Assessment of soil microbial activity by non- or low invasive methods

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Contents

AC	activated carbon
AFM-nTA	atomic force microscopy - nano thermal analysis
BTEX	benzene, toluene, ethyl-benzene, xylene
CARD-FISH	catalaysed reporter deposition - fluorescence in situ hybridisation
CSIA	compound specific stable isotope fractionation analysis
DAPI	4',6-diamidino-2-phenylindole
DNA	desoxyribonucleic acid
DNAPL	dense non aquous phase liquids
DOC	dissolved organic carbon
DOM	dissolved organic matter
DP	Direct-Push
EDX	energy dispersive x-ray analysis
ENA	enhanced natural attenuation
ESEM	environmental scanning electron microscopy
FA	fatty acids
FAME	fatty acid methyl ester
FE-SEM	field emission - scanning electron microscopy
FISH	fluorescence in situ hybridisation
GC-C-IRMS & GC-IRMS	gas chromatography - combustion - isotope ratio mass spectrometry
GC-MS	gas chromatography - mass spectrometry
HISH-SIMS	halogen in situ hybridisation - secondary ion mass spectrometry
LIF	laser induced fluorescence

LNAPL	light non aquous phase liquids
MCB	mono-chloro-benzene
MIP	membrane interface probe
MLPS	multi-level packer systems
MNA	monitored natural attenuation
MTBE	methyl-*tert*-butyl-ether
NA	natural attenuation
NanoSIMS	nano-scale secondary ion mass spectrometry
NER	non extractable residues
PAH	polycyclic aromatic hydrocarbons
PDB	Vienna Pee-Dee Belemnite
PLFA	phospho-lipid fatty acid
PTFE	poly-tetra-fluoro-ethylene (Teflon®)
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscopy
SMOW	standard mean ocean water
SOM	soil organic matter
TEA	terminal electron acceptor
tFA	total lipid fatty acid
TMCS	tri-methyl-chloro-silane
TOC	total organic carbon
WHC	water holding capacity

1 Introduction

In spite of their relatively small thickness compared to the earth crust or the atmosphere (Blume et al., 2010), soils and sediments are an essential compartment of the global carbon, nitrogen and phosphorous cycles (Seinfeld and Pandis, 2006). On the one hand, they directly affect the climate on the global scale and ecosystem properties such as the plant cover on the local scale. On the other hand, soil formation depends on the climate, the litter input by the plant cover, the type of parent rock material and the landscape configuration (Blume et al., 2010). Resulting from these partly interrelated, partly independent features, soil ecosystems exhibit a high degree of structural heterogeneity and complexity on several scales (Odum, 1969). This non-homogenous distribution of features has direct impact on the functionality of soils, which is, moreover, directly related to the patchy distribution of living microorganisms (Franklin and Mills, 2003; Becker et al., 2006; Lehmann et al., 2008). This heterogeneous distribution not only allows the coexistence of microorganisms with varying growth rates (Dechesne et al., 2007), but also leads to a non-homogeneous distribution of non-living soil organic matter (SOM) (Kögel-Knabner, 2002).
As soils are, apart from rock surfaces, forming the interface between the earth crust and the atmosphere, and, thereby, are the human habitat and the basis for human nutrition, they are prone to anthropogenic alteration by land management (Bellamy et al., 2005; Schulze and Freibauer, 2005). This in the past usually resulted in ecosystem degradation by soil threats according to the EU soil thematic strategy (Commission of the European Communities, 2006). Soil threats comprise physical processes like erosion, which is relocating nutrients and organic matter, and chemical processes like mineralisation of soil organic matter (Bellamy et al., 2005; Schulze and Freibauer, 2005) or soil pollution with hazardous organic substances due to industrial processes. These processes are threatening the soil functions, such as being drinking water ressources, cultivable land, carbon storages and living environments for microorganisms and plants (Blume et al., 2010). In order to maintain the ecosystem services provided by soils, their functions should be protected in intact soil systems and improved in already degraded systems by means of sustainable land management practices (Blume et al., 2010).
As Bellamy et al. (2005) and Schulze and Freibauer (2005) point out, soil microorganisms are, under sufficient nutrient supply, capable of degrading almost any organic structure (Gleixner et al., 2001; Schulze and Freibauer, 2005), even SOM, which was previously assumed to be realcitrant. This exemplifies the important role of microorganisms in soil systems and moreover gives strong reason for the degradative potential of soil microorganisms towards hazardous organic xenobiotics. *The assessment of the importance of soil bacteria for degradation processes of organic molecules in the water saturated (aquifer) and water unsaturated (vadose) zones of soils and sediments will, thus, be the general focus of this thesis.*

