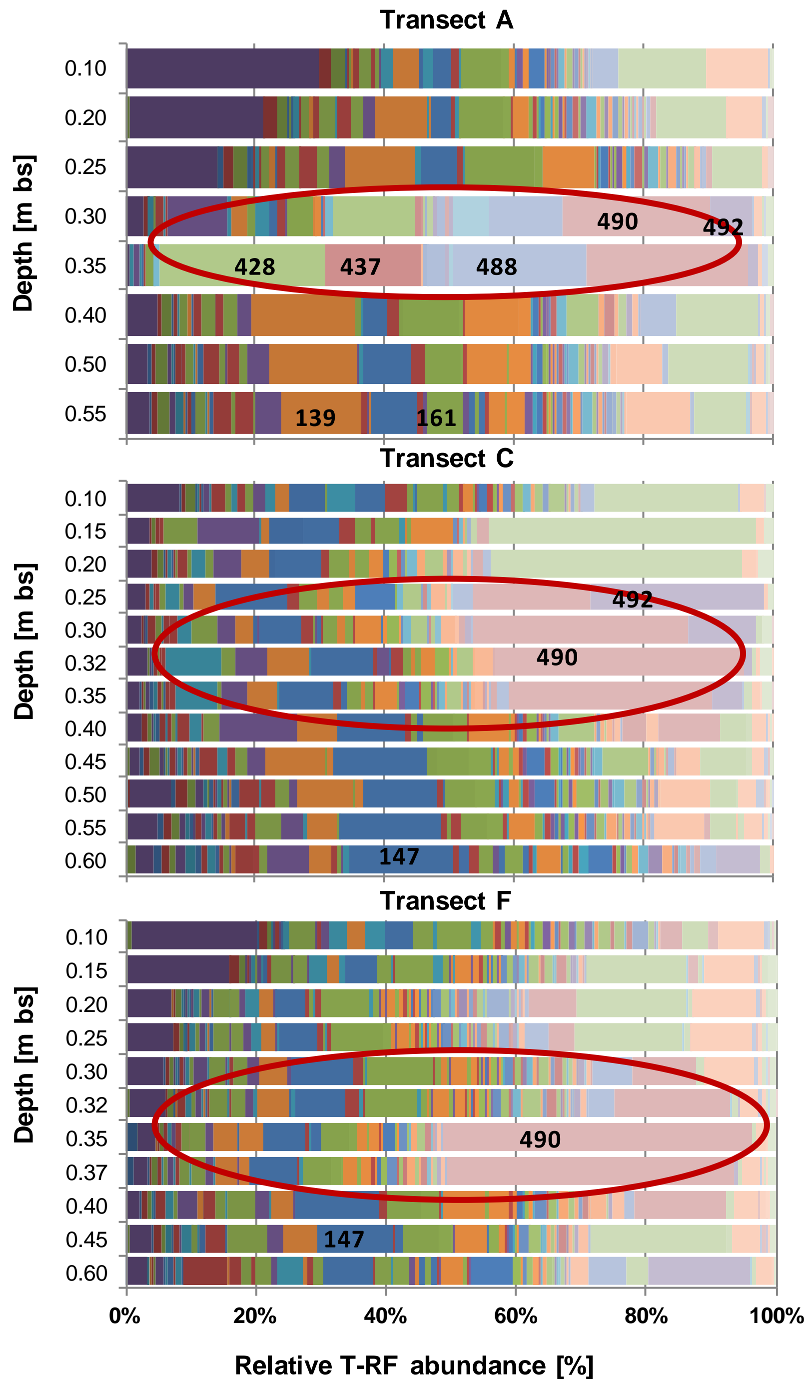

Fig. 3.3 Vertical patterns of bacterial community composition from T-RFLP fingerprinting in the sediments at transect A, C and F after 16 days of continuous toluene injection. The position of the toluene plume at the respective transect is indicated with the red oval. Dominant T-RFs inside and outside of the impacted area are highlighted.

3.3.4 Mid-term response – Day 63

The water and sediment sampling campaign 9 weeks after the switch on of the continuous toluene injection revealed an unexpected picture. Toluene, at day 16 being decreased by almost 95% at transect A, and not detectable further downgradient, now was high at transect A, with a maximum concentration of 21.24 ± 2.14 mg L^{-1}. Downgradient transects exhibited lower toluene concentrations of 1.40 ± 0.01 mg L^{-1} at transect C and 0.19 mg L^{-1} at transect F. Since day 44, oxygen measurements pointed at microaerophilic and locally anoxic conditions in the contaminated areas of the aquifer (Fig.3.1). At day 63 even a slight decline in nitrate was observed in the plume core hinting at the initiation of denitrification activities and anaerobic toluene degradation.

Sediment bacterial community analyses revealed less pronounced differences in degrader community composition compare to the community patterns at day 16. However the T-RFs 490 bp and 488 bp still constituted the key degrader population in the plume. T-RF abundances of 28%, 10% and 13% were observed for 490 bp peaks in the toluene plume at transect A, C and F respectively, whereas the 488 bp T-RF was present at maximum frequency of 10%, 3% and 2%. At day 63, a new prominent T-RF 77 bp appeared in the plume area at transect A, with a considerable abundance of 4 %. The Shannon-Wiener diversity *H*` was very similar with depth exhibiting no relevant deviation between contaminated and non-contaminated areas. However, bacterial diversity differed along the longitudinal gradient revealing for transect A the lowest bacterial diversity of $H' = 2.63 \pm 0.28$ and an average diversity of $H' = 3.59 \pm 0.19$ at transects C and F.

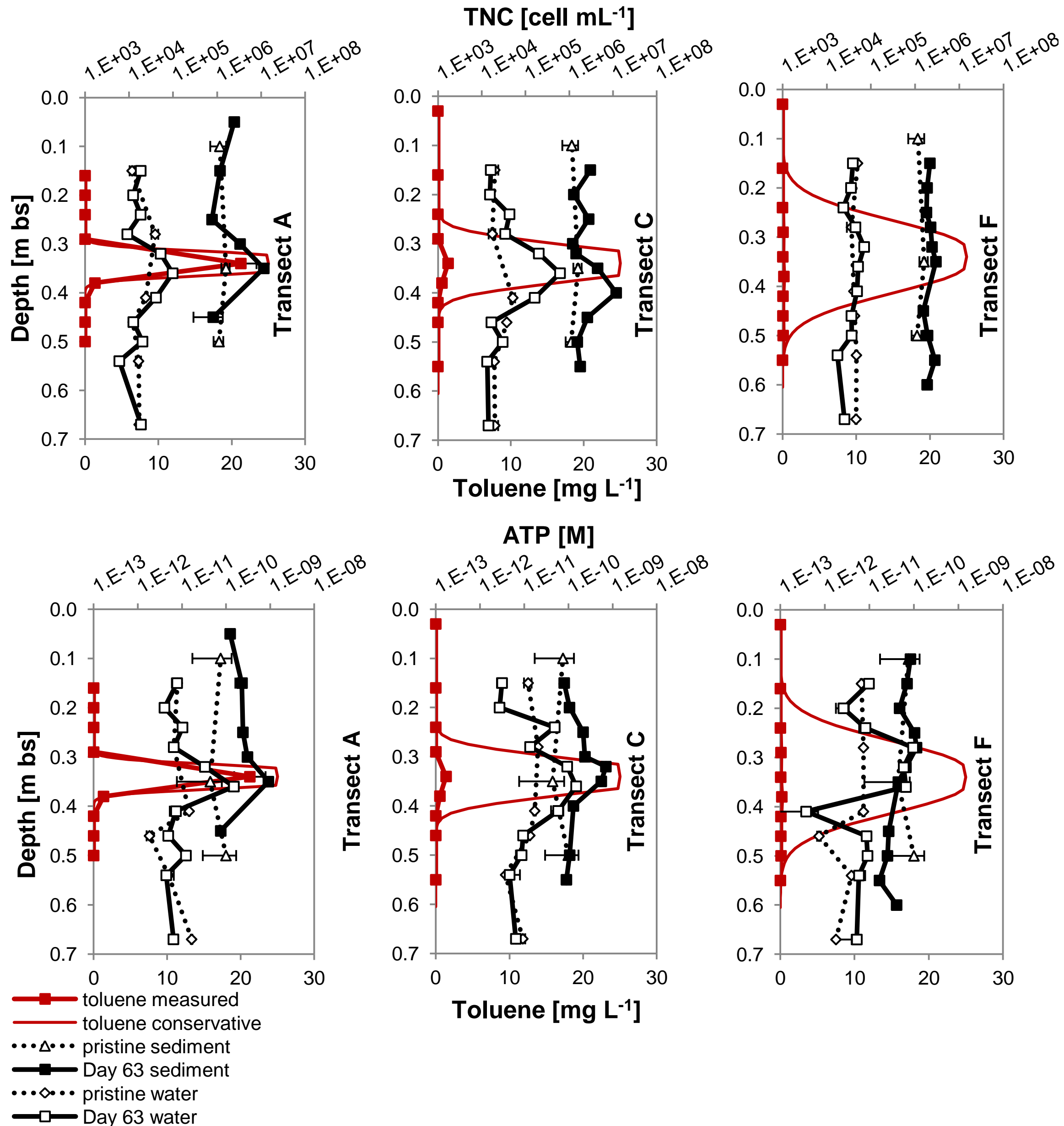

Fig. 3.4 Depth profiles of toluene (measured and modelled concentration for conservative transport), total bacterial cell numbers (TNC) and ATP concentration of attached and suspended cells at transect A, C and F after 63 days of constant toluene injection.

At day 63, a significant, positively related to toluene, increase of bacterial cells in the contaminated zone was found in water and sediment fraction at transect A ($r = 0.72$, $p \leq 0.01$) and C ($r = 0.79$, $p \leq 0.01$) (Fig.3.4). No change in cell numbers were observed at transect F.

Suspended cells were on average 1.7 to 13.8 times more abundant in the presence of toluene, with the highest cell numbers at transect C and lowest at transect F (Fig.3.4). All three sediment liners showed a trend of decreasing cell numbers with the distance to the contaminant source. Inside the plume the number of attached cells was highest at transect A exhibiting a mean value of $7.44 \pm 4.75 \times 10^6$ cells mL^{-1} which was on average 5.7 fold higher than in the non-contaminated area and was found to be positively correlated to toluene ($r = 0.91$, $P \leq 0.01$). Downgradient, a decrease in cell numbers was observed with $5.10 \pm 4.35 \times 10^6$ and $2.28 \pm 0.55 \times 10^6$ cells mL^{-1}, and a calculated ratio between cells inside and outside of the toluene plume was 2.6 and 1.1 at transect C and F respectively. The same trend could be seen with the ATP concentrations pointing at a higher proportion of active cells in the plume. In details, ATP concentration at transects A and C were on average 0.61 ± 0.35 nM and 0.46 ± 0.27 nM respectively. Bacterial carbon production of the attached bacterial community revealed doubling times of 67 days in the toluene plume, compared to 251 days in non contaminated areas.

3.3.5 The accidental toluene spill – Day 137

Regular monitoring of toluene and oxygen before and during the accidental toluene spill showed fast increasing concentrations of toluene accompanied by rapid oxygen consumption in the contaminated areas. Zones above and below the toluene plume remained fully oxic. Concomitant with the complete depletion of oxygen, a decrease in nitrate was observed.

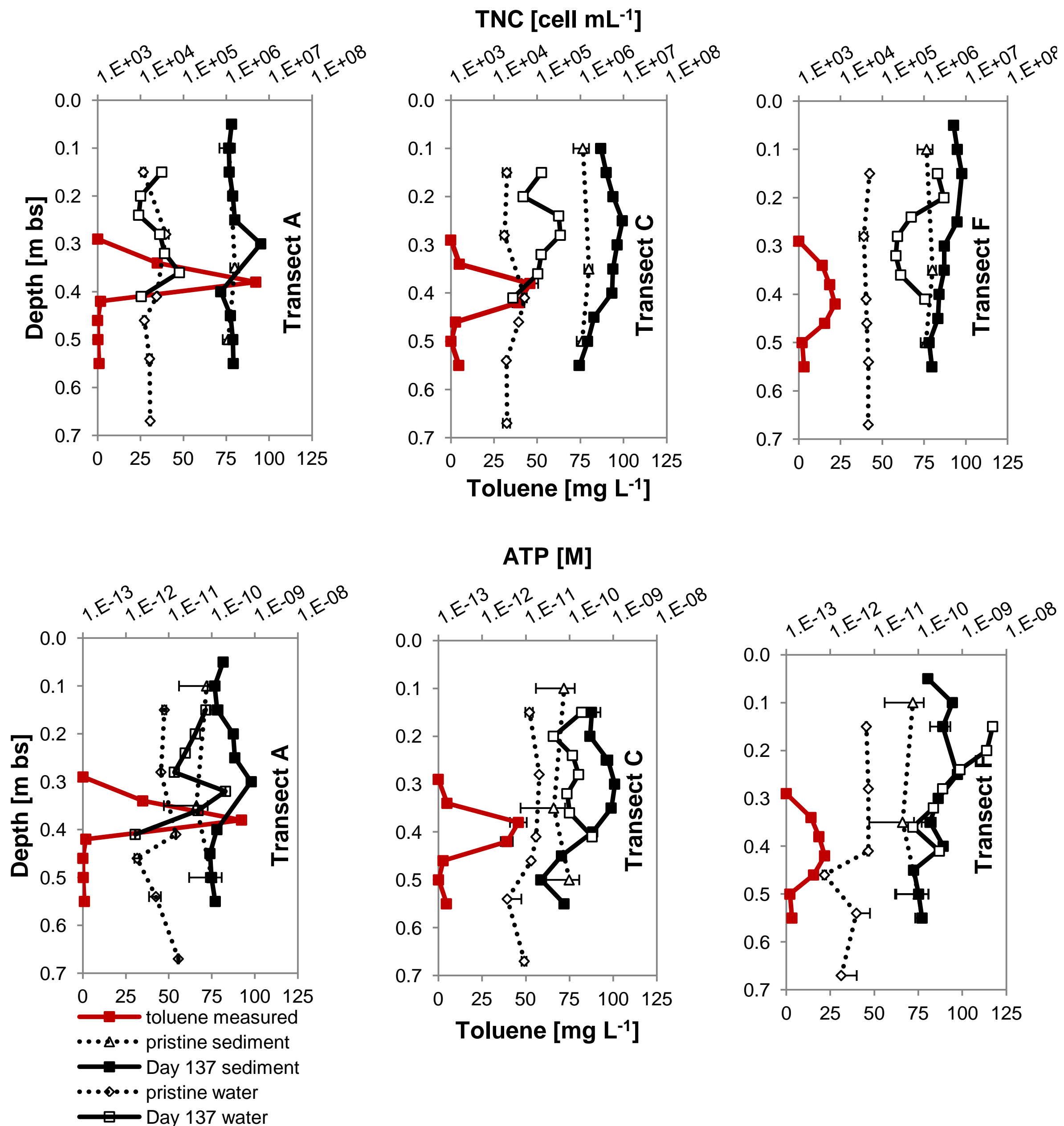

Fig. 3.5 Depth profiles of toluene, the total bacterial cell numbers (TNC) and ATP concentration of attached and suspended cells at transect A, C and F during the accidental toluene spill at day 137.

The injection of 200 mL pure toluene over a period of 6 hours resulted in widening of the plume (Fig.3.5). As a consequence, areas being previously unaffected, now faced toluene and turned anoxic exhibiting elevated cell numbers, ATP concentrations and changes in the bacterial community composition. The aquifer zones exposed to the continuous toluene

source from the very beginning, showed no changes in bacterial cell numbers during the toluene spill. Despite very high toluene concentrations ranging from 92.26 ± 1.1 mg L^{-1} to 31.29 ± 18.77 mg L^{-1} at 0.15 m (transect A) and 4.25 m (transect F) distance from the source, no direct effect on bacterial cell numbers could be seen. Only for transect F, the numbers of suspended and attached cells differed from day 63. The total number of suspended cells at transect A and C (0.15 and 0.95 m from the source) ranged from 3.91 ± 2.78 x 10^4 to 1.51 ± 1.27 x 10^5 cells mL^{-1}, respectively. At transect F an average number of 4.41 ± 3.73 x 10^5 cells mL^{-1} was observed, a nine fold increase of suspended cells compare to day 63. Contaminated sediments from the different transects showed a similar number of attached cells, however with a slight decrease towards the end of the indoor aquifer ranging from 6.39 ± 0.4 x 10^6 cells mL^{-1} at transect A to 3.64 ± 1.63 x 10^6 cells mL^{-1} at transect F. In the contaminant-free areas of the aquifer, the average total cell numbers remained low with 1.44 ± 0.44 x 10^6 cells mL^{-1} of sediment, similar to the value reported for day 63. High intracellular ATP concentrations in the contaminated zones unequivocally pointed at highly active bacterial communities. The ATP content in the contaminated sediment samples was 4 to 14 times higher compared to non-contaminated sediments. In fact, ATP was highest at transect A with 0.83 ± 0.18 nM and lowest at F with 0.40 ± 0.24 nM.

As seen from the 16s rRNA fingerprinting profiles, toluene impacted zones of the aquifer became now dominated by new key organisms. The greatest impact of toluene on taxonomic diversity was found in the areas not contaminated before, mainly in the upper part of the aquifer. The average Shannon-Wiener diversity *H`* in the toluene affected parts of the aquifer varied between 2.62 ± 0.22 and 2.32 ± 0.35 at transect C and F, respectively. In comparison, the toluene-free areas exhibited stable Shannon values all over the indoor aquifer with a mean of 3.20 ± 0.08. Such a drop in diversity was not seen at transect A. Exposure to high toluene concentration resulted in an enrichment of the T-RF 77 bp, representing the

degrader population that survived and maintained best in the toluene plume. More than 25% of all T-RFs in the plume belonged to this operational taxonomic unit (OTU). It was not found outside of the toluene plume and not in the pristine aquifer, as well as at day 16. T-RF 490 bp, being most abundant in the toluene plume during the continuous injection was still evident, especially at transect C representing up to 14 % of all the sequences.

To comparatively assess the effect of toluene contamination on sediment bacterial community, a correspondence analysis (CA) of the T-RFLP dataset was conducted. Ordination of samples revealed three distinct clusters, one composed of non-plume and pristine samples, second of toluene plume samples from day 16 and 63, a third cluster composed of toluene contaminated samples from the toluene spill at day 137 (Fig.3.7).

3.3.6 Recovery phase – Day 231

Only 35 days after the accidental toluene spill most of the toluene disappeared from the indoor aquifer. 94 days after the spill an extensive sampling campaign was carried out in order to assess the status of recovery of the indoor aquifer.

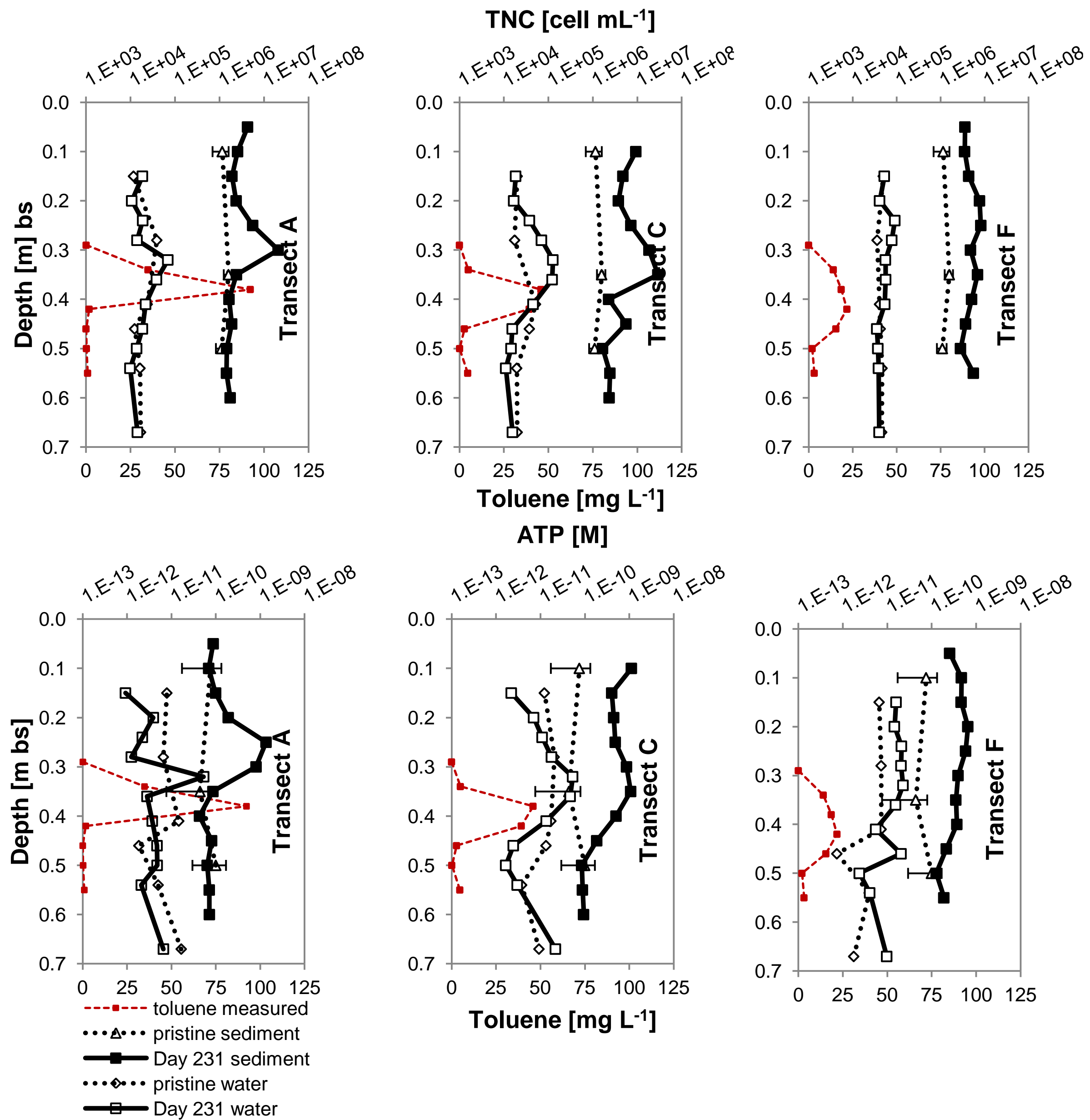

Fig. 3.6 Depth profiles of the total bacterial cell numbers (TNC) and ATP concentration of attached and suspended cells at transect A, C and F during the recovery phase at day 231. Toluene concentrations were below the detection limit. Former toluene plume (day 137) is marked with a dashed line.

At that time no more toluene was found in the system. Concentrations of dissolved oxygen at day 231were high again, close to saturation. The numbers of suspended bacterial cell have decreased considerably back to the pristine or near-pristine level. In contrary, the total

number of attached cells was still above the pristine level (Fig.3.6). The average cell number in the sediment at transect A and C was approximately one order of magnitude higher than at pristine conditions. Farther downgradient, the difference turned smaller with cell counts only 3-folds above the background. ATP measurements of attached and suspended microbes exhibited a similar trend. A notable decrease of microbial activity was observed in the water fraction, once toluene was gone. However, still a high amount of active biomass was found in the sediment (Fig.3.6).

T-RFLP profiles of attached bacterial communities revealed less pronounced differences in community composition than before in the areas of the former plume when compared to areas outside. T-RFs that were previously enriched in the plume core remained dominant, however, without causing drastic alterations in diversity and evenness when compared to the non contaminated parts of the aquifer. That hints at retrieval of sensitive members of the bacterial community, whereas dominant degraders stay. At transect A, no evident distinction could be seen between T-RF profiles of formerly contaminated and unaffected parts of the sediment, with an average Shannon value of $H' = 3.38 \pm 0.1$. However, the T-RFs 77 bp and 490 bp were abundant and clearly outlined in the area of the former toluene plume at transects C and F. Thus, despite complete toluene degradation and re-establishment of fully oxic conditions, the indoor aquifer did not recover in terms of sediment biomass and community composition. As seen with the CA ordination plot (Fig.3.7) samples from 94 days after termination of contaminant input did not cluster neither with those from before the contamination nor from non-contaminated areas of the system. They were clearly scattered between two clusters composed of toluene impacted samples.