Traditionally, microorganisms from environmental compartments are extracted and characterised in pure cultures in the laboratory in order to assess their metabolic and physiological parameters (Amann et al., 1995). Even for water samples, this approach has been demonstrated to be flawed by the limited cultivability of extracted microorganisms

in artifical laboratory systems (Amann et al., 1995). For example, Torsvik et al. (1990) estimated that only 0.3% of the total diversity of soil microbes can be assessed by means of these methods. Therefore, Brock (1987) and Amann et al. (1995) strongly recommended applying culture independent methods for a direct assessment of the whole microbial community under in situ conditions in order to overcome the need for cultivation. While the proposed methods, such as fluorescence in situ hybridisation (FISH; e.g. Bauman et al., 1980), usually adequately perform in water samples, they frequently fail for soil samples, due to the relatively high natural background fluorescence of soil components (Tischer et al., 2012). These methods are, moreover, requiring a priori information on the available microorganisms for the selection of suitable RNA-probes and are only capable of indirectly detecting active microorganisms via the proxy of their rRNA content (Bulgarelli et al., 2012). Direct proof and quantification of microbial activity requires the application of isotopic tracers or the use of isotopic fractionation techniques. *However, in order to directly assess soil microbial activity under in situ conditions, methods independent of a priori knowledge are, therefore, favourable.*

This especially holds true for the clean-up of contaminated soils and sediments, where the self cleaning potential of soil by indigenous microbes was recently explored in the framework of the natural attenuation method (NA; US-EPA, 1999; UK-EA, 2000; SYKE, 2006). While the natural attenuation term includes a variety of naturally occuring processes, such as dispersion, advection and volatilisation of contaminants, all leading to lower contaminant concentrations in the system of interest (Wiedemeier et al., 1999), only microbial contaminant degradation activity leads to a true reduction of the contaminant mass (Bombach et al., 2010b). The NA method is generally regarded favourable with respect to more invasive methods like soil excavations or pump-and-treat methods (Declercq et al., 2012), due to its feasibility with respect to remediation costs and effort. However, a more widespread application is currently prevented by legal constraints (Declercq et al., 2012) along with a lack of suitable methods providing governmentally accepted evidence on the efficiency of NA as a remedy for a field site (Schurig and Kästner, 2012; Schurig et al., 2013a). One of the legally accepted methods for aquifers is the in situ microcosm approach (BACTRAP®; e.g. Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006; Bombach et al., 2010a; Schurig and Kästner, 2012). BACTRAPs detect the active part of the indigenous microbial community by exposing a sterilized activated carbon surface loaded with a isotopically labelled contaminant of interest (^{13}C, ^{15}N or ^{2}H) to groundwater in pre-installed monitoring wells at the level of the aquifer for a few weeks. During the incubation period indigenous microorganisms from the aquifer are actively colonising the AC, where degrader microorganisms can degrade the supplied labelled contaminants. Subsequent to the exposition, extractable labelled biomarkers provide the conclusive proof for ongoing microbial degradation of the supplied contaminant under in situ conditions at the field site. Although several authors report that the method was proven to be successful when investigating the microbial contaminant degradation potential towards benzene, toluene, mono-chlorobenzene (MCB; Nijenhuis et al., 2007; Stelzer et al., 2009) and methyl-tert-butyl-ether (MTBE; Busch-Harris et al., 2008), it was only applied under water saturated conditions mainly in groundwater monitoring wells at the level of the aquifer or in constructed wetlands (Braeckevelt et al., 2007). Within these water saturated conditions, the exposed

activated carbon surfaces are in direct contact with the soil water and, thus, bacterial colonisation of the activated carbon via the continuous water film is straight forward (Schurig et al., 2013a).

However, at field sites usually not just the aquifer is contaminated, but instead hazardous substances can also be found in the vadose zone, where their remediation via NA processes could only be investigated by ex situ microcosm studies in the laboratory (Strevett et al., 2002; Madsen, 2005). *In situ measurements of microbial activity with BACTRAPs in the vadose zone, thus, seem desireable due to the significance of soil protection and feasible clean-up of contaminated sites.* Fast and cost effcient sampling at varying soil depths is commonly done with the Direct Push method. Therefore, an approach based on the well established Direct-Push method (DP) was chosen to enable in situ vadose zone measurements of microbial activity (Schurig et al., 2013a). The term Direct-Push describes analysis or sampling of the subsurface with probes that are pushed into the soil by means of static pressure or hammering (Loxham et al., 1988; van Ree and Olie, 1993; US-EPA, 2005; Zschornack and Leven, 2012). While the method is suitable for unconsolidated materials, such as soils, it is not applicable on rock surfaces and the sampling depth is typically restricted to 25-35 m with a maximum of 50-60 m under ideal conditions (Zschornack and Leven, 2012).
Currently a suite of DP-probes is available, which mainly carry devices for geophysical and chemical measurements (Zschornack and Leven, 2012). These probes allow a geophysical preinvestigation, identifiying soil formations of interest, and subsequent chemical measurements at a contaminated field site (Kästner and Cassiani, 2009). For example, Grundl et al. (2003) developed a probe measuring soil contents of polycyclic aromatic hydrocarbons (PAH) along depth profiles by means of laser induced fluorescence (LIF). As PAH are hydrophobic with low solubility in water they form, depending on their density, which also depends on whether they are halogenated or not, either light or dense non-aquous phase liquids (LNAPL or DNAPL). Due to their hydrophobic nature, these liquids show a heteroeneous distribution in the subsurface and, thus, require a dense sampling network to adequately cover their distribution. By means of the DP-method this can be achieved at a higher resolution and speed at lower costs compared to drilling of conventional groundwater monitoring wells. Another method for measuring the distribution of more volatile contaminants is the membrane interface probe (MIP; Christy, 1998; van Ree et al., 1999), where the measurement principle relies on heat induced desorption of volatile organic compounds from soils or sediments, subsequent diffusion through a membrane and transport to a detector (photo-ionisation or mass-spectrometer) by means of an inert carrier gas (Christy, 1998; van Ree et al., 1999). The combination of the geophysical and the chemical data measured at similar scales later allows for the setup of a detailed site model (Kästner et al., 2012). In principle, this approach could also be applied to the vadose zone. However, until now microbial data, to assess the role of microorganisms in the framework of a NA site remediation approach, cannot be obtained at the same spots by means of the DP technique, because microbial methods depend on a network of expensive groundwater monitoring wells for the incubation of in situ microcosms and, therefore, cannot be applied in the vadose zone. The construction of a conclusive site model, thus, requires data interpolation and scaling, which leads into scaling related issues caused by the

high structural and functional diversity of soil and sediment ecosystems as compared to, for example, the atmosphere. *In order to exclude scaling related errors, an adequate DP probe measuring in situ microbial activity would thus be desirable.*

The multidimensional complexity and heterogeneity of soil systems also complicates quantification of microorganisms under in situ conditions. While enumeration of microbial cells by microscopy is a common method for quantification in water samples (Amann et al., 1995), this method fails in soil samples due to the opacity of the soil matrix and the high surface area available for microbial colonisation. However, visualisation of microbial cells together with providing information on their identity, for example by means of the FISH method, would provide insights into the ecology of contaminant degrading microbes. Tischer et al. (2012) developed a catalysed reporter deposition-FISH (CARD-FISH) approach for soil samples. This approach provides insights into the identity of the visualised microbes, however it demands an extraction of the microbial cells from the soil sample and later visualisation. Bombach et al. (2010a) and Miltner et al. (2012) presented images obtained by confocal laser microscopy and scanning electron microscopy (SEM) from BACTRAPs being exposed in groundwater monitoring wells. While the images presented by Miltner et al. (2012) show a diverse biofilm formation on the AC, the images of Bombach et al. (2010a) were obtained from an enrichment culture and show mainly rod-shaped bacteria. These findings could indicate that mainly rod shaped bacteria (Bombach et al., 2010a) are degrading the toluene in the laboratory culture. However it is not known how many of the visualised microbes from the field sample (Miltner et al., 2012) are degrading toluene and if those are culturable with the present methods in the laboratory. Therefore, the results from the laboratory are not readily comparable to the field results. *An in situ visualisation approach should help clarifying this issue.*
Yet, these approaches still just visualize and identify certain microorganisms, but fail to present data on the contaminant degradation activity of the visualised microbes. As Musat et al. (2008) presented for a lake in Switzerland, even microbes with low abundance can account for the majority of metabolic turnover. The authors filtered the lake water, amended it with isotopically enriched ammonium (^{15}N) and bicarbonate (^{13}C), and subjected it to a CARD-FISH procedure in order to deposit halogen labelled FISH-probes in active cells. Later the authors visualised and measured the cells by means of nano-scale secondary ion mass spectrometry (NanoSIMS) (e.g. Lechene et al., 2006). Inverse relationships between the quantity of bacteria and their activity are likely occuring also in soil systems and the potential of the NanoSIMS intrument in soil science is not yet fully explored (e.g. Herrmann et al., 2007b; Mueller et al., 2012; Behrens et al., 2012). *Therefore, a visualisation of the activity of the microbial community, which is colonising BACTRAPs under in situ conditions, with NanoSIMS can be regarded advantageous in order to identify active microorganisms.*