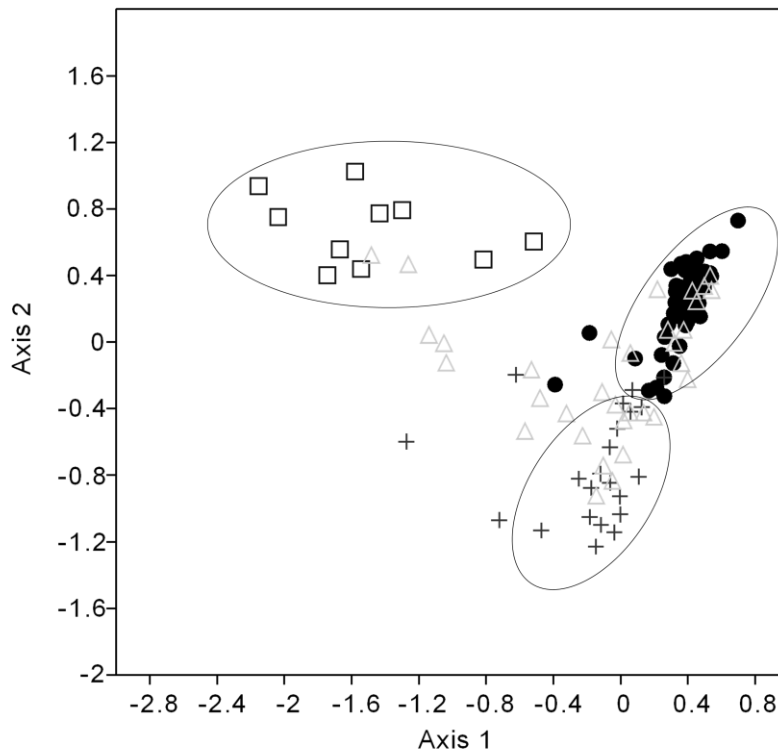

Fig. 3.7 Ordination diagram of a correspondence analysis (CA) based on T-RFLP data of sediment bacterial communities. Each point represents the average of 3 biological replicates. Black circles correspond to samples from pristine conditions and samples from outside the toluene plume; black crosses indicate samples from toluene plume during constant toluene injection (Day 16, Day 63), black rectangles correspond to samples from the plume after the toluene spill (Day 137) and grey triangles to samples from the recovery period (94 days after shut down of contaminant injection).

3.4 Discussion

The overall goal of our indoor experiment was to evaluate the short-term, mid-term and long-term response and recovery of a pristine, energy limited porous aquifer to a toluene contamination. The ecosystem reaction was followed by monitoring of selected microbiological variables such as bacterial abundance, active microbial biomass (ATP),

bacterial carbon production and composition of the bacterial communities in groundwater and sediment. Water chemistry, including toluene and oxygen concentrations was recorded and additionally a toluene transport model was carried out.

Despite the vast amount of literature on biodegradation of petroleum compounds, various oil products and individual aromatic hydrocarbons (in laboratory microcosms as well as different habitats (Haack and Bekins, 2000; Bekins et al., 2001; Cozzarelli et al., 2001; Head et al., 2006)), their degradation pathways and hydrocarbon degrading microbes itself, our ability to understand and predict the response of bacterial communities to selective pressure, such as hydrocarbon contamination, still remains in its infancy. Very few studies refer to the contamination of pristine habitats, especially groundwater ecosystems, therefore a paucity of information exists on their recovery potential – an essential concern at polluted sites undergoing NA. When studying contamination scenarios in the environment, in almost all cases one lacks information about the site conditions before contamination has occurred. Controlled lab experiments allow to follow effects of a contamination through the various phases from prior to contaminant appearance to the system´s recovery. Nevertheless, in most cases lab experiments are miniaturized and do not consider the appropriate scale. And without doubt, the transfer of knowledge about the processes, metabolites and community dynamics across different scales, from small (cm) to large (m) is not straightforward. In consequence, we designed an indoor aquifer system which fulfils a set of requirements: (a) it is small enough to be well controlled; (b) it allows the installation of dozens of sensors and water sampling ports vertically and horizontally distributed in a high spatial resolution; (c) dimensions of the system and sediment properties guarantee a reasonable water residence time; and finally (d) the system is big enough to treat it like a field site, i.e. to take sediment cores.

Prior to contamination, the indoor aquifer exhibited features typical for a pristine, oligotrophic groundwater ecosystem; poor in nutrients, well oxygenated and low in organic carbon. The pristine nature of the system was further underlined by low cell numbers in the water and sediment fractions, low microbial activities as underlined by long doubling times and low ATP concentration. Finally, the sediment bacterial communities showed a high diversity and evenness. Considering the pristine nature of the system, the aquifer was expected to be very vulnerable to the impact from toluene. However, switching on the toluene source revealed unexpected findings. Already, after one mean water residence time, a decrease in toluene concentration was observed coupled to turn down of oxygen (Fig.3.1). By day 16, 95% of the continuously introduced toluene was degraded. Toluene depletion was coupled to dramatic shifts in bacterial community composition along with a decrease in diversity in the plume. Those findings clearly hint at a surprisingly high, indigenous potential of the groundwater system, to response to and attenuate a continuous source of toluene pollution. The divergence in the structure of microbial communities associated with the selection of fast growing degraders in response to hydrocarbons as a carbon and energy source has been previously reported in several studies dealing with contaminated sites and in laboratory studies (Song and Bartha, 1990; Atlas et al., 1991; Haack and Bekins, 2000; Bundy et al., 2002; Röling et al., 2002; Röling et al., 2004; Bordenave et al., 2007; Anneser et al., 2010; Lekunberri et al., 2010). Few lab investigations of petroleum impact on microbial communities in pristine soil also reported fast changes in microbial community structure and biomass combined with a contaminant decrease after 10 - 12 (Shi et al., 1999) or 27 days (Bundy et al., 2002). On the contrary, Evans et al. (2004), although reporting an increase of biomass within 90 days after contaminant with toluene, reported no contaminant influence on the bacterial community structure.

Though an increase of microbial biomass upon contamination was reported in several studies, highly resolved water and sediment sampling at day 16 did not reveal changes in total numbers of cells and activity. Initial lack of change in microbial abundance upon contaminant input is explained by a balanced die-off of sensitive groups and proliferation of tolerant, contaminant-degrading strains. Also the influence of grazing and lyses by protozoa and phages, controlling the biomass of bacteria in our system must be considered (Novarino et al., 1997; Zarda et al., 1998; Kota et al., 1999; Ronn et al., 2002; Long et al., 2007). Preliminary results from the enumeration of VLPs and protozoa showed, that the indoor aquifer harbours a high viral and protozoan biomass forming a network of strong interactions, which become even tighter upon contamination. The ratio of bacteria to protozoa was found to be low in the contaminated area, as a result of high predators abundance compare to areas outside of the toluene plume (data not shown). Regarding the proportion of viral like particles (VLP) to bacteria, a higher ratio was found in the undisturbed areas in contrast to contaminated zones suggesting slower generation time or even viral-protozoa competition.

Surprisingly, toluene began to re-appear in the aquifer at day 63 at a concentration 10-times higher when compared to day 16, with only 30% of the incoming toluene being degraded at transect A. As can be seen from the oxygen profiles, the toluene increase concurred with the establishment of microaerophilic or anoxic conditions in the contaminant plume. At the same time the bacterial numbers and the active microbial biomass in groundwater and sediment from the toluene plume was found significantly increased, indicating active *in situ* growth. From now on, this toluene and oxygen patterns were maintained for the following 70 days, suggesting that aerobic toluene degradation must have balanced at a low level, as a consequence of oxygen depletion and insufficient replenishment to the plume core. We suspect that bacteria conducting initial aerobic toluene degradation quickly depleted the oxygen from the toluene plume and consequently restricted aerobic

degradation activities to the plume fringe zones (Bauer et al., 2008). In conclusion, initial aerobic degradation activity facilitated fast formation of an oxygen-free contaminant plume what in turn lowered down the overall biodegradation rate. A slight depletion of nitrate inside the toluene plume further hinted at the initiation of toluene degradation coupled to nitrate reduction. The concept of mixing controlled aerobic and anaerobic biodegradation in porous aquifer has been studied intensively (Thornton et al., 2001; Bauer et al., 2008; Bauer et al., 2009).

The short pulse of pure toluene phase showed that the microbial community was ready to withstand another dimension of contamination and maintained stable biomass level of attached and suspended bacteria. Obviously, the high toluene concentrations reached, had no direct harm to the pre-adapted, already well developed community of degraders, which, as indicated by ATP concentrations and BCP rates, surprisingly remained active when facing the pure toluene pulse. The threshold of toluene toxicity on microbial community was not part of this study. However it is known that pre-adapted mixed cultures degraded as high as 80 mg L^{-1} of toluene (Alvarez et al., 1994) and toluene degrading bacteria isolated from contaminated soil could grow on medium containing up to 540 mg L^{-1} of toluene (Hubert et al., 1999).

The re-establishment of the oxic conditions in the aquifer after switch-off of the source was surprisingly fast, as well as of, the toluene degradation, coupled with its partial wash-out. Abundance and activity of free-living bacteria soon returned to the initial concentration. Attached bacterial communities, however, seemed to need more time to recover from the disturbance. Differences in the recovery pattern of attached and suspended bacteria were most probably an outcome of the overall characteristics of this communities and a nature of the habitat they occupy. The vast majority of subsurface biomass is comprised of bacteria attached to sediment particles, compared to free-living bacteria representing only

a small fraction of this environment (Alfreider et al., 1997; Griebler et al., 2002). Studies of groundwater and surface water microbiota, emphasize substantial differences in the behavior and fate of unattached and attached bacterial communities, pointing at later ones being more stable, displaying a delayed reaction to changes (Marxsen, 1988; Hazen et al., 1991). In our indoor aquifer, biomass and activity of attached and free-living bacteria appeared to be different at day 231, when the system recovery from heavy toluene pollution was investigated. Suspended bacteria were responding first to the toluene introduction into the system. Similarly after toluene switch-off, the unattached bacteria were the first decreasing in abundance back to pristine levels, whereas the sediment cell numbers stayed elevated, exhibiting a temporal "memory effect". The attached community, except high level of biomass, exhibited also elevated ATP concentrations indicating that this community obviously stayed active for many weeks lacking a substantial source of organic carbon. At this stage of the experiment, disappearance of toluene from the system, and restoration of oxic conditions unequivocally document aquifer recovery on functional level, though the recovery of the sediment biomass and community composition could not be confirmed.

The T-RFLP analyses performed in this study, sought to explore the respond of the native community of an energetically limited aquifer to toluene contamination. The native microbial community right after toluene injection shifted immediately (day 16) to a stable community in the contaminant plume and remained different from that in the adjacent areas till the very end of the experiment. Despite the clear establishment of degrader populations, toluene was also cause for the disappearance of some of the T-RFs previously present in the pristine aquifer. These results emphasize that the injected toluene might have exerted a strong selective pressure on the microbial community, resulting in die-off of sensitive microbes and succession of populations associated with toluene degradation which became dominant in the plume. By day 63, differences between the plume community and non-impacted areas were

less pronounced, however still with a clear dominance of the key degrader T-RFs. We further could show that on day 63 a new group of bacteria, represented by T-RF 77 bp appeared which later on (day 137) highly dominated toluene impacted areas. We speculate that changes of electron acceptors availability and development of nitrate-reducing conditions could have cause these changes within the community. OTUs appearing on day 16, represented by T-RF 480 bp and less abundant T-RF 428 bp, 437 bp and 488 bp seemed to be associated with the early stage of hydrocarbon degradation, and predominating the sediment bacterial communities following exposure to toluene. Whereas the 77 bp related T-RF seemed to play a role in a late response to toluene contamination. By the end of the experiment, with the recovery of oxic conditions and the disappearance of toluene from the indoor aquifer, the abundance of the key degrader T-RFs started to decrease, however the overall community composition seemed to be altered compare to the pristine stage. The very few investigations undertaken to assess the impact of petroleum contamination on pristine systems, mainly based on lab studies (Evans et al., 2004; Bordenave et al., 2007), and the lack of pre-disturbance data from natural sites, makes it difficult to properly assess the recovery of the system. Laboratory and field studies have shown profound shifts of the overall microbial community structure due to hydrocarbon contamination (Haack and Bekins, 2000; Röling et al., 2004; Anneser et al., 2010; Lekunberri et al., 2010). Once the site is contaminated, microbial community will be changed, with respect to biomass and composition. The ecosystem might fast restore its functional stage after the anthropogenic disturbance faded, while the recovery of microbial communities in theirs composition, will require extended time periods (months to years) or might even never happen. Re-establishment of ecosystem processes, despite the presence of an altered community, might be explained by functional redundancy. Long term persistence of the altered community upon disturbance can be also understood as a memory effect and/or readiness of the bacteria to changing environmental

conditions, what in turn might explain the fast reaction of the bacterial communities towards disturbance with previous pollution history (Wikström et al., 2000).

3.5 Conclusions

In an almost one year lasting experiment, the resistance and resilience of a pristine model groundwater ecosystem to different scenarios of toluene contamination were investigated. From an ecological perspective, the aquifer´s resistance to the toluene contamination was, as expected, extremely low, underlined by immediate and significant changes in physical-chemical and microbial patterns. On the other hand, the extremely rapid and substantial rearrangements within the intrinsic microbial community, leading to a fast aerobic degradation of continuously introduced toluene, were astonishing. This indicates that oligotrophic, energy-limited groundwater systems harbour a catabolically versatile community containing hydrocarbon-degrading populations, and as such, have a very high intrinsic potential for NA. These indigenous microorganisms appeared to be ready to degrade toluene and subsequently grow fast, establishing populations of high relative abundance. Now our samples still await pyrotag sequencing for the identification of the key degraders within the microbial community. Complete disappearance of toluene from the system combined with return of fully oxic conditions unequivocally confirms the functional recovery of the site through natural attenuation. The study again hinted at limitations of contaminant degradation due to transverse dispersion. Furthermore, premise on the existence of a dynamic network of virus-bacteria-protozoa in the indoor aquifer calls for consideration of top-down regulation of groundwater ecosystems and their carrying capacity when assessing system´s reaction to disturbance. Last, but not least, generic findings of this study needs to be rendered into application when assessing NA at hydrocarbon contaminated sites.

3.6 Supplementary materials

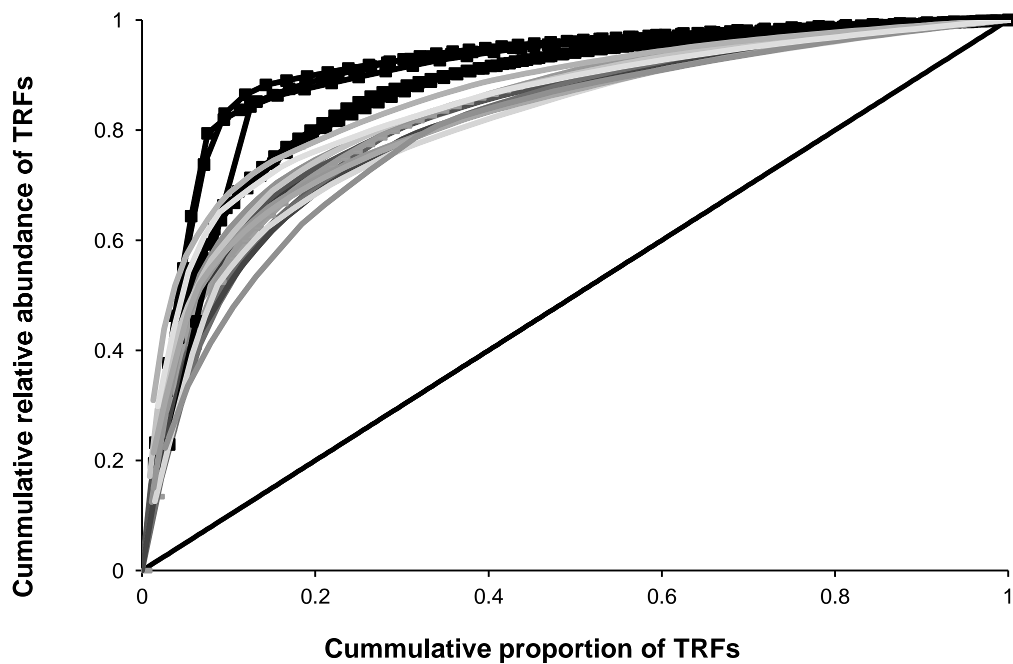

Fig. 3.S1 Pareto-Lorenz distribution curves for bacterial T-RF abundance patterns at transect A after 16 days of constant toluene input. Samples from the toluene plume (black squares) show the most uneven distribution of taxa, with samples from outside the contaminated area (gray lines) showing more even distribution. The diagonal black line indicates a theoretical even distribution.

Tab. 3.S1 Parameters used for the modeling

Parameter	Symbol [unit]	Value
mean water velocity	v [m/d]	1.61
longitudinal dispersivity	α_L [mm]	21.80
transversal dispersivity	α_T [mm]	0.40

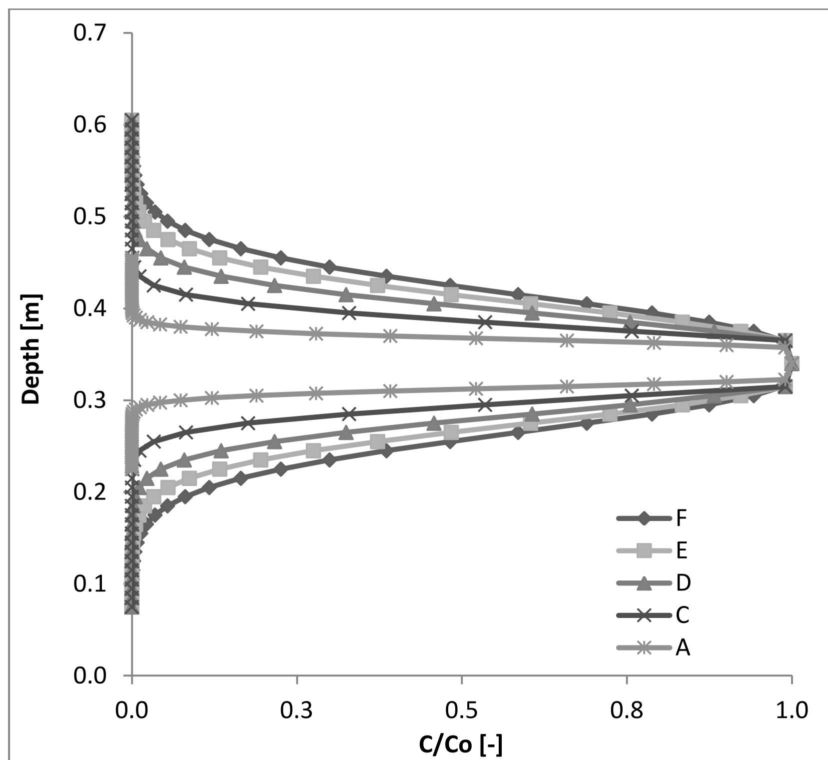

Fig. 3.S2 Modelled transversal – vertical distribution of toluene in the aquifer during constant injection calculated for the steady state at the different transects A-F.

Tab. 3.S2 Toluene degradation potential during continuous injection

	Degradation potential [%] *	
Transect	**Day 16**	**Day 63**
A	94.60	29.62
C	100.00	96.50
D	99.88	99.78
E	99.76	99.93
F	99.93	99.49

*Based on the results of the conservative transport model and determined by calculating the define integral under the computed theoretical and measured toluene concentration curve.

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4. Microbial indicators of groundwater ecosystem response to organic contamination – resistance, resilience and evaluation

4.1 Introduction

Groundwater systems today are subjected to an increasing number of threats caused by the introduction of contaminants. At the same time, aquifers produce and store one of mankind's most important resources, i.e. drinking water. Water purification and storage is an ecosystem service which undisputedly relays on the activity of intrinsic microbial communities. The same is true for the service of natural attenuation, including contaminant degradation. The subsurface forms the largest and the most diverse terrestrial habitat on earth, with groundwater ecosystems probably being the least explored and understood. Characterized by relatively stable environmental conditions (e.g. hydrology, temperature, pH), limited organic carbon and nutrient import, as well as the lack of light, groundwater ecosystems are an extreme environment to live in (Griebler and Lueders, 2009; Humphreys, 2009). With the absence of primary producers (chemolitoautotrophs) and important organic carbon sources, groundwater aquifers are generally energy-poor and in consequence vulnerable to disturbance from outside.

Management of drinking water resources as well as contaminated sites requires sophisticated assessment and monitoring schemes. To date, groundwater is evaluated exclusively for its chemical and quantitative status. However, as mentioned above, ecosystem functions and services likely depend on the living component which needs to be addressed considering biological and ecological criteria (Steube et al., 2009; Griebler et al., 2010; Stein et al., 2010). Moreover, the evaluation of the resistance and resilience of an ecosystem requires investigation of biological variables. Since groundwater ecosystems are predominantly inhabited by microorganisms and their leading role in natural attenuation of

organic contaminants was shown repeatedly (Wiedemeier et al., 1999; Röling and Verseveld, 2002; Smets and Pritchard, 2003; Meckenstock et al., 2004), selected features of microbial communities may well serve as indicators for ecosystem response upon disturbance.

Several assessment and monitoring strategies have been suggested that use microbial or faunal communities, or some of its patterns, as indicators of groundwater ecosystem quality, natural attenuation, response to disturbances and recovery. Parameters include: microbial biomass, the number and the ratio of attached and suspended bacterial cells, microbial diversity, evenness as well as taxonomic richness of individual groups (Griebler et al., 2002; Paerl et al., 2003; Marzorati et al., 2008; Wittebolle et al., 2009; Stein et al., 2010). Moreover prey-predator ratios (Kerr, 1974; Platt and Denman, 1977; Jeffries and Lawton, 1984), and changes in population density of viruses, bacteria and protozoa were also proposed as indicators of ecosystem performance (Madsen et al., 1991; Sinclair et al., 1993).

Chapter 3 of the thesis describes the response of the oligotrophic, indoor aquifer system to toluene contamination and its subsequent recovery, concentrating mainly on the intrinsic degradation capacity present, and factors limiting NA. To complete this picture, a set of abiotic and biotic variables has been chosen to exemplarily assess the disturbance of the ecosystem by the fate of the microbial communities facing toluene contamination in comparison to areas non-impacted. For this purpose samples were divided into two sets, accordingly to the presence or absence of toluene, and treated individually as a polluted site (toluene plume) and reference site (non-contaminated samples from above and below the plume). Additionally to some bacterial features, such as abundance, activity and diversity, the abundance of protozoa and viral-like particles (VLP) was analyzed with respect to their predator-prey and pollutant driven dynamics. The general working hypothesis was that in energy limited, oligoalimonic, low dynamic groundwater systems, microbial communities reflect the *in situ* environmental conditions. Exposed to anthropogenic impact such as the

toluene contamination, the indoor aquifer will not have the potential to withstand the disturbance, and the abundance, activity as well as the compositional structure of the microbial community may serve as sensitive indicators of ecosystem response, i.e. resistance and resilience.

Individual variables chosen in our study were the abundances and ratios of bacteria, protozoa and viruses, bacterial activity (ATP), as well as bacterial diversity (Shannon-Wiener index H'), evenness and richness. Biological variables have been completed with measurements of toluene and dissolved oxygen. The data presented originate from consecutive sampling campaigns conducted over 1 year, covering the initial, pristine phase (P), phase of constant toluene injection with moderate concentration (day 16 and day 63), followed by a toluene spill, simulated by the pulse injection of pure toluene (day 137), and finally the subsequent recovery of the aquifer (day 231).

4.2 Material and methods

4.2.1 Experimental set-up, water and sediment sampling

The experiment was carried out in the indoor groundwater system, which in details is described in the Introduction of this thesis. The water and sediment samples have been collected during a period of one year where the reaction of the initially pristine aquifer to toluene contamination as well as its potential for recovery was investigated. Extensive information about the experimental design and the sampling scheme is given in the Introduction section. Sediment data presented originate from samples collected during 5 sampling campaigns: P (pristine, before contamination), day 16, 63, 137 and day 231. Water data includes additionally samples collected 4 days after toluene injection (day 4). The

abundance of protozoa and virus-like particles (VLP) was assessed in water samples only at: P, day 63, day 137 and day 231.

4.2.2 Toluene and oxygen analysis

For quantification of toluene water samples were directly after collection preserved with NaOH (final concentration 100 mM) and spiked with ethylbenzene (final concentration 0.01mM) which was used as an internal standard. Toluene concentration as determined via headspace analysis on a Trace DSQ GC-MS instrument (Thermo Electro, Germany) equipped with a Combi PAL autosampler (CTC Analytics, Swizerland) as described by Anneser et al. (2008). Separation of compounds was done on a DB5 capillary column (J&W Scientific, USA) with helium used as carrier gas. Dissolved oxygen concentrations were measure *in situ* via optode sensors embedded in the sediment matrix (FIBOX3, PreSens GmbH, Regensburg, Germany).