Active microorganisms are playing a fundamental role for the physical stabilisation of soils (Forster, 1979), but more importantly they maintain the functional resilience of soil systems by degrading most organic substances entering the soil (Gleixner et al., 2001). However, what is happening to this living microbial biomass after cell death? Senesi (1992) claimed that organic xenobiotics, like pesticides and contaminants, are, irreversibly or reversibly, bound to humic substances by chemical or physical processes

and thereby form non-extractable residues consisting of residual contaminants. Recent results of Nowak et al. (2011), however, indicate that these organic xenobiotics are biodegraded and the non-extractable residues, previously considered to be residual substances or metabolites irreversibly bound to humic substances, are, in fact, partly microbial biomass residues. The quantity of living microbes in soil is generally assumed to be lower than 2% in terms of carbon storage (Blume et al., 2010), while their influence on SOM formation is usually not quantified, they form the most dynamic pool of soil organic matter. Moreover, according to the model of Petersen et al. (2005) microbes are processing most of the organic matter entering the soil and thereby change its molecular nature from plant derived material to microbial biomolecules (Miltner et al., 2012). These microbial residues have been described by the authors to have a size between 100 and 500 nm and to accompany microbial colonisation and proliferation in artificially controlled systems. *However, yet no evidence exists that these processes are important in the field and, moreover, no method exists for quantification of these fragments.*

1.1 Research questions

In order to address the previously identified gaps in knowledge the research questions for this thesis therefore were:

I Could the in situ microcosm approach (BACTRAP®) be adapted for field sites without groundwater monitoring wells?

II Is this new approach permitting better comparison to in situ conditions in the aquifer sediment?

III Could the new approach be used for vadose zone measurements of microbial activity?

IV How does the new method compare to commonly used approaches?

V Which new contaminants could be analysed with the new methodology?

VI Could microbial activity be visualised in situ with the new technique?

VII Is this visualisation approach working for living and dead microbial biomass in field samples?

1.2 Structure of the thesis

This section gives a short overview on the provided manuscripts, which had the task to find answers to the research questions.
Geyer et al. (2005) developed an in situ microcosm system to detect microbial contaminant degradation activity in aquifers accessible by groundwater monitoring wells. As these wells are not available at every field site and, moreover, cannot be regarded

representative for the aquifer without continuous pumping (Chapelle, 1993), Schurig and Kästner (2012, chapter 2.1) presented an adaptation of this microcosm approach to the DP technology platform. The field ready prototype was termed DP-BACTRAPs and was later filed a utility patent (Schurig et al., 2012).
Schurig et al. (2013a) presented data on the comparison of the 'classical' BACTRAP system to the newly developed DP-BACTRAPs, which was obtained during two field incubation periods in the BTEX-contaminated aquifer in Zeitz (Germany). The time needed for hydraulic equilibration of the AC with the surrounding soil was tested in several laboratory experiments, because the new probe should be applicable under vadose zone conditions, where microbes require a continous water film, or fungal hyphae, to migrate to the AC surfaces (Schurig et al., 2013a, chapter 2.2).
Soil contamination with hydrophobic organic substances arising from oil spills, like alkanes, frequently leads to formation of LNAPLs due to their low water solubility. These LNAPLs are floating on top of the groundwater table in the vadose zone, and therefore cannot yet be accurately assessed by in situ microcosms. However, alkanes are known to be readily biodegraded by microorganisms (Pirnik et al., 1974; Rojo, 2009). The only method for investigating the microbial mineralisation in these ecosystems are ex situ laboratory microcosm studies, which can be regarded biased with respect to in situ conditions and the microbial community. However, with the newly developed DP-BACTRAP system in situ measurements would become available. In order to test the applicability of the BACTRAP approach for alkanes, like hexadecane and pristane, and to compare it to the commonly used microcosm approach, a laboratory experiment was carried out with both methods under identical conditions (Schurig et al., 2013b, chapter 2.3).
Soil microbial diversity and cell numbers decline with increasing distance to the surface (Bone and Balkwill, 1988; Bekins et al., 1999) and more microorganisms are found in contaminated zones of soil compared to uncontaminated parts of similar depth (Ringelberg et al., 2008). Therefore, in otherwise oligotrophic contaminated aquifers, usually a higher fraction of the detected microorganisms is involved in contaminant degradation, whereas in nutrient richer parts of the vadose zone more microbes not being involved in contaminant degradation can be expected. For the DP-BACTRAP approach this means that the yield factors for the supplied stable-isotope labelled contaminants are decreasing upon application in the vadose zone and so does the enrichment of the extractable biomarkers, especially when only a small highly active fraction of microbes is degrading the contaminants. Easier identification of these degraders requires visualisation of microbial activity on the nano scale by means of NanoSIMS, as was exemplarily performed for DP-BACTRAPs incubated in an oligotrophic aquifer and in a constructed wetland (Schurig et al., 2013c, chapter 2.4).
Due to the rather long incubation time of in situ microcosms in the aquifer, some of the degrader microorganisms are already dead at the end of the incubation time. The visualisation approach provided the opportunity to investigate the contribution of this dead microbial biomass for SOM formation on the field scale (Schurig et al., 2013d, chapter 2.5), which was likely previously underestimated according to the theory of Miltner et al. (2012). This experiment highlights potential stabilisation mechanisms responsible for SOM and partly for the formation of non extractable microbial residues.