4.2.3 Measurement of microbial activity

Bacterial [^{3}H]-leucine incorporation and adenosine tri-phosphate (ATP) were used as estimators for microbial activity. The [^{3}H]-leucine incorporation method, modified after Chin-Leo and Kirchman (1988), was used to estimate the bacterial carbon production (BCP). In this chapter of the thesis, only BCP data from water are presented. A detailed description of the method can be found in Chapter 3.

The BacTiter-Glo Microbial Viability Assay (Promega Corporation, Madison, WI, USA), containing luciferase and cell lysis regent, was used to determine the microbial, cellular ATP concentration in water and sediment samples. The ATP concentration stands for

living and active biomass. Luminescence was measured in a Glomax luminometer (Turner Biosystems, Sunnyvale, CA). In principle the protocol of Hammes et al. (2010) was applied with slight modifications as explained in the material and method section of Chapter 3.

4.2.4 Determination of bacterial cell numbers and virus-like particles (VLP)

Groundwater and sediment subsamples dedicated for the determination of total bacterial counts were fixed with glutardialdehyde (final concentration 2.5%) and stored at +4°C prior to quantification. Counting of fluorescently labelled (SYBR Green I) cells was done in a flow cytometer (LSR II, BD, Germany). Detailed sample preparation and instrument settings followed the descriptions in (Hammes et al., 2008; Bayer et al., submitted) with minor modifications as described in Chapter 3. Final bacterial cell numbers were calculated from counts using fluorescence beads as internal standard and applying the formula given in Nebe-von-Caron et al. (2000).

For VLP enumeration, a water aliquot was fixed with glutardialdehyde (final concentration 1 %) and preserved at -80°C till further analysis. Prior to analysis, samples were prepared as described elsewhere (Marie et al., 1999; Brussaard, 2004). Briefly, 1mL sample was diluted in Tris-EDTA buffer (pH= 8), stained with SYBR Green I and incubated in a water bath at temperature of 80°C, in the darkness for 10 min. Counts of VLP were performed with a FACS Calibur flow cytometer (BD Sciences, USA) equipped with a 488nm air-cooled blue light laser. Parameters were collected on logarithmic scale and populations of VLPs were identified and distinguished from other particles and background noise by plotting sideward scatter events versus green fluorescence at 530 nm. Data analyses were performed using the CellQuest Pro Software (BD Bioscences, version 4.0).

4.2.5 Enumeration of protozoa by fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization was used to assess the abundance of protozoa, according to the protocol of Glöckner et al. (1996) and Manz et al. (1992) with the following modifications. Depending on the spatial and temporal origin of the sample, 15 – 120 mL of glutardialdehyde fixed (final concentration 2.5%) water was filtered through a polycarbonate filter with a pore size of 0.2 µm (Milipore Corp. USA). Subsequently, as a coating agent, low-gelling-point agarose (0.1 % w:v) was applied to both surfaces of the filter. The filter was then spotted onto a microscopic slide, air dried at 37°C and dehydrated in 3 x 2 min steps, of an ethanol concentration series (50%, 80% and 98% v:v). For better permeabilization of the cell´s peptidoglycan layer, the filter was additionally incubated in lysozyme (10 mg mL^{-1} in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0, Sigma, Austria) for 1h at 37°C. Consequently, triple washing in Mili-Q water was applied. An additional incubation in 0.01 M HCl for 20 min at room temperature was conducted in order to inactivate endogenous peroxidases. Thereafter, the washing step was repeated, the filter was dehydrated in ethanol and air dried at room temperature. Hybridization took place in a hybridization chamber for 90 min at 46°C at humid and dark conditions. Each filter was hybridized with 16 µL of hybridization buffer [0.9 mol L^{-1} NaCl, 0.02 mol L^{-1} 45% (v:v) formaldehyde, 0.02% (w:v) SDS] and 2 µL of the oligonucleotide probe EUK 1195 (5´-GGGCATCACAGACCTG-3`) labeled with Cy3 and Cy5 (3 ng μL^{-1}). After the hybridization the filter was transferred to a preheated washing buffer (20 mmol L^{-1} Tris-HCl, 5 mmol L^{-1} EDTA, 30 mmol L^{-1} NaCl0.01% SDS) and incubated for 20 min at 48°C. Subsequently the filter was washed 3 times in Mili-Q water and again air dried at room temperature. For additional visualization of bacterial cells, counterstaining with DAPI was performed and an antifading agent was applied (DAPI/Antifade Solution, Millipore, California, USA). As a

mounting medium CITIFLUOR Glycerol/PBS solution (Citifluor Ltd. London, United Kingdom) was used. Counting was done in an Axioplan 2 Zeiss epifluorescence microscopy, equipped with fluorescence illuminator HXP-120 LEj and the Axiocam Zeiss camera at a magnification of x63.

4.2.6 Analysis of bacterial community composition

A water volume of 1L, from each sampling point and time of sampling, was filtered through a 0.22 µm sterile, membrane filter (Neolab) and stored at -20°C till further processing. For total DNA extraction, filters were thawed and then cut aseptically into small pieces (approx. 2 mm^2), placed into bead-beating cups and further processed as described in (Winderl et al., 2008). The DNA extraction from sediment samples was done using an optimized protocol after (Gabor et al., 2003; Lueders et al., 2004) as described in Chapter 3 of the thesis.

T-RFLP analysis of bacterial 16s rRNA gene amplicons were primarily evaluated using the Gene Mapper 5.1 software (Applied Biosystems). Alignment of terminal restriction fragments (T-RFs) were conducted with the free access software T-REX as reported in Chapter 3. The Shannon-Wiener diversity, richness and evenness were calculated using PAST software.

4.2.7 Statistical analysis

Relationships between different experimental conditions and microbial community variables were explored by a One Way Analysis of Variance (ANOVA) in case of normal distribution and equal variance between the groups. If those criteria were not met, a Kruskal-Wallis ANOVA was applied. Normal distribution was checked with the Shapiro-Wilk test

and a p-value of ≤ 0.05 was set as the significance threshold. Correlations among the measured variables were explored by the Pearson product correlation test or the Spearman rank order correlation matrix test, according to the results of the normality test. Statistical analyses were performed using statistical package in SigmaPlot 12.0 for Windows. Differences in bacterial community composition of the samples originating from the toluene impacted areas and samples from outside of the toluene plume were analyzed by ANOSIM (analysis of similarities) based on a Bray-Curtis dissimilarity matrix. These analyses were performed in PAST software. A principal component analysis has been applied to explain the observed variances between the bacterial community composition from inside the plume and non-impacted areas of the indoor aquifer and to investigate the hypothetical T-RFs driving these divergences between the communities.

4.3 Results

4.3.1 Oxygen versus toluene

Dissolved oxygen is a fast and reliable indicator in oxic aquifers of the impact by organic contamination. In our experiment, we found a clear, negative correlation between toluene concentration and oxygen saturation (Tab.4.1). A decrease in oxygen was obvious already at day 4 of the continuous toluene input, and by day 137, was found to be completely depleted in toluene impacted zones. Later, the recovery of the aquifer from contamination was underlined by re-appearance of dissolved oxygen, reaching full saturation in the formerly contaminated areas at day 231 (Fig.4.1).

Tab. 4.1 Sperman rank correlation of toluene and oxygen during consecutive sampling campaigns. Dataset originates from measurements at transect A, D, and F.

Spearman rank correlation		TOLUENE day 4	day 16	day 63	day 137
OXYGEN	r	-0.51	-0.07	-0.66	-0.73
	P	≤ 0.05	0.76	**≤ 0.01**	**≤ 0.01**

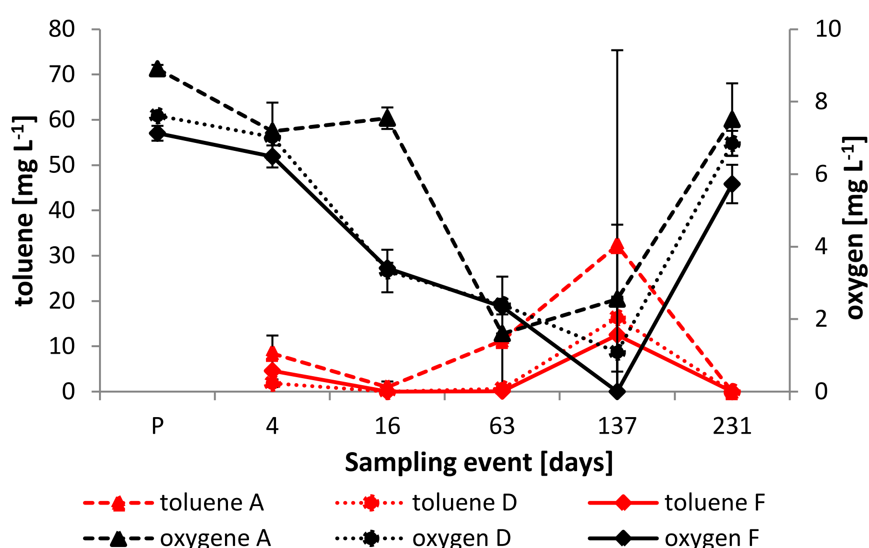


Fig. 4.1 Mean toluene and oxygen concentrations as measured during the consecutive sampling campaigns. Error bars show a standard deviation from the mean derived from transects A, D and F.

4.3.2 Abundance and activity of attached versus suspended bacteria

The average bacterial abundance (BA) in toluene impacted samples differed considerably from the values found with samples from the contaminant free areas. Water collected from the toluene plume contained minimum $3.95 \pm 3 \times 10^4$ and maximum $5.05 \pm 8.08 \times 10^5$ bacterial cells mL^{-1} within the time span of the experiment. Samples from outside of the plume showed considerable lower numbers, and displayed rather constant pattern

during the time of the experiment ranging from 1.53 ± 1.01 x 10^4 to 2.24 ± 1.05 x 10^4 bacterial cells mL^{-1} (Fig.4.2 A and B). Attached bacteria exhibited a similar divergence in cell numbers related to the toluene impact. Cell counts from the toluene plume zone varied considerably from 2.38 ± 0.74 x 10^6 to 8.72 ± 7.44 x 10^6 bacterial cells mL^{-1} of sediment, whereas the abundance in non-impacted sediment of the aquifer was between 1.65 ± 0.79 x 10^6 and 2.92 ± 1.38 x 10^6 bacterial cells mL^{-1} (Fig.4.2 C and D).

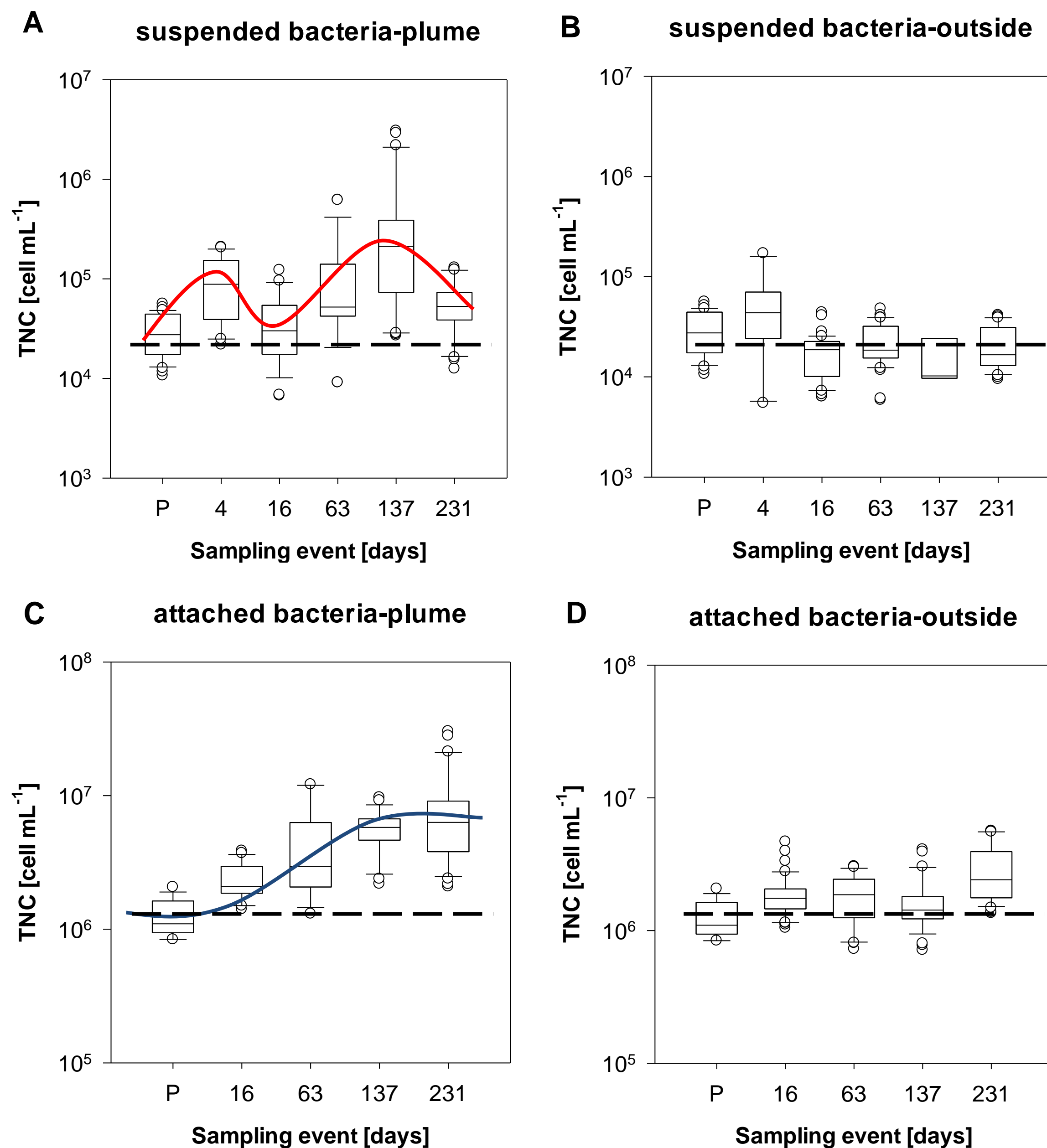


Fig. 4.2 Box plots showing total cell counts of suspended (water) and attached bacteria (sediment) in the toluene plume and outside, for the pristine aquifer (P), during injection of toluene (4-137) and the recovery phase (231). Each chart contains cell abundance at pristine stage as a reference. Dashed line was used to mark pattern in toluene-free areas and mirrored in the toluene plume.

Despite the spatial differences in bacterial abundance (BA) between contaminated and uncontaminated areas of the indoor aquifer, a different response to the toluene impact could be seen for the bacteria attached to the aquifer sediments and the bacteria suspended in groundwater. Water samples revealed a rapid response in bacterial cell densities, followed by considerable up and down fluctuations, whereas attached cells exhibited a delayed response of the toluene input as well as to its disappearance. However, for the time of contamination, the numbers of attached bacteria considerably increased (Fig.4.2 C). The number of suspended bacteria increased rapidly after toluene injection with a 3-fold higher mean value at day 4 than prior to contamination (ANOVA, $Q=4.35$, $p<0.05$) (Fig.4.2 A). After this early peak, the counts dropped back to values observed during pristine conditions (Day 16). Significantly higher cell numbers were found at day 63 (ANOVA, $Q=3.85$, $p<0.5$); followed by a further on average 4-times increase at day 137 (ANOVA, $Q=2.70$, $p<0.05$). This final increase coincided with the most dramatic expose of the aquifer´s microbial communities to contamination, i.e. a 6h pulse of pure toluene injected to the system (Fig.4.1). 94 days after the toluene pulse (day 231) a significant drop of suspended bacteria was observed with a mean ± standard deviation (SD) of $5.81 \pm 3.24 \times 10^4$ cells mL^{-1} (ANOVA, $Q=3.70$, $p<0.05$). The final values of suspended cells were comparable to the abundances from before the contamination, on average 2-times higher. A positive correlation between the number of suspended bacteria and the toluene concentration underlined the contaminant-induced changes observed in cell counts (Tab.4.2).

Sediment samples were characterized by higher total bacterial cell numbers when compared to water samples (Fig.4.2 A and C). The bacterial microbial community attached to

the sediments exhibited a delayed but more conservative reaction to the toluene input (Fig.4.2 C). In contrast to water samples, there was no early, significant increase in attached cells numbers. At day 63 analysis of sediment samples revealed total cell counts to be on average 4-times higher compared to the pristine phase (ANOVA, Q=3.89, $p<0.05$). The strong positive correlation with toluene at that point indicates the changes to be toluene-driven (Tab.4.2). The later pulse injection of pure toluene (day 137) resulted in a further increase (ANOVA, Q=2.30, $p<0.05$). At day 231, abundance of attached cells remained on the high constant level, showing, in the contrary to the plume water samples, no return to pre-contamination levels despite the complete disappearance of toluene. Total cell counts were 7-times higher compared to experiment´s starting conditions (ANOVA, Q=7.08, $p<0.05$).

With respect to active microbial biomass, as represented by ATP correlations, water and sediment samples from inside the toluene plume exhibited elevated values of cellular ATP compared to the toluene-free areas (Fig.4.3 A-D). The ATP concentration in contaminated water varied from $1.69 \pm 1.08 \times 10^{-11}$M to $6.85 \pm 0.14 \times 10^{-10}$ M, while water from outside the toluene plume contained between $5.91 \pm 6.31 \times 10^{-12}$ and $3.50 \pm 2.83 \times 10^{-11}$ M of ATP. Sediment samples from inside the toluene plume exhibited between $1.11 \pm 1.64 \times 10^{-10}$ to $6.16 \pm 3.41 \times 10^{-10}$ M of ATP whereas lower concentrations of ATP were measured outside the toluene plume, ranging from $5.15 \pm 7.37 \times 10^{-11}$ to $1.48 \pm 1.14 \times 10^{-10}$ M.

Tab. 4.2 Spearman rank correlation for the total number of suspended and attached cells, toluene and ATP in samples collected in pristine aquifer (P), during constant toluene injection at the days 4 - 63, during the pulse toluene injection – day 137, and the recovery phase – day 231.

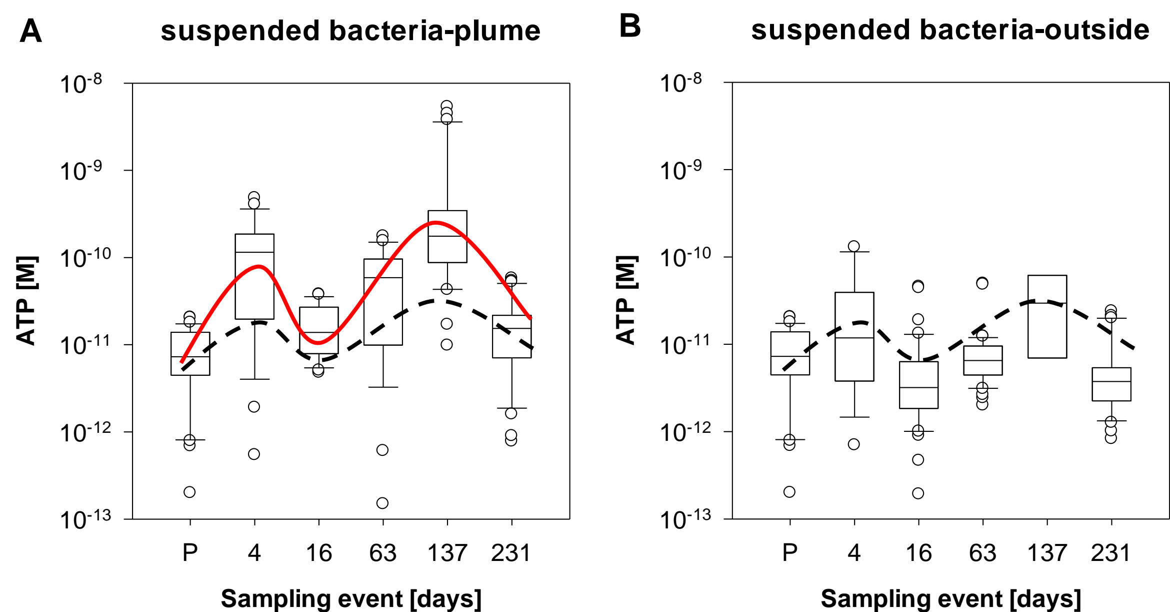
	sampling event	toluene	ATP
total number of suspended bacterial cells	**Pristine**		
	r		-0.14
	P		0.58
	Day 4		
	r	0.80	0.93
	P	**≤ 0.01**	**≤ 0.01**
	Day 16		
	r	0.34	0.56
	P	**0.05**	**≤ 0.01**
	Day 63		
	r	0.74	0.71
	P	**≤ 0.01**	**≤ 0.01**
	Day 137		
	r	0.33	0.74
	P	0.14	**≤ 0.01**
	Day 231		
	r		0.72
	P		**≤ 0.01**

	sampling event	toluene	ATP
total number of attached bacterial cells	**Pristine**		
	r		0.40
	P		0.10
	Day 16		
	r	0.09	0.16
	P	0.48	0.21
	Day 63		
	r	0.37	0.07
	P	**≤ 0.01**	0.61
	Day 137		
	r	0.53	0.72
	P	**≤ 0.01**	**≤ 0.01**
	Day 231		
	r		0.82
	P		**≤ 0.01**

The analysis of water samples showed ATP patterns following the total bacterial cell counts (Fig.4.3 A). At day 4, a significant, 15-fold increase of the ATP was observed

(ANOVA, Q=5.48, $p<0.05$). This increase was followed by a drop of ATP (day 16) and a consecutive 4- and additional 41-fold increase of active cells, at the day 63 (ANOVA, Q=4.05, $p<0.05$) and 137 (ANOVA, Q=8.31, $p<0.05$) respectively. The disappearance of toluene resulted in a significant decrease of the ATP concentrations (day 231) remaining on average 2-fold higher compared to the pristine conditions (ANOVA, Q=6.14, $p<0.05$).

ATP concentration measured from sediment samples again displayed similar patterns to the ones observed with bacterial counts. A significant increase of the ATP concentration upon toluene input was not observed until day 63 (Fig.4.3 C). At that time point, analysis of sediment samples showed ATP to be on average 4-times higher compared to the pristine phase (ANOVA, Q=2.53, $p<0.05$). The pulse injection of pure toluene resulted in a further increase of ATP (ANOVA, Q=2.68, $p<0.05$) which, in contrary to ATP measured from suspended cells, remained high, despite the complete disappearance of toluene from the indoor aquifer. The sampling campaign at day 231, revealed ATP concentrations exceeding those from pristine sediment by 8-folds (ANOVA, Q=8.80, $p<0.05$).



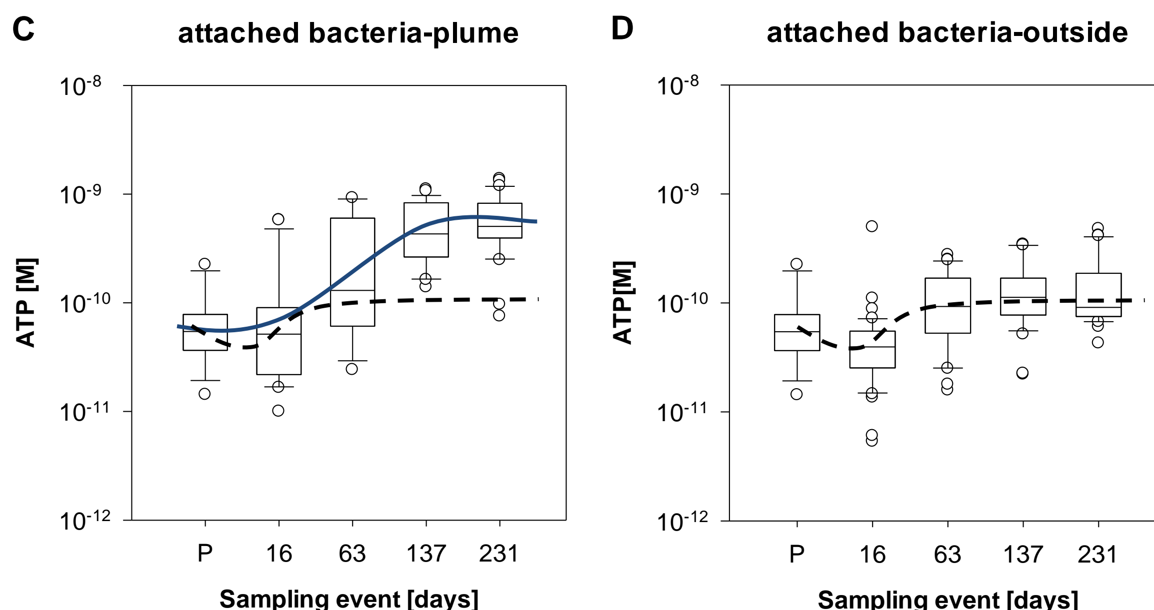


Fig. 4.3 Box and whiskers plots showing cellular ATP concentrations of suspended (A, B) and attached (C, D) bacteria in the toluene plume and outside, for the pristine aquifer (P), during injection of toluene (16-137) and the recovery phase (231).

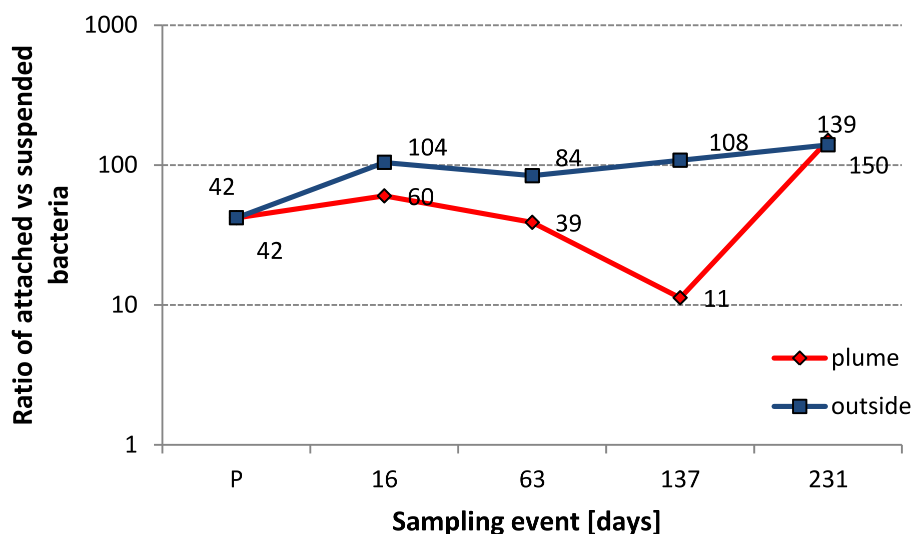


Fig. 4.4 Ratio of attached to suspended bacterial cells inside the toluene plume and outside, during pristine conditions (P), constant (days 16, 63) and pulse (day 137) injection of toluene and the recovery phase (day 231). The ratio was derived from the average cell number pooled from all transects (suspended) and liners (attached) which were separated according to presence or absence of toluene.

The ratio of attached to suspended bacterial cells showed clear different patterns during the consecutive phases of the experiment (Fig.4.4). As showed in figure 4 the pristine sampling campaign exhibited a relatively low ratio of attached to suspended bacteria equal to 42:1. Later, the curve drawn from samples outside of the toluene plume displayed rather constant ratios varying between 84 and 108. The highest ratio of attached to suspended cells in toluene-free samples was found at day 231. In contrast, the ratios from cells counts in sediment and water from inside the plume showed considerable changes during time of the experiment. The injection of toluene revealed a slight increase of the ratio, followed by a decrease of the ratio to 39:1 at day 63. The lowest value was obtained at day 137 as a response to the toluene spill. The sampling campaign conducted 94 days later, in an already toluene-free aquifer, revealed the highest ratio (150:1) caused by a remaining high density of attached cells and the drop back of suspended cell numbers to before contamination levels.

4.3.3 Abundance of virus-like particles (VLPs) and protozoa in groundwater

Groundwater samples collected from inside the toluene plume contained higher numbers of VLPs and protozoa, than groundwater from areas outside of the plume (Fig.4.5). The pristine aquifer harboured in its pore water on average $1.22 \pm 0.36 \times 10^6$ VLP mL^{-1} and $3.04 \pm 1.41 \times 10^4$ bacterial cells mL^{-1} with a ratio of 40: 1 (Fig.4.6 A). The abundance of those two groups was found to be strongly correlated ($r = 0.71$, $p \leq 0.01$). Quite variable was the protozoan population with a mean value of $6.74 \pm 6.25 \times 10^2$ cells mL^{-1}. The high standard deviation was caused by high protozoan numbers in three groundwater samples at transect A, containing many encysted-like protozoan cells. Excluding those three measurements, the recalculated mean protozoan abundance in the pristine aquifer was $2.91 \pm 1.24 \times 10^2$ cells mL^{-1} and the bacteria to protozoa ratio was 105: 1 (Fig.4.7).

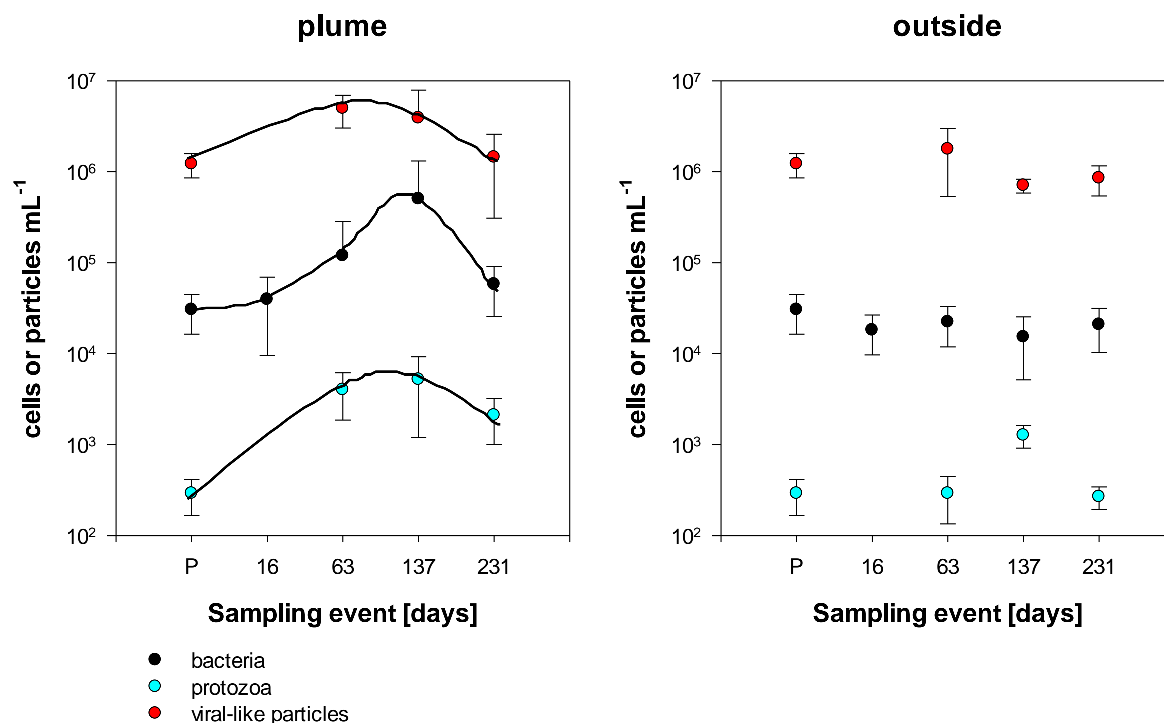
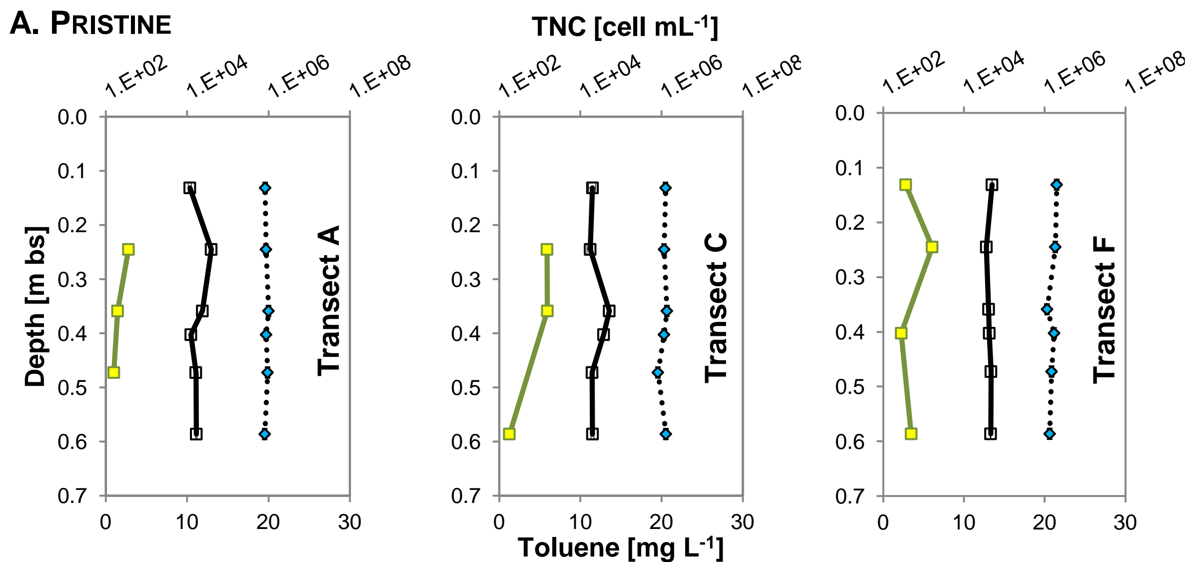
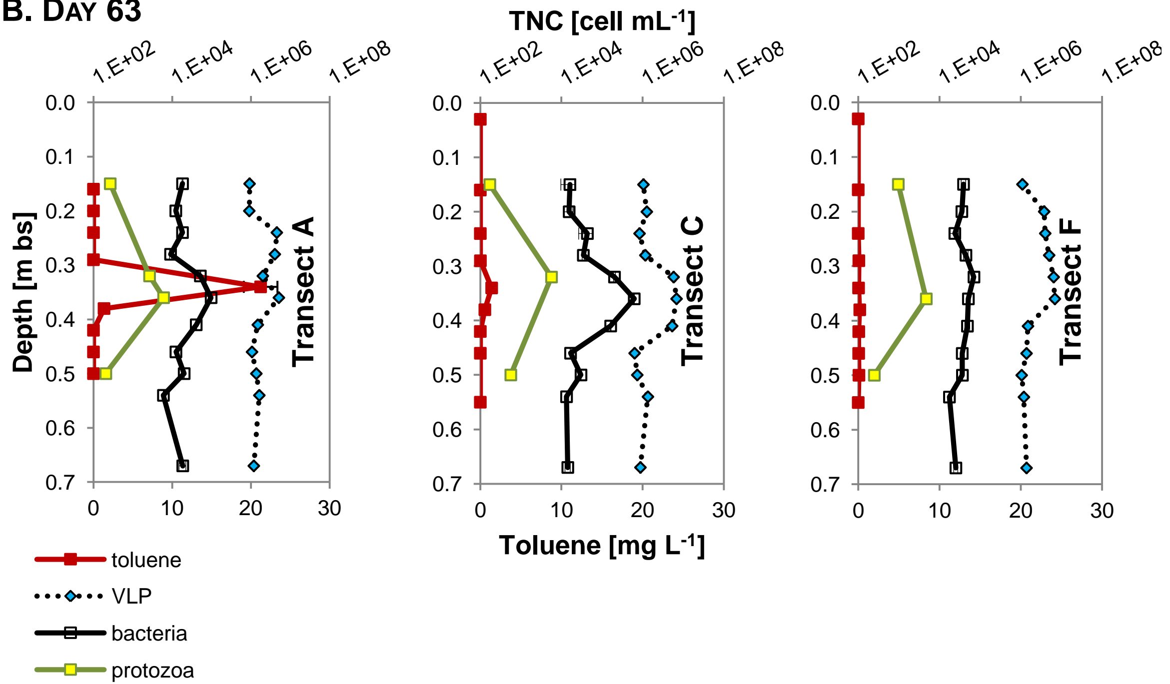


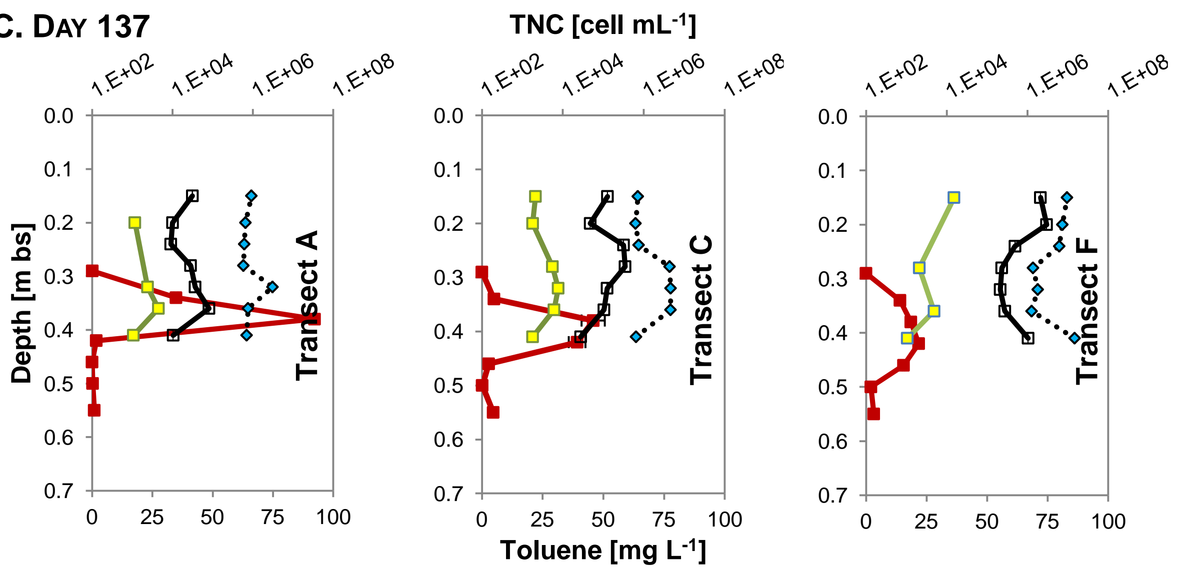
Fig. 4.5 Bacteria, protozoa and VLPs in groundwater from inside the toluene plume and from toluene-free areas of the indoor aquifer, at the pristine stage of the aquifer (P), during toluene contamination (days 16-137) and from the recovery phase (day 231). Values as means ± SD.

The presence of toluene in the aquifer as found by day 63 was accompanied by an increase of the protozoa and VLP abundance. In detail, the abundance of VLPs in the toluene plume, was 4-times higher, compared to the pristine stage, exhibiting a mean value of 4.99 ± 1.97 x 10^6 particles mL^{-1} (ANOVA, Q = 3.80, $p<0.05$) (Fig.4.6 B). Similarly, the toluene-induced increase of the bacterial abundance ($r = 0.74$, $p \leq 0.01$) was accompanied by about one order of magnitude higher protozoan cell counts ($r = 0.92$, $p \leq 0.01$) with a mean value of 4.78 ± 1.2 x 10^3 cells mL^{-1} and on average bacteria to protozoa ratio of 31:1. The injection of pure toluene was followed by a slight decrease of protozoa and VLP numbers, except for the transect F where an exceptional high increase of bacterial abundance occurred and protozoa counts increased by 1.6 fold (Fig.4.6 C). Excluding transect F, the overall ratio of VLP to

bacteria slightly decreased to 17:1 but remained unchanged for bacteria to protozoa. Ratios derived from transect F only, were 142 for bacteria and protozoa, and 4 for VLP and bacteria. At day 231, no more discrepancy was seen between transects (Fig.4.6 D). The toluene disappearance from the aquifer led to a rapid decrease of bacterial cell numbers followed by a decrease of VLPs to pristine or closely to pristine levels, resulting in consequence in an increase of their ratio (Fig.4.7). On the contrary, the protozoan abundance was found to be still, on average, 8-folds above the background (mean ± SD 2.24 ± 1.1 x 10^3 cells mL^{-1}) revealing a quite low bacteria to protozoa ratio when compared to the pristine situation.




B. Day 63
TNC [cell mL-1]
1.E+02
1.E+04
1.E+06
1.E+08
Depth [m bs]
Transect A
Transect C
Transect F
Toluene [mg L-1]
toluene
VLP
bacteria
protozoa


C. Day 137
TNC [cell mL-1]
1.E+02
1.E+04
1.E+06
1.E+08
Depth [m bs]
Transect A
Transect C
Transect F
Toluene [mg L-1]

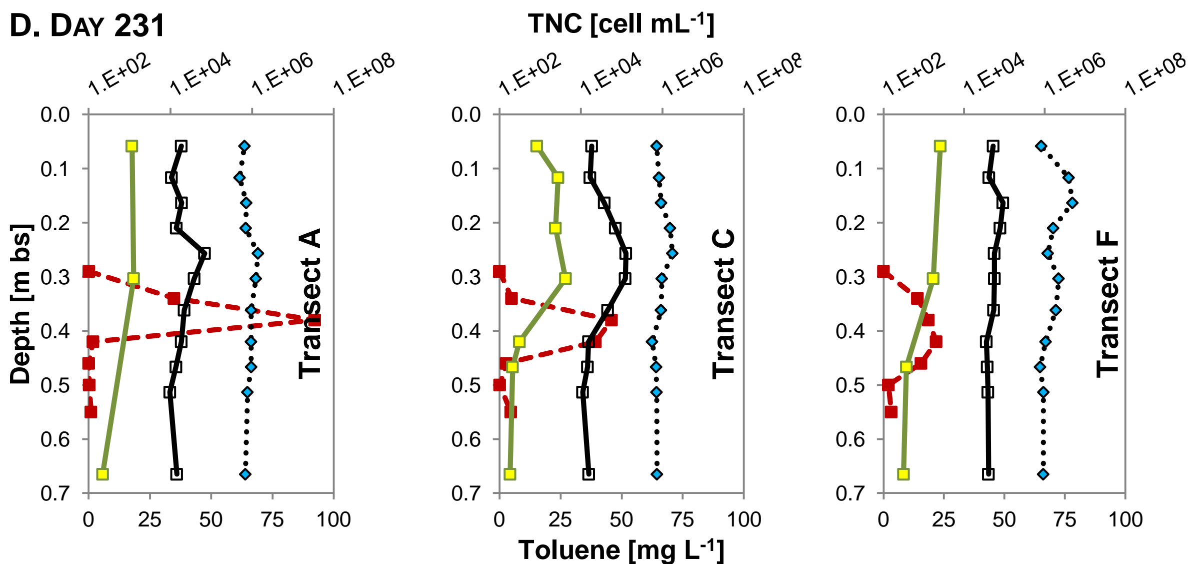


Fig. 4.6 Depth profiles of the total number of bacterial cells, virus like particles (VLP) and protozoa in water samples at pristine stage (A), during constant toluene injection (B), pulse toluene injection (C), and the recovery phase (D).

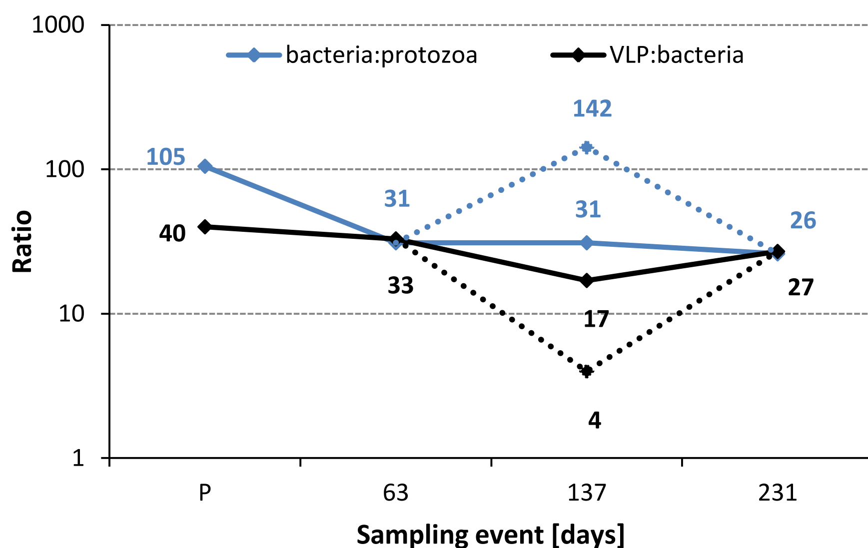


Fig. 4.7 Ratios between VLPs, bacteria and protozoa in groundwater from the pristine aquifer (P), during toluene contamination (63, 137) and the recovery phase (231). At day 137 solid lines indicate a ratio derived from transects A & C only; whereas the dotted lines show ratios observed at transect F.

4.3.4 Diversity and evenness of sediment bacterial communities

Sediment bacterial communities, as analyzed via T-RFLP fingerprinting, displayed statistically significant differences in community composition between samples from the toluene plume and from areas outside the plume (R= 0.46, p= 0.0001, ANOSIM). For a further evaluation of these detected differences, a principal component analysis (PCA) was conducted based on a variance-covariance matrix (Fig.4.8). As seen from the vectors, T-RFs 77, 490 accounted for the highest variance within the dataset and were found only in the toluene plume in high abundance. T-RFs 491 and 147 were present in toluene impacted and toluene free samples. As underlined by the Shannon diversity (H'), evenness and OTU richness, the bacterial community from outside the toluene plume showed a rather constant pattern, whereas the community inside the plume was characterized by contaminant-driven fluctuations (Fig.4.9). Moreover, the toluene impacted microbial community exhibited a considerably lower richness, diversity, and less even community at day 16 and 137 (Fig.4.9). The spill of pure toluene caused the strongest impact on the microbial community patterns, as shown by the decrease of T-RFs, the lower diversity and evenness. Disappearance of toluene from the system went along with the recovery of bacterial richness, diversity and evenness similar to the levels exhibited by the bacterial community outside the plume.

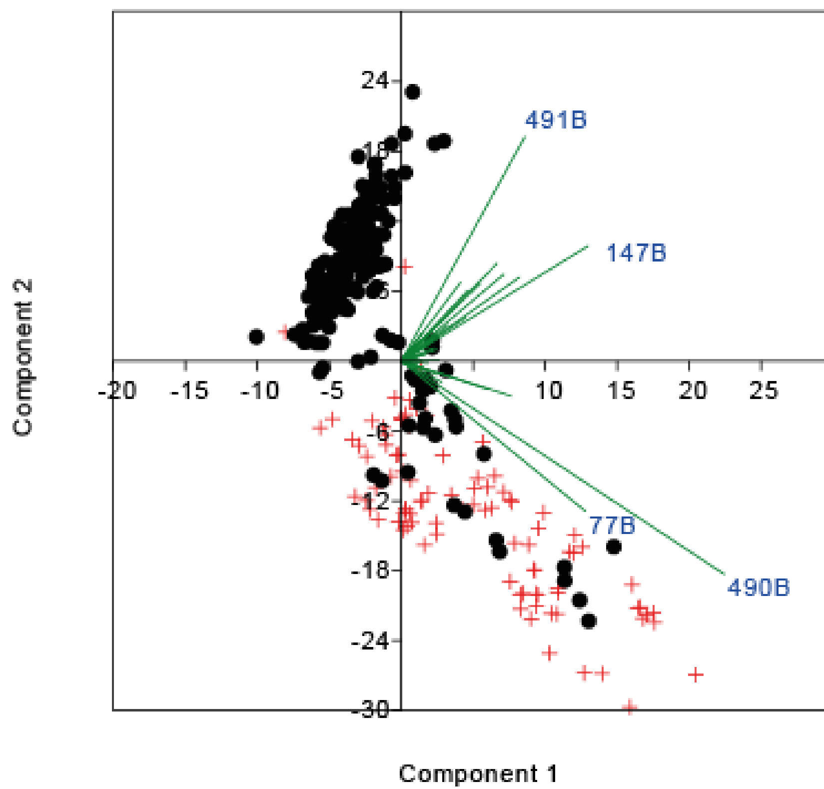
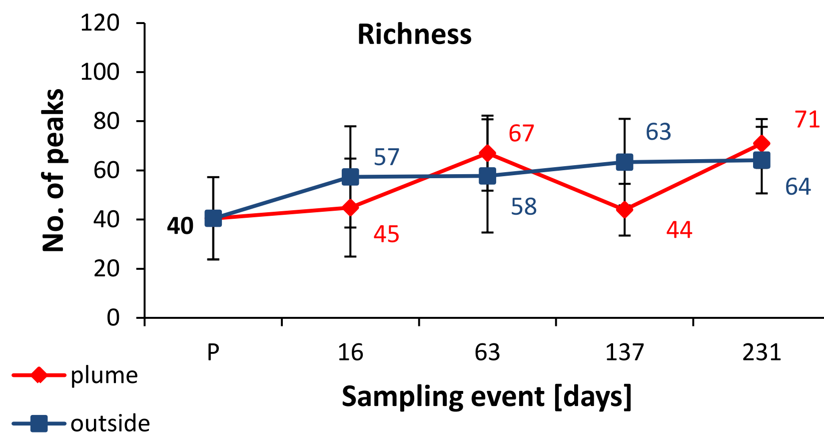


Fig. 4.8 Principal component analysis of T-RLFP fingerprints from sediment samples. Pooled dataset of T-RFs from inside the toluene plume and outside was analyzed.



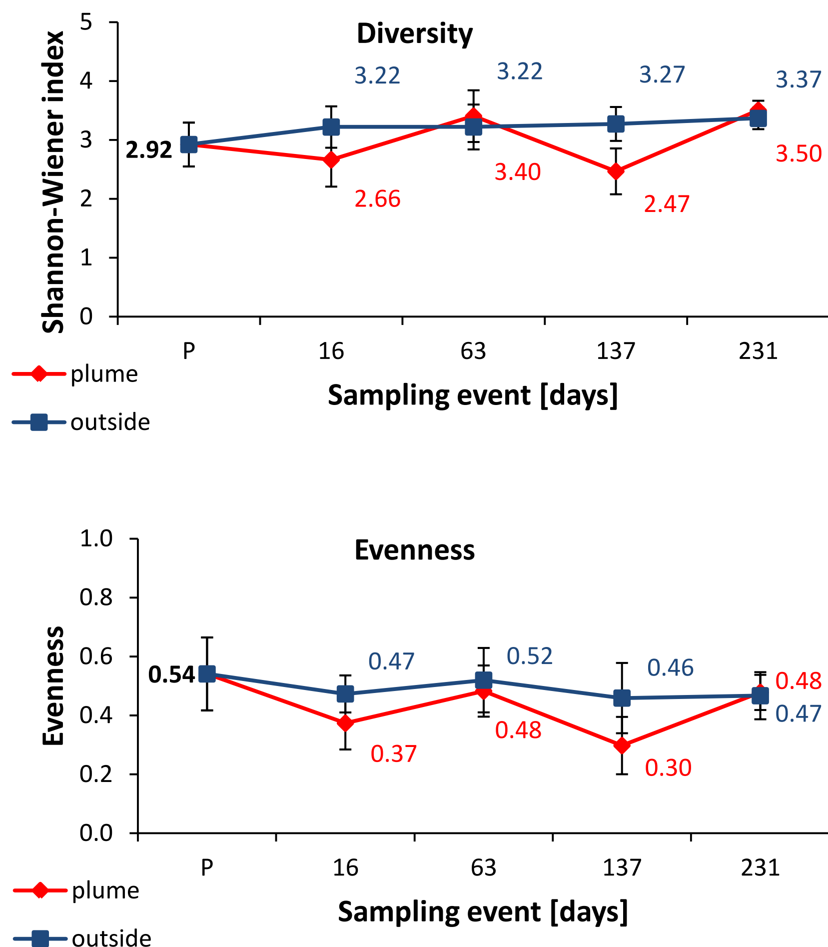


Fig. 4.9 Sediment bacterial community patterns, i.e. richness, Shannon diversity and evenness from outside and inside the toluene plume, as well as from the pristine aquifer prior to contamination.

4.4 Discussion

4.4.1 Resistance and resilience of pristine aquifers

Groundwater ecosystems are generally poor in organic carbon and energy, and therefore expected to be highly vulnerable to disturbances such as the load with organics. Due to the restricted mixing in porous sedimentary systems, organically contaminated aquifers readily switch from oxic to anoxic conditions. In consequence, we hypothesized that pristine aquifers have a low potential of ecosystem resistance and resilience. Following the ecosystem´s stability concept with resilience and resistance as its integral part (Holling,

1973b), two types of ecosystems are distinguished: (i) highly resistant and resilient ecosystems and (ii) ecosystems with a low resistance and resilience potential (Scheffer et al., 1993; Gunderson, 2000; Scheffer and Carpenter, 2003) (Fig.4.10). Lakes and rivers mainly belong to systems with a relatively high resistance and resilience potential, since it takes a powerful disturbance to change their state sustainably. These systems have a strong buffer capacity. This is especially true for larger lakes. Lotic systems on the other hand have an immense potential for a fast recovery. Low energy ecosystems such as oligotrophic aquifers characterized by a mainly laminar flow regime, a simple trophic structure of their communities and a narrow ecological amplitude of most of the species are generally not able to withstand disturbances without severe changes in their biological components, which in fact may go along with changes and loss of ecosystem functions and services (Boulton et al., 2008) as well as impairment of water quality . The input of organic carbon likely stimulates microbial activity and growth, increases cell sizes and biomass, and lead to changes in microbial community composition (Haack and Bekins, 2000; Goldscheider et al., 2006; Griebler and Lueders, 2009). Impact and persistence of an organic contamination in groundwater ecosystems depend on the type of contaminant, it´s concentration, the hydrogeological properties of the system, the physical-chemical conditions and potential degrader organisms present. With regards to the slow water flow, the comparable long water residence time, and the huge surface for sorption of contaminants, porous oligotrophic groundwater ecosystems when disturbed, will tend to persist, for considerable long time, in their altered state. And most likely removal of contamination from the system might not be enough to readily restore its biotic and abiotic components to the state from before the disturbance. This at least is the theory. To date, groundwater field studies and large scale experiments, with a focus on ecosystem resistance and resilience, are lacking. Moreover, since in most cases research follows the contamination, there is hardly any study that

included (i) characterization of the pristine stage, (ii) followed the contamination and (iii) its removal, evaluating the ecosystem´s recovery.

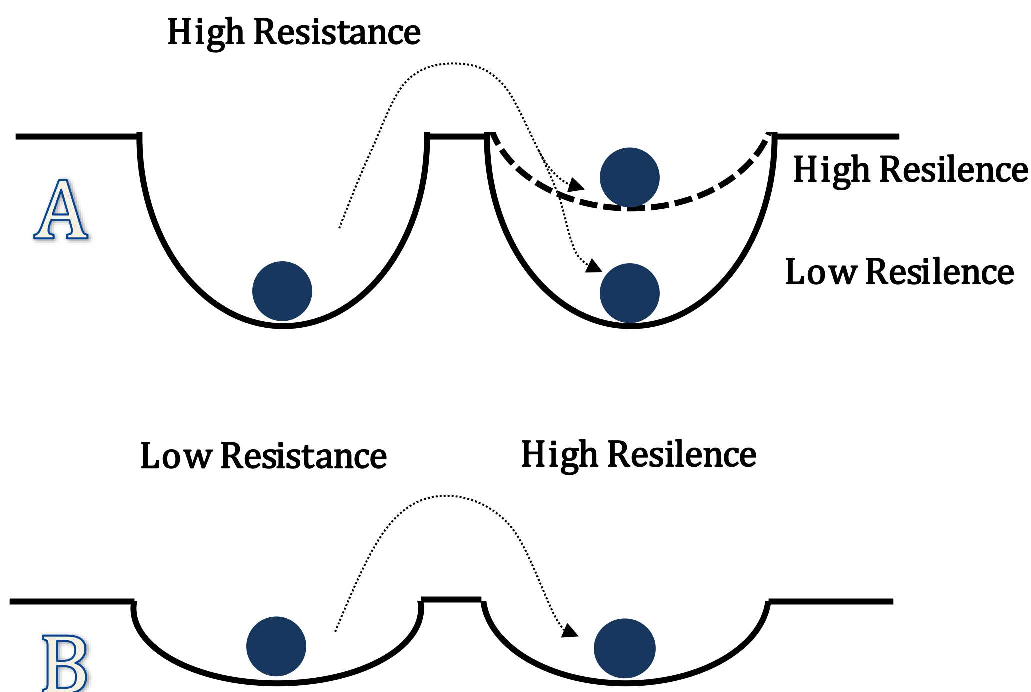


Fig. 4.10 Schematic representation of a high (A) and low (B) ecosystem´s resistance and resilience. Valley represents the stability domain whereas the ball stands for an ecosystem.

The oligoalimonic, energy poor indoor aquifer was, as initially hypothesized, very sensitive to the toluene contamination. Rapid oxygen depletion, an increase in the total number of bacteria, protozoa and VLPs as well as pronounced compositional reorganization of the bacterial community in the toluene impacted zones, indicated that the aquifer was not resistant to the organic contamination. On the other hand, very surprising was the unexpected high indigenous potential for aerobic degradation of toluene. Within only two weeks, more than 94% of toluene from a continuous 25mg L^{-1} source was mineralized within the travel distance of 4.40 m (see Chapter 3 of the thesis). In contrary, the intrinsic potential for a fast

anaerobic transformation of toluene was poor. With the expansion of oxygen depleted zones, the toluene concentrations in the aquifer went back up (see Chapter 3 of the thesis) with substantial biodegradation activities restricted to the plume fringes where oxygen was replenished from outside by transverse dispersion. In conclusion, continuous leaking of an organic contaminant will locally initiate a succession of changes, e.g. shifts of the dominant redox processes from aerobic mineralization to denitrification, iron and sulfate reduction (Wiedemeier et al., 1999; Christensen et al., 2000), always accompanied by changes in microbial community composition. It is worth mentioning that processes such as iron and sulfate reduction create, via the production of sulfide and ferrous iron, a long contamination legacy that prevents a fast return of the impacted areas to natural conditions after removal of the source.

As a follow up to the continuous toluene injection, the simulated short term (6h) toluene spill (day 137), was expected to create a long lasting source by NAPL phase trapped in the sediment pore space continuously releasing toluene to the groundwater forming a contaminant plume. Surprisingly, the 200 mL of toluene phase injected readily disappeared from the aquifer and was not anymore detectable 94 days after the spill. High oxygen concentrations at day 231 clearly pointed at a recovery of the system with respect to its redox status. However, a look at the biological, functional and compositional components of the aquifer, i.e. patterns of bacteria and protozoa did not allowed to draw the conclusion that the aquifer returned to its original status. While the bacterial abundance and activity in pore water at day 231 have returned to their initial levels, attached communities still exhibited patterns found during the presence of toluene, obviously requiring more time to recover from the disturbance. The same was seen for the abundance of protozoa, which despite the decrease of their prey (bacteria), was found to be significantly elevated at the end of the experiment. Since the abundance of protozoa depends on the abundance of bacteria, it is

suggested that protozoa in the sediment pore water at least partly grazed on bacteria attached to the sediment grains. Regarding the sediment bacterial community composition, no recovery could be found at day 231. To evaluate the time needed for the sediment community to recover another sampling campaign is recommended since the time span of 94 days obviously was too short. However we might also speculate that the bacterial community composition as revealed at day 231 provides a "final picture" of the toluene impact lasting for several years. However, given enough time, the lack of organic carbon and energy, should force at least the microbial biomass and cell numbers back down to initial, contaminant free conditions. The disappearance of toluene should promote the return of sensitive species; while for toluene degraders, the contaminant-free conditions might be stressful leading to a turn down of their reliable abundances to the level as seen exhibited prior to the contamination.

4.4.2 Indicators of groundwater disturbance and recovery

Over the past decades, human society has unequivocally introduced global degradation and disruption of groundwater systems to a degree that now requires assessment, intervention and remediation. Abuses such as diffusive loading with nitrate and pesticides, leaking sources of organic compounds and heavy metals as well as contamination with pharmaceutics, represent just some of the problems that impair ecosystem functions and threaten drinking water quality. Therefore, indicators for an appropriate and reliable assessment of (i) the current ecosystem status, (ii) its changes, (iii) trends over time, as well as (iv) the evaluation of the recovery progress are urgently needed. In fact, much of our knowledge on groundwater ecosystem response and recovery is incomplete and ecosystems complexities and variabilities in nature do not facilitate an easy choice of essential indicators

that would adequately characterize the system. Therefore the question about what to measure in order to describe the response and recovery of a system is often posed (Cairns et al., 1993; Niemeijer and de Groot, 2008). Ecosystems are composed of a structural (habitat characteristics), functional (major activities and processes) and compositional (community diversity, richness, evenness) level. Stress might cause changes at more than one level, e.g. changes in the composition of biocenoses as well as changes in organic carbon mineralization (Cairns and Niederlehner, 1993; Fraenzle, 2003; Griebler et al., 2010). Thus intelligent selection of monitoring criteria will consider more than one ecosystem level.

Measures of functional (bacterial carbon production, ATP) and compositional (total number of bacterial cells, abundance of protozoa and VLP, bacterial Shannon diversity) properties of the indoor aquifer, successfully provided insight into early stages of its response to toluene impact, the behaviour with prolonged and pulse contamination, as well as its progress of recovery.

4.4.2.1 Oxygen concentration

Oxygen was found to be a very good early warning indicator of aquifer disturbance by organic contamination. Since oxygen is the terminal electron acceptor in aerobic oxidation of organics, it is a “functional” indicator. A fast decrease in oxygen concentrations hints at pronounced activities. In our experiment, oxygen concentrations started to decrease immediately in areas where toluene was present. Oxygen, also rapidly retrieved its full saturation levels once toluene disappeared from the system (day 231). As almost exclusively aerobic degradation took place, oxygen concentrations reflected very well the presence and absence of toluene, however failed to indicate the biological status of the aquifer, since at day 231 its functional and compositional components were still distorted from the pristine stage.

The fast return of oxygen to values reported from before the contamination is a result of the hydrological properties of the indoor aquifer and the contaminant`s characteristics. Toluene is quite easily degradable and has a low sorption tendency.

4.4.2.2 Bacterial abundance, activity and ratios

Microbial variables such as the bacterial abundance (BA), the ratio of cells attached to sediment particles and suspended in the water, and the activity in terms of biomass production and active biomass, were found to be very good indicators of changes in the system`s status. Water and sediment samples collected from toluene impacted areas showed a clear increase in cell counts compared to toluene-free areas within the aquifer (Fig.4.2). Exactly the same pattern could be drawn from measurements of ATP concentrations, indicating more active microbial biomass in the toluene plume (Fig.4.3). The bacterial abundance found in groundwater and sediments from outside of the toluene plume was in the range typical for natural, contaminant free aquifers (Ghiorse and Wilson, 1988; Brielmann et al., 2009; Griebler and Lueders, 2009; Zhou et al., 2012) whereas samples from the toluene plume exhibited values as found in contaminant impacted aquifers (Haack and Bekins, 2000). The same was true for ATP concentrations (Aamand et al., 1989; Lautenschlager et al., 2010).

Attached and suspended cells, although both being distorted from the initial level, exhibited very different patterns upon disturbance and during recovery. Suspended bacteria showed an immediate response to toluene, acting therefore, similarly to oxygen, as an intrinsic early warning indicator. The temporal increased cell numbers in pore water at day 4 were most probably a result of cells reproducing and sensitive cells being either killed or actively leaving the sediment into the water fraction. A statistically significant increase of

suspended cells was fund starting from day 63, however, when toluene was no longer present in the aquifer, cells counts in water decreased immediately. The tight coupling between bacterial numbers in pore water and toluene, makes this variable an indicator of the aquifer`s early response to contamination. However, the variable was inadequate for assessing the system recovery. Patterns observed for total suspended bacteria were nicely mirrored by ATP concentration, suggesting its valuable role as easy and fast indicator at the ecosystems functional level. This is important in situation when an immediate estimation of the groundwater ecosystem status is needed, since ATP measurements are fast. Successful application of ATP measurements assessing stagnant tap water quality, looking at overnight growth of microbes, was shown by Lautenschlager et al. (2010).

When analyzing changes of bacterial total counts in sediment samples, a completely opposite response to toluene impact was found. The attached bacterial community displayed a delayed and more conservative reaction to toluene input with significant increase in cell density only at day 63. Later, the abundance stayed high even when toluene was no more detected in the aquifer. High levels of ATP detected at day 231 supported the long term survival of an abundant and active bacterial community in the sediment matrix. Attached bacteria have a high potential in integrating long-term disturbances to the system providing valuable information about the past. These two different response patterns of attached and suspended bacteria underline the importance of assessing the abundance of both. Sediment communities as long-term indicators are necessary in reflecting alterations at large temporal scales, showing in our case the lack of aquifer recovery despite the absence of contaminant, high oxygen concentrations and low cell numbers in pore water. Importance of sampling water and sediment for reliable information of processes in subsurface, was showed in several earlier studies, e.g. (Alfreider et al., 1997; Griebler et al., 2002; Anneser et al., 2010).

The ratio of attached to suspended bacteria was suggested to be a valuable indicator of

prevailing nutrient conditions in the environment, which a high ratio typical for carbon and nutrient poor conditions and a low ratio at elevated concentrations of carbon, e.g. (Harvey et al., 1984; Griebler et al., 2001; Griebler et al., 2002). Indeed, comparing ratios in the toluene plume with ratios from outside, different patterns can be seen. Samples from contaminant-free areas of the aquifer show rather constant ratios in comparison to the toluene plume, where ratios were considerably lower. Moreover, fluctuations of the ratio curve in the toluene plume, well depict the dynamic changes of the bacterial abundance at the start of the contaminant injections, when a decrease of the ratio was observed as well as after the exposure of the aquifer to the toluene spill, reflected in the lowest attached to suspended ratio. The previously discussed "memory effect" of the sediment was reflected by a steep increase of the ratio at day 231. Surprisingly low was the initial ratio in the pristine aquifer samples, and according to literature e.g. (Griebler et al., 2002; Goldscheider et al., 2006; Griebler and Lueders, 2009) higher values are expected. Repeated measurements of the surplus sediment samples from pristine stage, revealed 2-fold higher bacterial abundance and a consecutive increase of the ratio to 82:1, instead of 42:1, similar to what have been later observed in the toluene free areas, during the phases of contamination and recovery.

4.4.2.3 Bacterial community biodiversity indices

Bacterial diversity, richness and evenness, common measures of community structure, were found to be good indicators of a community response to the toluene impact. The theoretical relationship between community diversity (inferred from species richness and evenness) and ecosystem stability (Chapin et al., 1997; Chapin et al., 2000; Wittebolle et al., 2009; Downing and Leibold, 2010), have repetitively led to the opinion that a high diversity stands for a healthy ecosystem, whereas a decrease in diversity alerts ecosystem deterioration

and decline in ecosystem integrity (Briones and Raskin, 2003; Girvan et al., 2005; Bruelheide and Luginbuehl, 2009; Clements and Rohr, 2009). However, it is worth to mention that using bacterial community structure as an indicator, one might be limited in early management decisions. By the time changes in microbial community composition are detected, a considerable harm to the ecosystem may have already occurred.

As previously hypothesized, the microbial community of the low energy and low dynamic indoor aquifer reacted abruptly to the toluene impact. Bacterial community composition inside the toluene plume was shown to be significantly different compared to the community outside. This is underlined by the two separate clusters depicted on the PCA plot (Fig.4.8) as well as the patterns observed for bacterial richness, diversity and evenness (Fig.4.9). Although a contamination, in terms of degradable organic compounds, when introduced to an oligotrophic, groundwater ecosystem is believed to cause an increase in microbial diversity (Horner-Devine et al., 2004), in our case, a pronounced decrease was observed. Bacterial diversity, evenness and taxonomic richness in non-contaminated sediment was found to be relatively low, as typically shown for oligotrophic groundwater ecosystems (Goldscheider et al., 2006; Brielmann et al., 2009; Griebler and Lueders, 2009; Griebler et al., 2010) and exhibited rather constant patterns during the time of the experiment. Significantly different patterns were derived when analyzing samples from inside the toluene plume (Fig.4.9). Fluctuations observed, reflected the different toluene injection modes and concentrations. Initial exposition of bacterial community to toluene impact resulted in a pronounced bacterial community reorganization, as shown by the first deflection on the curves. Further recovery of the bacterial richness, diversity and evenness, occasionally exceeding even values reported from non-impacted areas are due to growth and establishment of toluene degrading species facing constantly incoming toluene. Later, during the spill of pure toluene again pronounced shifts in the community of degraders occurred, as showed by

a new drop in richness, diversity and evenness. As discussed in details in Chapter 3 of the thesis, the accidental spill of toluene led to the decrease of TR-Fs still present or even established during the early response to toluene contamination and, on the other hand, an increase in relative abundance of individual TR-Fs which were more adapted to high toluene concentrations.

Disappearance of toluene from the aquifer and the recovery of oxic conditions went along with an increase of bacterial richness, evenness and diversity to pristine or almost pristine levels indicating recovery of the microbial community. In fact, the community composition at day 231 was significantly different compare to the pre-contamination stage, hinting at a high resilience in terms of diversity and evenness but at a low resilience in terms of community composition. In fact, the key degraders started to decrease in abundance, however at that time they still constituted a dominant part of the community. Retrieved richness, diversity and evenness to the levels from before the contamination, could then only be explained by re-appearance of toluene sensitive members of the community.

The highly abundant T-RFs detected only in the toluene plume i.e. TR-Fs 490 bp and 77 bp, as seen from the PCA plot, may serve as indicator species. However due to only 1 bp difference between TR-F 490 bp (present only in the plume) and 491 bp (present in pristine sediments) this needs future evaluations, since they might represent the same OTU (Lueders and Friedrich, 2003). Using TRFs as indicators would also ask for further identification of the key strains by sequencing.

4.4.2.4 Food web dynamics

Food web dynamics are the basis of ecosystem functioning and therefore can be directly related to ecosystem health (DeAngelis et al., 1989; Thompson et al., 2012). In fact,

analysis of food web dynamics contribute important information in the assessment of ecosystem status, since the food web structure integrates various direct and indirect impacts of stressors over time (Rohr et al., 2006). The indoor aquifer harbored a considerable abundance of virus-like particles and protozoa. Microscopically examined samples, revealed that not only flagellated protozoa were present, but also ciliates, as judged by their morphology. In pristine groundwaters, protozoa generally exhibit a rather low abundance, ranging from $< 10^0$ to10^2 cells g dw^{-1} (Novarino et al., 1997). Their numbers might, however, increase by several orders of magnitude due to organic contamination (Kinner et al., 1998; Zarda et al., 1998). Protozoan density in the pristine indoor aquifer was in the range of 10^2 cells mL^{-1} and, in some samples, a high portion of cells were encysted. The ratio of bacteria to protozoa ranged from 45-105 depending whether cysts have been accounted for or not. It is known that protozoa appear to produce cysts due to unfavorable environmental conditions such as, high temperature, shortage of food, extreme pH, or lack of oxygen (Sleigh, 1989). In our case, the encysted cells in the pristine aquifer can be related to the energy-poor conditions.

Virus-like particles were found in quite high abundances in our indoor aquifer. The ratio of VLPs to bacteria at pristine conditions was 40:1, which was found to be considerably higher compare to ratios reported in groundwater or marine aquifers, i.e. 3 – 6 (Corinaldesi et al., 2003), 6 (Roudnew et al., 2012), 12 (Kyle et al., 2008). Studies on the relationship between VLPs and bacteria in aquatic ecosystems emphasize the important role of viruses in bacterial mortality (Wommack and Colwell, 2000; Weinbauer, 2004). Therefore the elevated VLPs to bacteria ratio in the pristine samples hint at the potential importance of viruses under these conditions. A high correlation between VLPs and bacterial abundance may indicate that viruses actively control the population size of bacteria. On the other hand, the lack of correlation between VLPs and bacterial carbon production (BCP), as well as VLPs and ATP,

and taking into account the low values of bacterial active biomass and BCP, we could speculate that viruses had a rather minor impact on bacterial abundance at that phase of the experiment. Bacteria in pristine aquifer are generally in the state of low metabolic activity, and as such, less attractive to viral infection.

Injection of toluene resulted in an increase of the numbers of predators of bacteria, i.e. protozoa and bacteriophages (viruses). A strong coupling to the bacteria was proven by strong statistical correlations. In the areas of the aquifer where toluene was not detected, no dynamics became visible during the entire time of the experiment (Fig.4.5), with one exception - an increase of protozoan abundance at day 137. Toluene driven changes of aquifer´s food web was well depicted by the decrease of ratios (Fig.4.7). Facing the contamination, bacterial counts, increased 4 times compared to pristine phase, whereas protozoan counts increased by factor of 7. A link between protozoan abundance and organic matter input is frequently reported in the literature. Their elevated numbers indicate rapidly growing bacterial populations obviously stimulated by input of organic carbon. Monitoring of protozoan abundance well indicated the increase of bacterial cell numbers, and indirectly - disturbance of the indoor aquifer. Use of protozoa as bioindicators of water and sediment contamination is documented and discussed in the literature (Cairns Jr. et al., 1972; Madsen et al., 1991; Berger et al., 1997; Foissner, 1999). Elevated protozoan counts in contaminated groundwater samples were also shown by e.g. (Sinclair et al., 1993; Kinner et al., 1998; Kota et al., 1999).

In the pore water of the toluene free aquifer, bacteria decreased fast in abundance, approaching background levels at each transect. This response was not observed in protozoan abundance. Despite the obvious decrease of their prey, protozoan abundance stayed elevated, suggesting protozoa grazing on attached bacteria, which abundance was still high at that time. On a long run protozoa should contribute to decrease in attached bacterial biomass and then

decline in numbers themselves or encysts. In summary, protozoan density reacting rapidly to toluene impact, served as a very good early warning signal of aquifer disturbance. Moreover, their elevated numbers still present 94 days after toluene disappearance makes them long-term indicators of ecosystem disturbance.

The abundance of VLPs closely followed the patterns of bacterial cells in groundwater during the toluene impact, as well as during contaminant disappearance and system´s recovery, suggesting a close coupling between bacteriophages and bacteria. Moreover, high correlation between VLPs and bacterial abundance, bacterial carbon production and ATP upon toluene input indicate, as mentioned elsewhere (Fuhrman, 1999; Wommack and Colwell, 2000; Weinbauer, 2004) preferential viral infection of active, living bacterial cells over those exhibiting low metabolic activity. As already shown for protozoa, the discrepancy in the VLPs to bacteria ratio at day 137 was mainly due to the high bacterial abundance at transect F. In summary, the host-depended mode of reproduction of viruses makes them react quickly to changes in prey abundance offering a valuable indicative power of this variable. The high correlation of VLPs with bacterial ATP and bacterial carbon production hint at possible role of viruses in controlling the abundance of bacteria and most probably key degrader population in contaminated aquifers, and should thus be taken in consideration when assessing natural attenuation. Moreover, despite the very strong correlation between biological variables (e.g. ratio of bacteria and VLPs abundance), no collapse of biodegradation was observed, which reflect the carefully maintained trade-off between prey and predators, as well as predators themselves.

4.4.3 Evaluation of used indicators

As underlined in the introduction, the assessment of groundwater ecosystem services and health, prevention from ecosystems disturbance and collapse of its functions as well as

evaluation of remediation progress, needs proper multilayered assessment schemes and sets of indicators. Without them, judgement on ecosystem status, response to disturbance and recovery is incomplete. Our study could show that from the presented set of variables, some have a clear indicative value with respect to toluene impact on the indoor aquifer and its recovery.

A key aspect in prediction of site-specific response and recovery is a profound understanding of ecosystem behaviour. Thus, understanding the interplay of ecosystem`s biological components and biogeochemical processes is required in identifying reliable indicators. Moreover, it is of immense importance to conduct long-term ecosystem studies, since they are the only “way/vehicle” by which the ecosystem performance can be understood. Secondly, defining clear statements of recovery of “what” with reference to “what” we are looking for, will help in development of general guidelines and models on response and recovery assessment.

4.5 Supplementary material

Tab. 4.S1 Spearman rank correlation of selected parameters in pristine aquifer (A), during constant toluene injection at the day 63 (B), during pulse toluene injection – day 137 (C), and recovery phase – day 231 (D).

A).

PRISTINE		VLPs	protozoa	ATP	BCP
bacteria	r	**0.71**	0.52	-0.14	0.20
	p	**≤ 0.01**	0.14	0.58	0.41
VLPs	r		0.62	-0.11	0.20
	p		0.07	0.65	0.41
protozoa	r			0.33	0.23
	p			0.36	0.52

B).

Day 63		VLPs	protozoa	ATP	BCP	toluene
bacteria	r	0.52	0.92	0.71	0.19	0.74
	p	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	0.29	**≤ 0.01**
VLPs	r		0.70	0.43	0.38	0.71
	p		**≤ 0.05**	**≤ 0.05**	**≤ 0.05**	**≤ 0.01**
protozoa	r			0.84	0.58	0.85
	p			**≤ 0.01**	0.07	**≤ 0.01**

C.)

Day 137		VLPs	protozoa	ATP	toluene
bacteria	r	0.79	0.78	0.74	0.71
	p	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**
VLPs	r		0.51	0.71	0.42
	p		0.06	**≤ 0.01**	0.06
protozoa	r			0.21	0.68
	p			0.46	**≤ 0.01**

D).

Day 231		VLPs	protozoa	ATP
bacteria	r	0.78	0.74	0.67
	p	**≤ 0.01**	**≤ 0.01**	**≤ 0.01**
VLPs	r		0.62	0.67
	p		**≤ 0.05**	**≤ 0.01**
protozoa	r			0.38
	p			0.17

4.6 References

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5. Final discussion

Hydrocarbon pollution of Earth has been a problem ever since man began to use fossil fuels. For certain types of hydrocarbons and under certain environmental conditions, naturally occurring physical, chemical and biological processes collectively termed as “natural attenuation” (NA) result in substantial degradation or transformation of contaminants. Biodegradation is performed by naturally occurring microorganisms or lab strains and consortia released to the subsurface, and is of major relevance since it leads to the removal of pollutant mass from the environment (Wiedemeier et al., 1999; Röling and Verseveld, 2002; Smets and Pritchard, 2003) and in the best case, restoration of ecosystem status and functions. The hydrological and geochemical properties, and the microbial activities of the system are strongly coupled (Lovley and Chapelle, 1995; Chapelle, 2000). In consequence, NA is not always effective. Successful NA requires the spatio-temporal integration of complex interactions that occur between the contaminants, the subsurface environment, and the indigenous microbial populations at each site. That is, integration of relevant processes operating at scales ranging from 10^{-6} m (microbial cell diameter) up to dozens or thousands meters (field site) (Fig.5.1) (Dobbins et al., 1992; Smets et al., 2002; Schirmer and Butler, 2004). Impairment or modification of one of those processes influence consecutively the overall response, the progress of NA and the recovery of the groundwater systems from contamination at a given site.

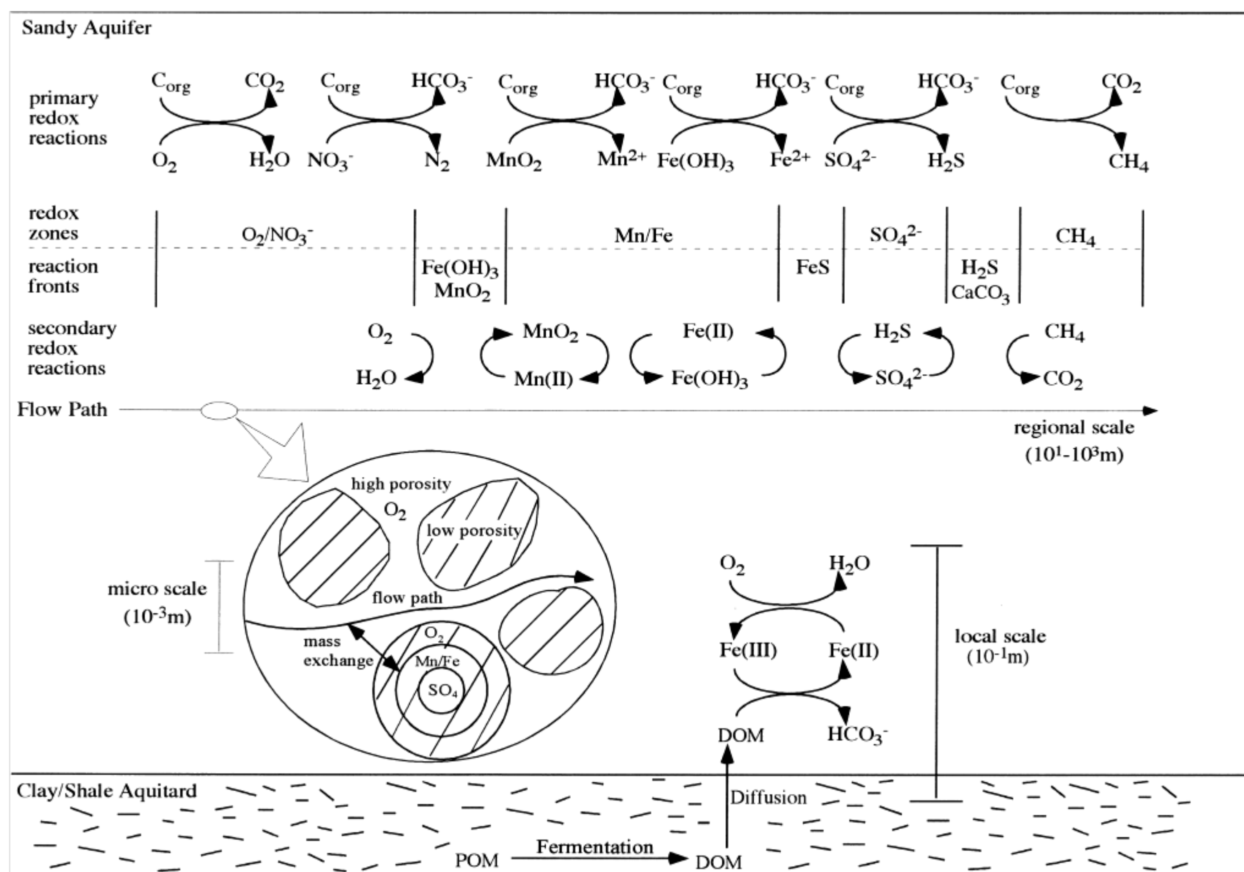

Fig.5.1 Sketch of a porous aquifer system showing redox reactions and zonations at a variety of spatial scales, modified after (Hunter et al., 1998).

Groundwater ecosystems, when compared to surface aquatic systems, constitute, due to their geochemical, hydrological and biological properties, special and extreme habitats for living. This is important also from the perspective of NA potential and degradation services. Poor in organic carbon and energy, characterized by low microbial diversity, a simple trophic structure of their communities and narrow ecological amplitude of most of the species, oligotrophic groundwater aquifers are considered to be very sensitive and vulnerable to input of organics (Goldscheider et al., 2006; Griebler and Lueders, 2009). Pristine, organic-poor shallow groundwater aquifers have typically considerable amounts of dissolved oxygen. When contaminated - aerobic biodegradation of organic compounds in those aquifers may occur very rapidly. For microorganisms, aerobic respiration is the most energetically favourable reaction (Chapelle, 1993; Lovley and Chapelle, 1995). However, because of the

restricted mixing and slow replenishment of oxygen, aquifers contaminated with organics, such as hydrocarbons, become quickly depleted in oxygen (Christensen et al., 2000; Bauer et al., 2008; Bauer et al., 2009). Aquifers, once anoxic, are consequently believed to persist in their altered stage for a long time. They will become oxic again, only when the contamination is gone and reduced chemical species have been re-oxidized. However, due to the long water residence times as well as the structure of porous aquifers containing a huge sediment surface for sorption of contaminants, it´s generally believed that a severe contamination leads to irreversible changes at the functional (e.g. biomass, grow rate, ratio of prey to predators) and compositional (e.g. diversity, evenness) level of the groundwater ecosystem, interpreted as a lack of resilience. Bearing this in mind, one aim of this PhD project was to assess the spontaneous, intrinsic biodegradation potential of a pristine oligotrophic and oligoalimonic aquifer when contaminated with toluene. As presented in Chapter 2, our indoor aquifer system when subjected to a 30h pulse of 486 mg of toluene, revealed over a distance of 4.2 m and in a time period of 150h, on average a 40% toluene reduction, attributed to spontaneous intrinsic microbial degradation. Mainly estimated from the break through curves of toluene and deuterated water as a conservative tracer, toluene specific carbon isotope analysis showed an overall enrichment of heavy isotopes, confirming the degradation of toluene by the indigenous microbial community. Later, as is discussed in Chapter 3, the aquifer when subjected to the same toluene input concentration, but injected continuously, showed a 95% reduction of toluene at day 16 after contamination switch on. Follow up sampling campaigns revealed, however, a surprising picture. At day 63 of the experiment, toluene concentrations measured in the aquifer were 10 times higher than at day 16, accounting for only 30% toluene removal. This re-appearance of toluene co-occurred with a change from oxic to anoxic conditions. The above described reaction of the indoor aquifer to the toluene pulse, the continuous toluene source, as well as the spill put emphasize that pristine groundwater

systems, although being energy limited posses a very high and rapid intrinsic potential for the biodegradation of toluene, a monoaromatic hydrocarbon. The early natural attenuation (NA) of the contaminant was exclusively related to aerobic bacteria. Since mean doubling times of bacteria in oligotrophic aquifers are generally long, ranging from days to months or even years, the relatively short pulse of toluene lasting for only 30 hours may be assumed not long enough to activate dormant cells and induce in situ growth of specific degraders. In consequence, toluene degradation was most probably carried out mainly, if not explicitly, by native, naturally occurring hydrocarbon degraders in the pristine, non-contaminated aquifer which in fact are expected to be ubiquitous, since specific hydrocarbons, such as toluene, occur naturally in the environment. The 194 mg of toluene being biodegraded within that short time points at an enormous buffering capacity of aquifers. The subsequent application of constant toluene contamination revealed even more interesting aspect. Degradation of 95% of continuously incoming toluene, clearly showed, that the microbial community have an even higher NA capacity. However, with the depletion of oxygen in contaminated zones, toluene biodegradation activities slowed down, what was reflected in re-appearance of toluene at day 63. As commonly observed in subsurface aquifers, demonstrated in laboratory studies and proven in modelling scenarios, low solubility of oxygen in the water and restricted transversal mixing, of not only oxygen but all dissolved terminal electron acceptors, exert control on biodegradation rates (Cirpka et al., 1999a; Thornton et al., 2001; Jose et al., 2004; Bauer et al., 2008; Bauer et al., 2009; Rolle et al., 2009; Grathwohl et al., 2011). In our indoor aquifer, aerobic bacteria, having control on the initial toluene degradation, after some time depleted the oxygen in the core of the contaminant plume and consequently, restricted themselves from being active in this area giving way to anaerobic microorganism. However, these reacted pretty slowly. A stable anoxic toluene plume established providing some evidence for denitrifying processes during the next 70 days. The aerobic toluene degradation

was probably only confined to the plume fringes facilitated by oxygen replenishment from ambient water. This observation clearly implies that (i) groundwater systems have a big potential for the spontaneous biodegradation of easy degradable hydrocarbons, (ii) and the temporal dimension of this potential is determined by the supply and availability of dissolved oxygen, in porous systems, controlled by transverse mixing. The microbial community present and the transport processes exert the control of natural attenuation processes in a connected way. The majority of hydrocarbon contaminations in aquifers, by the time they are discovered, are already at steady-state with anaerobic degradation processes dominating. An overlapping, different redox processes (denitrification, manganese reduction, iron reduction, sulphate reduction, methanogenesis) e.g. (Davis et al., 1999; Christensen et al., 2000; Bekins et al., 2001; Cozzarelli et al., 2001; Anneser et al., 2008) and their small scale distribution in plumes may lead to steep, physical-chemical and microbial gradients. The availability of a large pool of potential electron acceptors suggests a quick and large biodegradation potential accomplished by anaerobes, but in fact, the residence time of contaminant plumes very often reaches dozens or even hundreds of years. As reduction of electron acceptors with electrons from available organic contaminant follows a certain sequence determined by the respective redox potentials, the energy yields which bacteria get from various processes differ. As long as oxygen is present, aerobic oxidation is the most energetically favourable degradation process, supporting fast growth of microbes and fast aerobic degradation of contaminants. Anaerobic processes, however, are considered inferior to aerobic degradation regarding their kinetics and capacities providing far less energy from substrate turnover. Consequently, anaerobes produce less biomass per substrate molecule and they grow slowly compare to aerobes. Additionally, factors such as (i) transverse dispersion, restricting mixing of electron donors and acceptors (shown by the example of oxygen depletion in the indoor aquifer, Chapter 3), (ii) transient hydraulic conditions, (iii) contaminant inaccessibility (e.g. due to

sorption), (iv) contaminant toxicity, (v) lack of nutrients (i.e. P, N) and (vi) unfavourable conditions, such as temperature, salinity, pH, have direct influence on contaminant transformation. Moreover, microbially-driven oxidation of hydrocarbons may lead to precipitation of reduced reaction products, as is observed during Fe(III) and sulphate reduction sustaining formation of iron sulphide (Fig.5.1). Precipitation of solid phases alters the chemical and physical characteristics of the porous aquifer, and delay or even impair the recovery of the aquifer to natural conditions after removal of the source.

Generation of anoxic conditions with iron (III) or sulphate reduction as prevailing process was not achieved in the indoor aquifer. Even, the simulated short term (6h) toluene spill which (day 137) was expected to create a NAPL phase trapped in the sediment pore space continuously releasing toluene to the groundwater, did not form a long-lasting contaminant plume. Surprisingly, the 200 mL of toluene phase injected, readily dissolved into the groundwater, disappeared from the aquifer and was not anymore detectable 94 days after the spill. This fact provided a few interesting aspects on aquifer recovery. High oxygen concentrations at day 231 clearly pointed at a recovery of the system with respect to its redox status. A similar conclusion was drawn from bacterial abundance and activity measures from pore water, which have been returned to the levels from before the contamination. However, the sediment samples still exhibited microbial patterns found during the presence of toluene underlying more time required for sediment communities to recover from the disturbance. The general lack of long-term studies on groundwater ecosystem recovery from disturbance, as well as the general absence of pre-disturbance data, makes it difficult to extrapolate findings from day 94 to a later time point and draw some final conclusions on the indoor aquifer´s resilience potential. We might, therefore, speculate that with respect to abundance and activity of attached bacteria, the disappearance of organic carbon source will entail a decline of their density and activities to the pristine levels. Regarding bacterial community

composition, toluene disappearance might facilitate the return of sensitive species, however, toluene degraders will remain at a higher relative abundance probably for many months and years. The community therefore might then express a new equilibrium state, stable, but different from the initial state. Such new compositional structure of the microbial community would provide a functional advantage to the ecosystem in case of a repeated stress, enabling fast reaction, e.g. (Wikström et al., 2000).

In search of reliable indicators for the assessment of ecosystem status, we observed significant differences in functional and compositional response and recovery of the indoor aquifer from toluene contamination. Chapter 4 describes the changes of the ecosystem on different levels of its biological organisation in detail, emphasizing the distinct patterns of aquifer response to the toluene injection as well as the toluene disappearance and ecosystem recovery. Based on dissolved oxygen measurements and determination of the abundance of suspended bacteria, the current status of the system was well depicted, due to a fast reaction of these two variables to toluene input and its disappearance. The incorporation of the sediment samples into our assessment, although expressing retarded reaction to the stress applied, revealed a not yet recovery of the bacterial community attached to the sediment, showing a "long memory". Based on these two contradictory pictures, we underlined the importance of collecting water and sediment samples when assessing the ecosystem state. Apart from changes in bacterial abundance, the decrease in ratio of attached to suspended bacteria upon toluene contamination and its subsequent recovery provided additional valuable insights into the system´s response to toluene and its disappearance. When following the compositional changes of the sediment bacterial community inside the toluene plume, dynamic changes of community diversity, evenness and richness, clearly indicated a fast reorganization of the community with toluene degraders increasing in relative abundance. Another aspect of ecosystem changes induced by toluene was the variations in the ratio of

viruses (bacteriophages) to bacteria and bacteria to protozoa. In fact, analysis of microbial food web dynamics contributed important information in the assessment of aquifer response to and recovery from toluene contamination. Changes in viruses and protozoa abundance served as early warning signals of aquifer disturbance and elevated numbers of protozoa still after toluene disappearance, served as long-term indicators of ecosystem disturbance. Undoubtedly, abiotic parameters only provided us with full picture of the ecological and functional aspects of aquifer response and recovery to toluene impact when complemented with biological measures. As such, successful assessment of groundwater ecosystem services, prevention from disturbance and evaluation of undertaken remediation actions, depends not only upon an understanding of microbial degradation processes, but also upon an understanding of the complex interactions that occur between the contaminants, the subsurface environment, and the indigenous microbial populations at each polluted site. For assessment purposes it therefore needs indicators that represent all levels of an ecosystem i.e. the structural, functional, and compositional level (Dale and Beyeler, 2001). Following the same line of arguments, since groundwater systems are the main source of drinking water, which is provided by purifying activities of microorganisms, sustainable management, restoration and protection of these resources calls for consideration of biotic criteria in water quality monitoring (Steube et al., 2009; Griebler et al., 2010; Stein et al., 2010).

The investigated indoor aquifer, with its data set collected at the pristine stage, during different scenarios of toluene contamination and upon termination of contaminant input, constitutes a unique experiment. Its size, the spatially highly resolved sampling and the use of a simple model contaminant applied, allowed an in depth ecological understanding of a pristine aquifer´s response to organic impact and its recovery. The resistance to the toluene stress, understood as the degree to which the system and its compartments (accounting for 3 ecosystem levels) remains unchanged, was shown to be poor implying its vulnerability and

thus raising the need for preventing groundwater ecosystem from deterioration. On the other hand, the energy poor system possessed a surprisingly high spontaneous degradation potential strictly linked to aerobic populations. The insufficient replenishment of oxygen to the porous sediment was shown however, to considerably slow down the natural attenuation capacities of the aquifer calling for further evaluation of factors limiting anaerobic NA. The reliable assessment of the indoor aquifer´s resistance and resilience was only possible due to set of indicators including both, biotic and abiotic variables as well as water and sediment samples. Within the time frame of 231 days (94 days after the contaminant switch off) the system has not fully recovered.

An important part of the work, still to be done is to translate our generic findings into application to enhance NA at sites contaminated with hydrocarbons and maybe with a more complex contamination.

5.1 References

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Appendix

Tab. A1. Toluene concentrations, total bacterial cell numbers and ATP concentrations of suspended cells in the pristine aquifer, during injection of toluene (Day 4- Day 137) and the recovery phase (Day 231).

sampling campaign	Sample name	z-coordinate [m]	toluene [mg/L]		bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD	mean	SD
PRISTINE	A_2_1	0.03	NA	NA	1.69E+04	9.90E+02	1.66E-11	6.36E-13
	A_2_4	0.16	NA	NA	1.64E+04	2.26E+03	5.01E-12	1.53E-12
	A_2_6	0.24	NA	NA	1.24E+04	1.06E+03	1.84E-12	2.55E-13
	A_2_7	0.29	NA	NA	2.38E+04	4.24E+02	1.46E-11	3.54E-13
	A_2_10	0.42	NA	NA	3.91E+04	6.86E+03	6.55E-12	1.20E-13
	A_2_13	0.55	NA	NA	1.17E+04	1.41E+03	7.87E-12	8.91E-13
	C_2_1	0.03	NA	NA	2.00E+04	3.82E+03	9.32E-12	1.22E-12
	C_2_4	0.16	NA	NA	1.95E+04	6.36E+02	3.80E-12	4.21E-12
	C_2_6	0.24	NA	NA	3.75E+04	2.12E+02	1.34E-11	2.12E-13
	C_2_7	0.29	NA	NA	5.16E+04	6.58E+03	1.73E-11	9.90E-13
	C_2_10	0.42	NA	NA	1.75E+04	3.04E+03	2.05E-11	1.41E-13
	C_2_13	0.55	NA	NA	2.00E+04	3.96E+03	1.23E-11	2.40E-12
	F_2_1	0.03	NA	NA	4.61E+04	1.56E+03	1.77E-12	2.23E-12
	F_2_4	0.16	NA	NA	4.69E+04	2.05E+03	3.94E-12	4.06E-12
	F_2_6	0.24	NA	NA	4.27E+04	2.62E+03	7.38E-13	7.07E-14
	F_2_7	0.29	NA	NA	4.11E+04	3.25E+03	7.34E-12	4.24E-13
	F_2_10	0.42	NA	NA	3.62E+04	7.64E+03	7.34E-12	3.54E-14
	F_2_13	0.55	NA	NA	4.99E+04	2.62E+03	6.61E-12	2.69E-13
Day 4	A_2_6	0.24	0.07	0.00	5.62E+03	1.93E+02	1.13E-12	6.02E-13
	A_2_7	0.29	0.10	0.00	2.55E+04	8.68E+02	7.12E-12	1.40E-12
	A_2_8	0.34	5.67	0.03	4.79E+04	6.60E+03	2.89E-11	4.62E-12
	A_2_9	0.38	11.21	0.05	1.73E+05	2.73E+04	1.60E-10	3.62E-11
	A_2_10	0.42	0.08	0.00	2.33E+04	2.12E+03	1.22E-12	9.62E-13
	A_2_11	0.46	0.07	0.00	2.32E+04	3.33E+03	3.92E-12	1.41E-13
	A_2_12	0.50	0.07	0.00	2.29E+04	3.37E+03	3.64E-12	1.02E-13
	C_2_6	0.24	0.39	0.00	6.19E+04	6.75E+03	3.79E-11	1.15E-12
	C_2_7	0.29	1.68	0.05	1.65E+05	5.50E+03	1.76E-10	1.41E-12
	C_2_8	0.34	2.81	1.96	1.32E+05	7.76E+03	1.02E-10	7.26E-12
	C_2_9	0.38	2.07	0.01	4.05E+04	4.05E+03	1.87E-11	3.87E-13
	C_2_10	0.42	0.77	0.00	3.94E+04	8.68E+02	2.26E-11	1.12E-12
	C_2_11	0.46	0.17	0.00	3.71E+04	4.34E+02	1.18E-11	1.12E-12
	C_2_12	0.50	0.10	0.00	4.36E+04	4.68E+03	4.76E-12	8.20E-13
	F_2_6	0.24	0.22	0.00	5.52E+04	2.42E+02	1.99E-11	1.72E-12
	F_2_7	0.29	2.84	0.01	8.28E+04	5.28E+03	1.95E-10	1.39E-11

sampling campaign	Sample name	z-coordinate [m]	toluene [mg/L]		bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD	mean	SD
	F_2_8	0.34	4.33	0.01	1.48E+05	5.71E+03	4.45E-10	5.27E-11
	F_2_9	0.38	5.56	0.01	2.07E+05	1.79E+03	3.03E-10	1.56E-11
	F_2_10	0.42	9.35	0.07	1.03E+05	1.84E+04	1.29E-10	9.75E-12
	F_2_11	0.46	3.45	0.02	8.80E+04	9.34E+03	4.27E-11	2.25E-12
	F_2_12	0.50	1.90	0.01	1.64E+05	9.64E+03	1.21E-10	1.21E-11
Day 16	A_2_1	0.03	NA	NA	8.29E+03	5.81E+02	6.83E-12	7.21E-13
	A_2_4	0.16	0.00	0.00	2.50E+04	7.75E+02	4.50E-11	9.48E-13
	A_2_5	0.20	0.00	0.00	9.31E+03	0.00E+00	1.73E-12	5.20E-13
	A_2_6	0.24	0.00	0.00	8.59E+03	1.02E+03	7.21E-12	1.12E-12
	A_2_7	0.29	0.00	0.00	1.18E+04	3.39E+02	7.37E-12	4.88E-13
	A_2_8	0.34	0.10	0.02	5.48E+04	8.72E+02	1.75E-11	2.65E-12
	A_2_9	0.38	1.85	0.06	5.34E+04	1.74E+03	3.44E-11	4.65E-13
	A_2_10	0.42	0.00	0.00	6.78E+03	9.68E+01	5.18E-12	5.56E-13
	A_2_11	0.46	0.00	0.00	6.54E+03	2.42E+02	2.00E-12	7.87E-13
	A_2_12	0.50	0.00	0.00	1.30E+04	7.26E+02	2.77E-12	4.67E-14
	A_2_13	0.55	NA	NA	6.81E+03	6.29E+02	2.14E-12	6.36E-13
	C_2_1	0.03	0.00	0.00	2.14E+04	2.03E+04	9.53E-13	7.00E-14
	C_2_4	0.16	0.00	0.00	1.86E+04	1.68E+04	6.33E-13	6.24E-13
	C_2_5	0.20	NA	NA	2.20E+04	2.00E+04	1.90E-12	8.95E-13
	C_2_6	0.24	0.00	0.00	1.88E+04	1.60E+04	6.54E-12	5.38E-12
	C_2_7	0.29	0.00	0.00	4.06E+04	4.40E+04	1.55E-11	2.74E-13
	C_2_8	0.34	0.00	0.00	1.22E+05	1.09E+05	3.77E-11	3.61E-13
	C_2_9	0.38	0.00	0.00	8.98E+04	8.74E+04	2.47E-11	3.03E-12
	C_2_10	0.42	0.00	0.00	3.73E+04	3.66E+04	8.08E-12	1.48E-13
	C_2_11	0.46	0.00	0.00	4.12E+04	4.26E+04	1.61E-11	3.92E-12
	C_2_12	0.50	NA	NA	1.68E+04	1.67E+04	5.82E-12	3.18E-12
	C_2_13	0.55	0.00	0.00	2.06E+04	1.95E+04	2.35E-12	8.36E-13
	F_2_1	0.03	0.00	0.00	2.45E+04	7.75E+02	1.29E-12	1.16E-12
	F_2_4	0.16	0.00	0.00	2.66E+04	2.32E+03	4.07E-12	2.55E-14
	F_2_5	0.20	0.00	0.00	2.15E+04	1.84E+03	6.21E-12	1.70E-13
	F_2_6	0.24	0.00	0.00	1.91E+04	4.84E+01	3.62E-12	2.27E-13
	F_2_7	0.29	0.02	0.00	1.99E+04	3.20E+03	8.23E-12	1.14E-12
	F_2_8	0.34	0.00	0.00	2.13E+04	3.39E+03	1.28E-11	1.68E-12
	F_2_9	0.38	0.01	0.00	3.01E+04	9.68E+01	2.85E-11	1.79E-12
	F_2_10	0.42	NA	NA	2.24E+04	3.68E+03	7.42E-12	3.30E-12
	F_2_11	0.46	0.00	0.00	1.65E+04	7.26E+02	1.23E-11	1.96E-12
	F_2_12	0.50	0.01	0.00	2.02E+04	2.42E+03	4.08E-12	5.85E-13
	F_2_13	0.55	0.00	0.00	2.19E+04	3.53E+03	2.96E-12	1.09E-12
Day 63	A_2_1	0.03	NA	NA	1.86E+04	3.63E+03	6.42E-12	8.08E-13
	A_2_4	0.16	0.00	0.00	5.99E+03	1.45E+02	4.42E-12	6.05E-14
	A_2_5	0.20	0.00	0.00	2.02E+04	1.11E+03	1.23E-11	3.17E-14
	A_2_6	0.24	0.00	0.00	1.25E+04	2.91E+02	4.90E-12	5.43E-14

sampling campaign	Sample name	z-coordinate [m]	toluene [mg/L]		bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD	mean	SD
	A_2_7	0.29	0.00	0.00	4.08E+04	1.26E+03	7.34E-12	2.03E-12
	A_2_8	0.34	21.24	2.14	9.76E+04	5.33E+02	1.51E-10	3.54E-11
	A_2_9	0.38	1.34	0.09	5.20E+04	2.91E+02	3.32E-11	6.89E-12
	A_2_10	0.42	0.00	0.00	9.11E+03	0.00E+00	6.52E-12	4.14E-14
	A_2_11	0.46	0.00	0.00	1.83E+04	4.84E+02	1.03E-11	1.00E-12
	A_2_12	0.50	0.00	0.00	1.24E+04	9.20E+02	4.02E-12	4.30E-13
	A_2_13	0.55	NA	NA	1.84E+04	1.84E+03	7.79E-12	1.87E-12
	C_2_1	0.03	0.00	0.00	1.43E+04	2.13E+03	6.49E-12	8.01E-13
	C_2_4	0.16	0.00	0.00	1.33E+04	4.36E+02	4.68E-12	7.94E-13
	C_2_5	0.20	NA	NA	3.02E+04	3.44E+03	8.74E-12	2.23E-12
	C_2_6	0.24	0.00	0.00	1.67E+04	4.84E+02	9.45E-12	4.91E-13
	C_2_7	0.29	0.01	0.02	1.63E+05	1.13E+04	5.86E-11	1.81E-11
	C_2_8	0.34	1.40	0.01	6.19E+05	2.18E+03	1.50E-10	8.04E-12
	C_2_9	0.38	0.56	0.15	2.02E+05	1.68E+04	9.40E-11	8.94E-12
	C_2_10	0.42	0.00	0.00	3.45E+04	3.63E+03	1.36E-11	5.63E-13
	C_2_11	0.46	0.00	0.00	4.34E+04	6.49E+03	4.90E-11	1.06E-12
	C_2_12	0.50	NA	NA	1.54E+04	4.84E+01	2.77E-12	4.72E-13
	C_2_13	0.55	0.00	0.00	1.61E+04	4.84E+01	3.16E-12	7.39E-13
	F_2_1	0.03	0.00	0.00	2.53E+04	2.86E+03	5.22E-12	7.36E-13
	F_2_4	0.16	0.00	0.00	1.75E+04	1.21E+03	5.82E-12	2.59E-13
	F_2_5	0.20	NA	NA	3.66E+04	6.78E+03	9.06E-12	3.78E-13
	F_2_6	0.24	0.00	0.00	3.62E+04	4.16E+03	8.61E-12	8.88E-13
	F_2_7	0.29	0.08	0.03	4.86E+04	1.60E+03	3.78E-13	3.24E-13
	F_2_8	0.34	0.04	0.01	5.13E+04	5.71E+03	6.58E-11	2.33E-12
	F_2_9	0.38	0.19	0.07	7.03E+04	5.57E+03	5.88E-11	2.49E-12
	F_2_10	0.42	0.07	0.00	4.46E+04	5.33E+02	9.54E-11	1.45E-12
	F_2_11	0.46	0.06	0.01	2.38E+04	4.94E+03	8.14E-12	2.25E-12
	F_2_12	0.50	0.09	0.02	3.54E+04	1.07E+03	2.60E-12	8.36E-13
	F_2_13	0.55	0.00	0.00	3.90E+04	4.84E+01	9.75E-12	3.12E-13
Day 137	A_2_7	0.29	0.12	0.02	1.05E+04	5.07E+02	1.66E-12	7.23E-14
	A_2_8	0.34	34.82	0.82	8.12E+04	8.10E+02	4.78E-11	1.46E-12
	A_2_9	0.38	92.26	1.11	3.67E+04	3.04E+02	2.08E-10	7.95E-12
	A_2_10	0.42	1.77	0.08	2.83E+04	6.08E+02	1.34E-11	5.11E-12
	A_2_11	0.46	0.04	0.00	9.10E+03	8.10E+02	2.41E-11	1.82E-12
	A_2_12	0.50	0.25	0.01	1.01E+04	4.05E+02	4.13E-11	1.06E-11
	A_2_13	0.55	0.93	0.03	3.14E+04	3.85E+03	7.28E-11	9.97E-12
	C_2_7	0.29	0.02	0.00	2.77E+04	1.22E+03	3.33E-10	3.25E-10
	C_2_8	0.34	4.99	0.24	1.04E+05	3.95E+03	1.01E-10	1.15E-11
	C_2_9	0.38	45.88	4.78	1.25E+05	2.03E+02	8.85E-11	3.42E-12
	C_2_10	0.42	39.26	3.44	3.46E+05	2.18E+04	1.63E-10	5.97E-12
	C_2_11	0.46	2.81	0.17	3.17E+05	4.32E+04	1.18E-10	2.00E-11
	C_2_12	0.50	0.14	0.00	4.77E+04	4.36E+03	4.32E-11	7.76E-13

sampling campaign	Sample name	z-coordinate [m]	toluene [mg/L]		bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD	mean	SD
	C_2_13	0.55	4.67	0.04	1.27E+05	5.98E+03	1.92E-10	1.11E-11
	F_2_7	0.29	0.01	0.00	1.04E+06	3.65E+03	3.01E-10	1.33E-11
	F_2_8	0.34	14.05	0.22	2.76E+05	2.33E+03	7.55E-11	1.77E-11
	F_2_9	0.38	18.54	1.14	2.12E+05	1.37E+04	2.21E-10	4.05E-11
	F_2_10	0.42	21.78	1.06	2.32E+05	3.75E+03	3.59E-10	1.24E-10
	F_2_11	0.46	15.51	0.44	4.94E+05	3.51E+04	8.86E-10	9.97E-11
	F_2_12	0.50	2.04	0.12	2.98E+06	1.08E+05	3.59E-09	3.02E-10
	F_2_13	0.55	3.21	0.38	2.10E+06	1.24E+05	4.90E-09	6.36E-10
Day 231	A_2_1	0.03	0.00	0.00	1.433E+04	1.72E+03	6.596E-12	3.79E-12
	A_2_2	0.08	0.00	0.00	NA	NA	NA	NA
	A_2_3	0.12	0.00	0.00	NA	NA	NA	NA
	A_2_4	0.16	0.00	0.00	9.742E+03	3.04E+02	2.097E-12	1.17E-12
	A_2_5	0.20	0.00	0.00	1.375E+04	1.32E+03	4.763E-12	4.95E-15
	A_2_6	0.24	0.00	0.00	1.855E+04	2.43E+03	4.726E-12	1.24E-12
	A_2_7	0.29	0.00	0.00	2.185E+04	8.10E+02	3.692E-12	1.58E-13
	A_2_8	0.34	0.00	0.00	3.839E+04	1.72E+03	2.815E-12	2.88E-12
	A_2_9	0.38	0.00	0.00	6.898E+04	8.10E+02	5.317E-11	6.62E-12
	A_2_10	0.42	0.00	0.00	1.404E+04	2.13E+03	1.248E-12	4.93E-13
	A_2_11	0.46	0.00	0.00	1.884E+04	6.08E+02	2.208E-12	3.19E-13
	A_2_12	0.50	0.00	0.00	1.067E+04	4.05E+02	3.904E-12	3.11E-13
	A_2_13	0.55	0.00	0.00	1.855E+04	3.04E+03	9.195E-13	1.32E-13
	C_2_1	0.03	0.00	0.00	1.562E+04	3.75E+03	2.182E-11	3.11E-12
	C_2_2	0.08	0.00	0.00	NA	NA	NA	NA
	C_2_3	0.12	0.00	0.00	NA	NA	NA	NA
	C_2_4	0.16	0.00	0.00	1.103E+04	1.01E+02	3.014E-12	1.73E-12
	C_2_5	0.20	0.00	0.00	1.454E+04	1.22E+03	1.660E-12	2.76E-13
	C_2_6	0.24	0.00	0.00	1.547E+04	1.01E+02	2.459E-12	4.64E-14
	C_2_7	0.29	0.00	0.00	4.541E+04	2.94E+03	1.357E-11	1.23E-13
	C_2_8	0.34	0.00	0.00	1.223E+05	2.03E+03	4.685E-11	2.17E-13
	C_2_9	0.38	0.00	0.00	1.266E+05	4.46E+03	5.331E-11	1.06E-12
	C_2_10	0.42	0.00	0.00	7.013E+04	3.24E+03	1.779E-11	3.10E-12
	C_2_11	0.46	0.00	0.00	3.761E+04	2.23E+03	1.098E-11	1.58E-13
	C_2_12	0.50	0.00	0.00	1.669E+04	6.08E+02	7.046E-12	9.29E-13
	C_2_13	0.55	0.00	0.00	1.841E+04	2.03E+03	2.214E-12	9.66E-14
	F_2_1	0.03	0.00	0.00	4.02E+04	1.70E+03	9.784E-12	5.72E-13
	F_2_2	0.08	0.00	0.00	NA	NA	NA	NA
	F_2_3	0.12	0.00	0.00	NA	NA	NA	NA
	F_2_4	0.16	0.00	0.00	3.89E+04	1.56E+03	4.050E-12	7.91E-13
	F_2_5	0.20	0.00	0.00	3.76E+04	1.56E+03	2.364E-12	2.54E-13
	F_2_6	0.24	0.00	0.00	3.56E+04	2.12E+03	2.055E-11	8.27E-13
	F_2_7	0.29	0.00	0.00	5.40E+04	3.68E+03	5.529E-12	2.51E-12
	F_2_8	0.34	0.00	0.00	5.66E+04	5.87E+03	1.544E-11	4.22E-13

sampling campaign	Sample name	z-coordinate [m]	toluene [mg/L]		bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD	mean	SD
	F_2_9	0.38	0.00	0.00	5.59E+04	3.68E+03	2.278E-11	1.64E-12
	F_2_10	0.42	0.00	0.00	7.79E+04	5.16E+03	2.056E-11	2.31E-12
	F_2_11	0.46	0.00	0.00	9.15E+04	6.01E+03	2.098E-11	4.98E-13
	F_2_12	0.50	0.00	0.00	4.12E+04	1.13E+03	1.456E-11	1.75E-12
	F_2_13	0.55	0.00	0.00	5.27E+04	1.26E+04	1.587E-11	1.12E-12

Tab. A2. Total bacterial cell numbers and ATP concentrations of attached cells of the pristine aquifer, during the injection of toluene (Day 16- Day 137) and the recovery phase (Day 231).

sampling campaign	Sample name	z-coordinate [m]	bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD
PRISTINE	C1520	0.20	1.28E+06	1.68E+05	4.93E-11	1.39E-11
	C3035	0.35	1.63E+06	3.27E+03	2.73E-11	5.66E-13
	C4550	0.60	1.55E+06	7.27E+05	1.21E-10	1.03E-10
	F1520	0.20	1.20E+06	3.60E+05	1.82E-10	5.94E-11
	F3035	0.35	1.88E+06	0.00E+00	8.74E-11	2.76E-11
	F4550	0.60	8.91E+05	7.35E+04	5.44E-11	7.07E-14
	E020	0.20	8.41E+05	7.07E+02	6.75E-11	1.63E-12
	E2035	0.35	1.26E+06	4.30E+05	1.71E-11	3.89E-12
	E3560	0.60	1.01E+06	1.48E+04	5.08E-11	1.06E-12
Day 16	A010	0.15	2.31E+06	3.06E+05	9.92E-11	1.55E-11
	A1020	0.20	1.10E+06	6.67E+04	3.73E-11	1.21E-11
	A2025	0.30	1.22E+06	1.02E+05	2.73E-10	3.17E-10
	A2530	0.35	1.46E+06	6.67E+04	2.44E-10	4.24E-12
	A3035	0.40	1.97E+06	5.27E+04	5.78E-10	7.07E-13
	A3545	0.45	1.57E+06	8.43E+04	3.34E-11	2.04E-11
	A4555	0.50	1.67E+06	2.92E+05	3.44E-11	9.19E-13
	A5565	0.60	1.79E+06	5.62E+04	3.56E-11	2.75E-11
	C010	0.10	1.36E+06	1.41E+05	1.98E-11	8.77E-12
	C1015	0.15	1.18E+06	4.92E+04	1.45E-11	1.29E-11
	C1520	0.20	1.84E+06	1.44E+05	3.40E-11	9.62E-12
	C2025	0.25	1.63E+06	3.51E+03	5.47E-11	1.43E-11
	C2530	0.30	3.03E+06	4.50E+05	5.05E-11	1.48E-11
	C3035	0.35	2.92E+06	4.92E+04	4.62E-11	9.19E-12
	C3540	0.38	1.91E+06	1.26E+05	7.39E-11	7.85E-12
	C4045	0.40	2.34E+06	6.32E+04	8.21E-11	1.48E-11
	C4550	0.45	3.53E+06	4.60E+05	7.22E-11	2.46E-11
	C5055	0.50	2.52E+06	3.83E+05	2.95E-11	6.36E-12
	C5560	0.55	1.89E+06	2.00E+05	4.85E-11	1.56E-11
	C6070	0.60	1.63E+06	0.00E+00	6.38E-11	1.19E-11
	F010	0.15	1.93E+06	1.69E+05	2.94E-11	1.46E-11

sampling campaign	Sample name	z-coordinate [m]	bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD
	F1015	0.20	2.17E+06	1.93E+05	3.65E-11	3.10E-11
	F1520	0.25	1.39E+06	9.13E+04	2.26E-11	2.34E-11
	F2025	0.30	2.09E+06	1.12E+05	4.98E-11	5.66E-13
	F2530	0.33	1.69E+06	2.67E+05	2.23E-11	1.41E-13
	F3035	0.35	2.93E+06	7.03E+04	1.87E-11	2.97E-12
	F3540	0.38	3.57E+06	1.97E+05	1.39E-11	5.52E-12
	F4045	0.40	1.75E+06	1.76E+05	2.29E-11	7.64E-12
	F4550	0.45	1.35E+06	3.44E+05	3.40E-11	2.58E-11
	F5055	0.50	1.81E+06	5.27E+04	2.69E-11	1.98E-12
	F5560	0.55	2.13E+06	1.79E+05	4.55E-11	1.03E-11
	F6070	0.60	4.33E+06	5.06E+05	5.91E-11	1.54E-11
Day 63	A010	0.15	NA	NA	NA	NA
	A1020	0.25	8.17E+05	1.76E+04	7.58E-11	6.29E-12
	A2030	0.35	1.15E+07	5.27E+05	9.10E-10	1.41E-11
	A3035	0.40	3.33E+06	3.16E+05	3.06E-10	6.51E-11
	A3540	0.45	7.68E+05	5.97E+04	2.41E-10	1.27E-11
	A4050	0.55	1.17E+06	1.16E+05	2.29E-10	6.65E-11
	A5060	0.65	2.46E+06	4.92E+04	1.24E-10	6.36E-12
	A6070	0.70	NA	NA	NA	NA
	C010	0.10	NA	NA	NA	NA
	C1015	0.15	1.79E+06	2.42E+05	9.01E-11	7.07E-14
	C1520	0.20	1.54E+06	2.11E+04	1.07E-10	1.61E-11
	C2025	0.25	2.61E+06	NA	NA	NA
	C2530	0.30	1.19E+07	3.02E+05	1.30E-10	0.00E+00
	C3035	0.35	4.47E+06	2.88E+05	5.55E-10	2.19E-11
	C3540	0.38	1.44E+06	1.93E+05	7.04E-10	1.70E-11
	C4045	0.40	1.21E+06	6.32E+04	2.37E-10	1.41E-11
	C4550	0.45	2.80E+06	1.19E+05	2.15E-10	7.78E-12
	C5055	0.50	1.28E+06	1.51E+05	1.06E-10	2.83E-12
	C5560	0.55	3.05E+06	1.41E+04	8.04E-11	1.34E-11
	C6070	0.60	NA	NA	NA	NA
	F010	0.10	1.87E+06	3.22E+05	4.07E-11	3.54E-13
	F1015	0.15	2.79E+06	3.15E+05	1.68E-11	1.41E-12
	F1520	0.20	1.91E+06	3.66E+03	2.54E-11	5.66E-13
	F2025	0.25	1.53E+06	9.16E+04	2.70E-11	3.96E-12
	F2530	0.35	2.95E+06	1.87E+05	4.45E-11	2.26E-12
	F3035	0.38	2.41E+06	2.09E+05	6.59E-11	1.41E-13
	F3540	0.42	2.24E+06	7.32E+03	1.14E-10	0.00E+00
	F4045	0.45	1.80E+06	2.20E+04	1.03E-10	7.07E-13
	F4550	0.50	1.86E+06	1.25E+05	4.75E-11	7.07E-14
	F5055	0.55	2.16E+06	4.03E+04	6.99E-11	2.12E-12
	F5560	0.60	NA	NA	8.33E-11	8.49E-13

sampling campaign	Sample name	z-coordinate [m]	bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD
	F6070	0.65	NA	NA	NA	NA
Day 137	A010	0.15	1.46E+06	2.64E+05	1.20E-10	1.70E-11
	A1015	0.20	1.43E+06	0.00E+00	9.86E-11	2.59E-11
	A1520	0.25	1.26E+06	2.97E+04	9.03E-11	9.48E-12
	A2025	0.30	7.46E+05	4.46E+04	1.31E-10	1.06E-11
	A3035	0.40	6.39E+06	4.09E+05	8.25E-10	1.48E-10
	A3540	0.45	1.59E+06	2.56E+05	3.42E-10	4.24E-12
	A4045	0.50	1.42E+06	1.49E+04	3.15E-10	2.55E-11
	A4550	0.55	1.18E+06	1.26E+05	1.34E-10	4.38E-11
	A5055	0.60	1.14E+06	4.83E+04	1.16E-10	3.63E-11
	A5560	0.65	1.34E+06	1.97E+05	1.83E-10	1.77E-11
	C010	0.15	9.56E+05	2.23E+05	7.59E-11	9.90E-13
	C1015	0.20	1.48E+06	5.57E+04	2.23E-11	2.83E-13
	C1520	0.25	2.08E+06	2.56E+05	6.64E-11	2.69E-12
	C2025	0.30	5.43E+06	1.67E+05	3.44E-10	2.76E-11
	C2530	0.35	5.72E+06	6.13E+05	9.08E-10	1.13E-11
	C3035	0.40	7.18E+06	6.24E+05	1.09E-09	2.83E-11
	C3540	0.45	9.45E+06	3.34E+05	7.58E-10	2.11E-10
	C4045	0.50	5.74E+06	1.67E+05	2.97E-10	4.17E-11
	C4550	0.55	4.01E+06	1.00E+05	3.23E-10	2.26E-11
	C5055	0.60	2.99E+06	6.32E+04	NA	NA
	F010	0.15	1.54E+06	2.20E+04	1.21E-10	3.56E-11
	F1020	0.20	1.32E+06	7.32E+04	1.02E-10	7.02E-11
	F2030	0.25	2.14E+06	2.82E+05	7.84E-11	1.58E-11
	F3035	0.30	2.28E+06	1.39E+05	3.69E-10	9.90E-12
	F3540	0.35	3.04E+06	5.13E+05	1.85E-10	6.51E-11
	F4045	0.40	3.06E+06	2.82E+05	2.84E-10	4.24E-11
	F4550	0.45	6.17E+06	6.63E+05	7.78E-10	4.38E-11
	F5055	0.55	8.02E+06	3.08E+05	3.61E-10	1.75E-10
	F5560	0.60	6.24E+06	4.17E+05	5.96E-10	4.24E-12
	F6065	0.65	5.09E+06	5.13E+04	1.65E-10	2.12E-12
Day 231	A010	0.10	1.74E+06	1.76E+05	7.14E-11	5.09E-12
	A1015	0.15	1.43E+06	7.35E+04	7.10E-11	2.05E-12
	A1520	0.20	1.45E+06	1.36E+05	6.38E-11	4.67E-12
	A2025	0.25	1.88E+06	1.47E+05	8.03E-11	1.34E-12
	A2530	0.30	1.63E+06	5.88E+04	4.28E-11	1.41E-13
	A3035	0.35	2.40E+06	4.45E+05	8.58E-11	1.47E-11
	A3540	0.40	2.07E+07	8.20E+05	8.05E-10	2.26E-11
	A4045	0.45	5.56E+06	7.39E+05	1.35E-09	3.54E-11
	A4550	0.50	2.36E+06	7.72E+04	1.90E-10	7.07E-12
	A5055	0.55	1.90E+06	1.84E+05	9.88E-11	1.32E-11
	A5560	0.60	2.54E+06	9.92E+04	6.89E-11	1.98E-12

sampling campaign	Sample name	z-coordinate [m]	bacterial abundance [cell/mL]		ATP [M]	
			mean	SD	mean	SD
	A6065	0.65	4.26E+06	7.72E+04	8.72E-11	8.34E-12
	C010	0.10	2.34E+06	9.56E+04	9.39E-11	7.00E-12
	C1015	0.15	2.43E+06	8.45E+04	8.85E-11	3.54E-12
	C1520	0.20	1.66E+06	5.15E+04	8.51E-11	3.32E-12
	C2025	0.25	5.58E+06	2.57E+04	1.86E-10	1.41E-12
	C2530	0.30	2.28E+06	1.51E+05	5.05E-10	7.07E-13
	C3035	0.35	2.92E+07	1.54E+06	1.08E-09	9.90E-11
	C3540	0.40	1.84E+07	1.43E+05	8.71E-10	6.58E-11
	C4045	0.45	7.20E+06	2.87E+05	4.86E-10	6.15E-11
	C4550	0.50	3.77E+06	9.92E+04	4.48E-10	4.31E-11
	C5055	0.55	4.77E+06	4.04E+04	4.05E-10	9.19E-12
	C5560	0.60	9.32E+06	3.31E+04	1.12E-09	1.06E-10
	F010	0.15	5.59E+06	7.35E+04	1.89E-10	7.78E-12
	F1015	0.20	2.85E+06	3.79E+05	1.27E-10	8.49E-12
	F1520	0.25	3.72E+06	3.01E+05	2.15E-10	1.34E-11
	F2025	0.30	5.11E+06	1.21E+05	3.71E-10	3.32E-11
	F2530	0.35	6.97E+06	6.14E+05	3.52E-10	5.87E-11
	F3035	0.40	4.82E+06	1.69E+05	3.95E-10	2.26E-11
	F3540	0.45	8.27E+06	3.38E+05	5.84E-10	9.83E-11
	F4045	0.50	7.73E+06	6.43E+05	6.55E-10	1.98E-11
	F4550	0.55	4.38E+06	2.21E+04	4.63E-10	4.95E-11
	F5055	0.60	3.53E+06	5.15E+04	4.69E-10	3.57E-11
	F5560	0.65	3.60E+06	2.94E+04	2.56E-10	1.03E-11

Tab. A3. Mean abundance of virus-like particles and protozoa in pore water in the pristine aquifer, during toluene injection (Day 63 & 137) and the recovery phase (Day 231).

sampling campaign	Sample name	z-coordinate [m]	VLPs/mL	protozoa/mL
PRISTINE	A_2_1	0.03	8.11E+05	NA
	A_2_4	0.16	9.52E+05	1.59E+02
	A_2_6	0.24	8.75E+05	NA
	A_2_7	0.29	1.00E+06	1.97E+02
	A_2_10	0.42	8.69E+05	3.63E+02
	A_2_13	0.55	8.31E+05	NA
	C_2_1	0.03	1.28E+06	1.79E+02
	C_2_4	0.16	8.15E+05	NA
	C_2_6	0.24	1.15E+06	NA
	C_2_7	0.29	1.36E+06	1.55E+03
	C_2_10	0.42	1.16E+06	1.51E+03
	C_2_13	0.55	1.26E+06	NA

sampling campaign	Sample name	z-coordinate [m]	VLPs/mL	protozoa/mL
	F_2_1	0.03	1.34E+06	4.97E+02
	F_2_4	0.16	1.50E+06	NA
	F_2_6	0.24	1.72E+06	2.82E+02
	F_2_7	0.29	1.15E+06	NA
	F_2_10	0.42	1.84E+06	1.64E+03
	F_2_13	0.55	2.00E+06	3.63E+02
Day 63	A_2_1	0.03	1.19E+06	NA
	A_2_4	0.16	1.64E+06	NA
	A_2_5	0.20	1.38E+06	2.06E+02
	A_2_6	0.24	1.07E+06	NA
	A_2_7	0.29	1.50E+06	NA
	A_2_8	0.34	5.14E+06	6.06E+03
	A_2_9	0.38	2.01E+06	2.66E+03
	A_2_10	0.42	4.06E+06	NA
	A_2_11	0.46	4.56E+06	NA
	A_2_12	0.50	9.12E+05	NA
	A_2_13	0.55	9.27E+05	2.67E+02
	C_2_1	0.03	8.80E+05	7.23E+02
	C_2_4	0.16	1.35E+06	NA
	C_2_5	0.20	7.37E+05	5.64E+02
	C_2_6	0.24	6.33E+05	NA
	C_2_7	0.29	5.34E+06	NA
	C_2_8	0.34	6.83E+06	NA
	C_2_9	0.38	5.79E+06	5.69E+03
	C_2_10	0.42	1.16E+06	NA
	C_2_11	0.46	8.38E+05	NA
	C_2_12	0.50	1.27E+06	NA
	C_2_13	0.55	1.04E+06	1.72E+02
	F_2_1	0.03	1.38E+06	NA
	F_2_4	0.16	1.19E+06	NA
	F_2_5	0.20	1.05E+06	2.49E+02
	F_2_6	0.24	1.38E+06	NA
	F_2_7	0.29	1.50E+06	NA
	F_2_8	0.34	6.89E+06	4.72E+03
	F_2_9	0.38	6.43E+06	NA
	F_2_10	0.42	4.98E+06	NA
	F_2_11	0.46	3.99E+06	NA
	F_2_12	0.50	3.75E+06	NA
	F_2_13	0.55	1.10E+06	9.67E+02
Day 137	A_2_7	0.29	7.01E+05	1.07E+03
	A_2_8	0.34	7.58E+05	4.49E+03
	A_2_9	0.38	3.09E+06	2.41E+03
	A_2_10	0.42	5.82E+05	NA

sampling campaign	Sample name	z-coordinate [m]	VLPs/mL	protozoa/mL
	A_2_11	0.46	6.12E+05	NA
	A_2_12	0.50	6.56E+05	1.16E+03
	A_2_13	0.55	9.21E+05	NA
	C_2_7	0.29	6.46E+05	1.80E+03
	C_2_8	0.34	4.60E+06	6.07E+03
	C_2_9	0.38	4.61E+06	7.69E+03
	C_2_10	0.42	4.36E+06	5.55E+03
	C_2_11	0.46	7.48E+05	NA
	C_2_12	0.50	6.26E+05	1.80E+03
	C_2_13	0.55	7.15E+05	2.11E+03
	F_2_7	0.29	1.48E+07	1.06E+03
	F_2_8	0.34	1.27E+06	4.88E+03
	F_2_9	0.38	1.81E+06	NA
	F_2_10	0.42	1.38E+06	2.10E+03
	F_2_11	0.46	6.15E+06	NA
	F_2_12	0.50	7.35E+06	NA
	F_2_13	0.55	9.59E+06	1.52E+04
Day 231	A_2_1	0.03	6.87E+05	2.21E+02
	A_2_4	0.16	7.73E+05	NA
	A_2_5	0.20	9.56E+05	NA
	A_2_6	0.24	9.46E+05	NA
	A_2_7	0.29	9.53E+05	NA
	A_2_8	0.34	1.24E+06	1.26E+03
	A_2_9	0.38	1.40E+06	NA
	A_2_10	0.42	7.03E+05	NA
	A_2_11	0.46	7.23E+05	NA
	A_2_12	0.50	5.53E+05	NA
	A_2_13	0.55	6.56E+05	1.15E+03
	C_2_1	0.03	7.37E+05	1.83E+02
	C_2_4	0.16	7.20E+05	NA
	C_2_5	0.20	7.04E+05	2.10E+02
	C_2_6	0.24	5.58E+05	3.11E+02
	C_2_7	0.29	9.25E+05	NA
	C_2_8	0.34	9.65E+05	4.20E+03
	C_2_9	0.38	1.76E+06	NA
	C_2_10	0.42	1.54E+06	2.37E+03
	C_2_11	0.46	9.31E+05	NA
	C_2_12	0.50	8.27E+05	2.74E+03
	C_2_13	0.55	7.26E+05	8.28E+02
	F_2_1	0.03	9.18E+05	3.15E+02
	F_2_4	0.16	9.30E+05	NA
	F_2_5	0.20	7.68E+05	3.72E+02
	F_2_6	0.24	1.05E+06	NA

sampling campaign	Sample name	z-coordinate [m]	VLPs/mL	protozoa/mL
	F_2_7	0.29	1.89E+06	NA
	F_2_8	0.34	2.20E+06	1.71E+03
	F_2_9	0.38	1.19E+06	NA
	F_2_10	0.42	1.62E+06	NA
	F_2_11	0.46	4.87E+06	NA
	F_2_12	0.50	3.92E+06	NA
	F_2_13	0.55	8.20E+05	2.57E+03

Tab. 4A. Mean bacterial Shannon diversity, richness and evenness in the sediment in the pristine aquifer, during toluene injection (day 16-137) and the recovery phase (day 231)

Sampling campaign	Sample name	Richness		Shannon diversity		Evenness	
		mean	SD	mean	SD	mean	SD
PRISTINE	C020	39.00	16.97	3.24	0.34	0.69	0.07
	C2035	49.00	6.00	3.19	0.06	0.50	0.03
	C3560	25.00	4.24	2.23	0.16	0.38	0.00
	F020	46.00	4.24	2.94	0.35	0.43	0.18
	F2035	36.50	10.61	3.11	0.32	0.63	0.02
	F3560	43.00	45.25	2.82	0.85	0.62	0.30
Day 16	A010	59.00	8.19	3.31	0.10	0.47	0.02
	A1020	51.00	3.61	3.19	0.03	0.48	0.05
	A2025	43.00	12.12	3.09	0.22	0.53	0.04
	A2530	22.33	2.31	1.94	0.11	0.31	0.02
	A3035	37.33	14.19	2.74	0.13	0.45	0.15
	A3545	53.33	15.70	3.21	0.15	0.48	0.08
	A4555	70.00	24.04	3.31	0.27	0.40	0.03
	A5565	39.67	18.56	2.68	0.14	0.42	0.17
	C010	57.00	0.00	3.53	0.00	0.60	0.00
	C1015	71.50	19.09	3.63	0.18	0.54	0.05
	C1520	86.00	0.00	3.70	0.00	0.47	0.00
	C2025	72.50	10.61	3.53	0.16	0.47	0.01
	C2530	54.67	10.21	3.14	0.09	0.44	0.13
	C3035	51.00	1.41	2.91	0.10	0.36	0.04
	C3540	23.50	16.26	2.35	0.53	0.52	0.11
	C4045	40.33	12.01	2.75	0.31	0.40	0.00
	C4550	22.50	10.61	2.27	0.46	0.46	0.01
	C5055	35.50	2.12	2.68	0.21	0.41	0.06
	C5560	36.50	10.61	2.79	0.38	0.45	0.04
	C6070	50.33	12.10	3.21	0.11	0.51	0.08
	F010	40.00	24.04	2.97	0.87	0.55	0.12

Sampling campaign	Sample name	Richness		Shannon diversity		Evenness	
		mean	SD	mean	SD	mean	SD
	F1520	46.50	28.99	3.08	0.64	0.52	0.02
	F2025	54.50	14.85	3.40	0.23	0.56	0.03
	F2530	51.67	2.52	2.65	0.02	0.27	0.01
	F3035	45.33	1.53	2.46	0.13	0.26	0.03
	F3540	82.00	13.08	3.51	0.18	0.41	0.03
	F4045	84.00	8.49	3.56	0.04	0.42	0.03
	F4550	90.33	14.05	3.48	0.06	0.36	0.04
	F5055	81.00	24.04	3.41	0.12	0.39	0.07
	F5560	75.00	10.00	3.38	0.05	0.40	0.03
	F6070	58.00	2.83	3.45	0.03	0.54	0.01
Day 63	A1020	23.00	12.53	2.55	0.47	0.64	0.11
	A2030	35.00	3.46	2.43	0.07	0.33	0.02
	A3035	42.33	12.02	2.87	0.20	0.46	0.02
	A3540	34.00	11.31	2.70	0.14	0.48	0.22
	A4050	24.00	8.49	2.74	0.15	0.68	0.14
	A5060	24.00	1.41	2.51	0.05	0.52	0.06
	C1015	54.00	33.94	3.27	0.36	0.56	0.17
	C1520	67.33	11.59	3.50	0.10	0.50	0.04
	C2025	64.33	8.39	3.64	0.08	0.60	0.03
	C2530	70.33	9.07	3.49	0.07	0.47	0.03
	C3035	76.50	2.12	3.78	0.04	0.57	0.01
	C3540	76.67	5.51	3.76	0.06	0.56	0.03
	C4045	72.67	19.55	3.52	0.06	0.49	0.11
	C4550	52.33	19.76	3.49	0.33	0.66	0.06
	C5055	45.33	9.29	3.36	0.11	0.64	0.07
	C5560	41.33	7.77	3.26	0.17	0.64	0.03
	F010	79.00	13.00	3.74	0.08	0.54	0.05
	F1015	83.67	3.21	3.58	0.01	0.43	0.02
	F1520	70.00	5.00	3.41	0.03	0.44	0.04
	F2025	75.50	3.54	3.60	0.04	0.48	0.00
	F2530	74.67	5.51	3.32	0.11	0.37	0.02
	F3035	65.33	9.07	3.38	0.09	0.46	0.06
	F3540	81.33	10.07	3.77	0.06	0.53	0.03
	F4045	70.33	13.43	3.18	0.44	0.35	0.08
	F4550	78.00	11.36	3.37	0.11	0.38	0.01
	F5055	72.67	9.61	3.30	0.12	0.38	0.02
Day 137	A010	70.00	4.24	3.46	0.09	0.46	0.01
	A1015	56.33	10.69	3.43	0.12	0.56	0.05
	A1520	72.50	6.36	3.32	0.13	0.38	0.02
	A2025	66.00	11.31	3.54	0.02	0.53	0.08
	A3035	41.33	6.03	3.05	0.05	0.51	0.05
	A3540	65.00	7.55	3.44	0.05	0.48	0.03

Sampling campaign	Sample name	Richness		Shannon diversity		Evenness	
		mean	SD	mean	SD	mean	SD
	A4045	72.33	22.68	3.43	0.22	0.44	0.03
	A4550	77.67	6.66	3.48	0.07	0.42	0.04
	A5055	75.67	10.79	3.29	0.14	0.36	0.01
	A5560	50.67	23.46	2.85	0.23	0.37	0.08
	C010	83.00	12.29	3.80	0.13	0.54	0.02
	C1015	72.00	2.00	3.35	0.03	0.39	0.02
	C1520	62.00	4.24	2.88	0.09	0.29	0.01
	C2025	44.67	7.23	2.47	0.16	0.27	0.02
	C2530	45.00	9.00	2.52	0.06	0.28	0.04
	C3035	52.33	19.50	2.88	0.15	0.37	0.09
	C3540	40.33	17.56	2.61	0.24	0.37	0.10
	C4045	43.00	12.17	2.96	0.15	0.47	0.08
	C4550	50.00	2.83	3.15	0.14	0.47	0.04
	F010	17.50	0.71	2.71	0.03	0.86	0.01
	F1020	73.50	16.26	3.61	0.12	0.51	0.05
	F2030	50.50	14.85	2.95	0.70	0.40	0.16
	F3035	70.00	7.07	3.28	0.09	0.38	0.00
	F3540	48.00	0.00	2.64	0.00	0.29	0.00
	F4045	49.00	4.24	2.57	0.09	0.27	0.00
	F4550	33.00	0.00	1.86	0.00	0.19	0.00
	F5055	34.00	0.00	1.88	0.00	0.19	0.00
	F6065	39.50	9.19	2.19	0.28	0.23	0.01
	F6570	62.50	9.19	3.22	0.06	0.41	0.08
Day 231	A010	54.50	14.85	3.44	0.12	0.59	0.09
	A1015	44.00	1.41	3.31	0.02	0.62	0.01
	A1520	57.50	10.61	3.27	0.04	0.46	0.06
	A2025	69.00	7.07	3.31	0.03	0.40	0.03
	A2530	58.50	6.36	3.27	0.06	0.45	0.02
	A3035	56.00	7.07	3.41	0.03	0.54	0.05
	A3540	65.50	3.54	3.50	0.02	0.51	0.02
	A4045	69.00	1.41	3.17	0.05	0.34	0.01
	A4550	64.00	8.49	3.38	0.03	0.46	0.05
	A5055	61.00	0.00	3.54	0.00	0.56	0.00
	A5560	63.00	0.00	3.20	0.00	0.39	1.00
	A6065	77.00	2.83	3.29	0.09	0.35	0.02
	C010	62.00	0.00	3.32	0.00	0.45	0.00
	C1015	53.50	17.68	3.20	0.77	0.50	0.21
	C1520	64.00	24.04	3.54	0.50	0.56	0.06
	C2025	89.50	6.36	3.76	0.03	0.48	0.05
	C2530	77.50	12.02	3.72	0.07	0.54	0.05
	C3035	75.50	2.12	3.45	0.03	0.42	0.00
	C3540	70.50	2.12	3.17	0.04	0.34	0.00

Sampling campaign	Sample name	Richness		Shannon diversity		Evenness	
		mean	SD	mean	SD	mean	SD
	C4045	88.00	5.66	3.63	0.05	0.43	0.01
	C4550	74.00	24.04	3.48	0.23	0.45	0.05
	C5055	71.00	19.80	3.44	0.33	0.45	0.02
	C5560	61.50	12.02	3.20	0.36	0.41	0.07
	F010	68.00	0.00	3.64	0.00	0.56	0.00
	F1015	60.00	19.80	3.29	0.16	0.46	0.08
	F1520	55.00	1.41	3.00	0.01	0.36	0.01
	F2025	78.00	0.00	3.75	0.00	0.55	0.00
	F3035	74.50	0.71	3.59	0.06	0.49	0.03
	F3540	65.00	0.00	3.40	0.00	0.46	0.00
	F4045	63.50	10.61	3.44	0.15	0.49	0.01
	F5055	67.50	9.19	3.58	0.07	0.53	0.03
	F5560	72.00	1.41	3.50	0.03	0.46	0.02

Authorship clarifications

Intrinsic potential for instantaneous biodegradation of toluene in a pristine, energy-limited aquifer

The idea and concept for the experimental design were carried out by Shiran Qiu, Dr. Martin Elsner and Dr. Christian Griebler. Shiran Qiu collected the samples and did the analysis with the support of the technical staff from Dr. Martin Elsner lab. Prof. Dr. Piotr Maloszewski performed the modeling and evaluated the results. PhD candidate, Dr. Christian Griebler, Shiran Qiu, Dr. Martin Elsner and Prof. Dr. Piotr Maloszewski interpreted and discusses the results together. The ecologically relevant aspect of this study was independently investigated and discussed by PhD candidate and Dr. Christian Griebler. The draft of the manuscript was written by the PhD candidate, Dr. Christian Griebler, Shiran Qiu and Prof. Dr. Piotr Maloszewski. The edition and improvement of the manuscript was done by PhD candidate whereas the correction and optimalization was done by Dr. Christian Griebler. The manuscript is prepared for the submission in the *Water Research* Journal.

Response and recovery of a pristine groundwater ecosystem impacted by toluene contamination – a meso scale indoor aquifer experiment

The concept for the experiment was developed by PhD candidate, Michael Larentis, Shiran Qiu, Dr. Christian Griebler, Dr. Tillmann Lüders, Dr. Martin Elsner and Prof. Dr. Rainer U. Meckenstock. PhD candidate, Shiran Qiu and Michael Larentis with the support of the technical staff organized sampling campaigns and collected the samples. All analyses were done by PhD candidate, except for the toluene (done by Shiran Qiu) and oxygen measurements (done by Sigrid Kaschuba) and measurements of physical-chemical parameters (co-share between the PhD candidate, Michael Larentis, Peter Knappet and technical staff). The evaluation and interpretation of results was done by PhD candidate and Dr. Christian Griebler. PhD candidate performed the statistical analyses and a graphical illustration of the data. The entire manuscript was written by PhD candidate, further on improved and corrected by Dr. Christian Griebler. The manuscript is prepared to be submitted in *Journal of Contaminant Hydrology.*

Microbial indicators of groundwater ecosystem response to organic contamination – resistance, resilience and evaluation

The idea of the experiment and the scientific design was developed by PhD candidate and Dr. Christian Griebler. Samples´ collection was managed by PhD candidate, Shiran Qiu and Michael Larentis. The assessment of the virus-like particles was done by Yuxiang Zhou and toluene analyses by Shiran Qiu. PhD candidate performed FISH, DNA extraction and T-RFLP analyses, measurements of bacterial abundance as well as data interpretation, statistics and their graphical illustrations. Together with Dr. Christian Griebler results were discussed and evaluated. PhD candidate wrote the entire manuscript, which was improved and corrected by Dr. Christian Griebler. The manuscript will be submitted to *Ecological Indicators* Journal.

Publications

A. Herzyk, P. Maloszewski, S. Qiu, M. Elsner, C. Griebler **Intrinsic potential for spontaneous biodegradation of toluene in a pristine, energy-limited aquifer.** *Water Research* (in preparation)

A. Herzyk, S. Qiu, P. Maloszewski, M. Hünniger, S. Kaschuba, M. Elsner, M. Larentis, T. Lüders, Rainer U. Meckenstock, Susanne I. Schmidt, C. Griebler **Response and recovery of a pristine groundwater ecosystem impacted by toluene contamination – a meso scale indoor aquifer experiment.** *Journal of Contaminant Hydrology* (in preparation)

A. Herzyk, S. Qiu, Y. Zhou, M. Elsner, C. Griebler **Microbial indicators of groundwater ecosystem response to organic contamination – resistance, resilience and evaluation.** *Ecological Indicators* (in preparation)

M. Larentis, **A. Herzyk**, M.S. Granitsiotis, S. Qiu, C. Griebler, M. Elsner, R.U. Meckenstock, T. Lüders. **Fast microbial community response and development of aerobic and anaerobic degrader populations upon toluene contamination of a pristine indoor model aquifer – distinct capacity of groundwater and sediment.** *Environmental Microbiology* (in preparation)

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Lebenslauf

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