2 Compendium of manuscripts

2.1 In-situ microcosms for demonstrating microbial contaminant degradation (BACTRAP®)

Schurig, C., Kästner, M., 2012. In situ microcosms for demonstrating microbial contaminant degradation (BACTRAP®).
In: Kästner, M., Braeckevelt, M., Döberl, G., Cassiani, G., Petrangeli Papini, M., Leven-Pfister, C., van Ree, D. (Eds.), Model-Driven Soil Probing, Site Assessment and Evaluation - Guidance on Technologies. Sapienza Universita Editrice, Rome, pp. 149–161.
ISBN: 978-88-95814-72-8

Abstract:
The book chapter was published in a handbook aimed for site managers and legal authorities and gives an introduction to the BACTRAP method, which formed the basis of this thesis. The chapter presents some background information and technical requirements of the 'classical' BACTRAP approach and the newly developed DP-BACTRAP approach. Moreover, a protocol for a succesful application is given, in addition to a analysis scheme, cost estimates and examples of field applications. Additional factsheets provide the reader with a fast overview of the techniques.[1]

Author contribution: 70%
My contribution to the publication was writing the manuscript and (re-)designing the figures. Matthias Kästner assisted with finalising the manuscript.

(signature of supervisor)

SECTION 3 - CHAPTER 8

IN SITU MICROCOSMS FOR DEMONSTRATING MICROBIAL CONTAMINANT DEGRADATION (BACTRAP®)

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[1] Factsheets are provided in the appendix p. XXXIII

8.1 INTRODUCTION

A large number of contaminated field sites exist in Europe, and monitored natural attenuation (MNA) might be an interesting remediation option in particular for financial reasons. However, very few methods are accepted by authorities as conclusive proofs of natural attenuation. One such method is the recently developed in situ microcosm approach (BACTRAP®). These in situ microcosms consist of a perforated cage system (Teflon tubes or stainless steel cages) filled with a carrier matrix depending on the target contaminant, e.g. the commonly used granulated activated carbon, or BioSep® beads, or comparable materials. Stable-isotope-labeled contaminants (BTEX, MTBE, MCB and all compounds available with stable isotope labels (^{13}C, ^{15}N)) are loaded on the activated carbon matrix and the microcosms are then exposed for about 4 - 8 weeks, either in groundwater wells or in Direct Push (DP) liners or probes, depending on the growth kinetics of the respective bacteria. During microbial degradation, the ^{13}C label of the contaminant is mineralized and a certain amount of the carbon (or nitrogen) is incorporated into the biomass of the contaminant degraders. The label can be detected in biomarkers, e.g. phospho-lipid fatty acids (PLFA), amino acids or DNA/RNA. The transformation of the C and N into biomolecules then provides the ultimate evidence that the contaminant has been degraded under the field conditions. As such, the functioning of the method is basically not considerably influenced by the absolute concentration of contaminant inside the aquifer; as long as indigenous contaminant degrading microbes are active, BACTRAPs are able to detect their activity. Based on this approach, monitored natural attenuation is accepted by many authorities as a site remediation measure[1].

Until 2010, the in situ microcosms were only applicable in pre-installed groundwater wells at the respective filtration level of the well within the aquifer. Hence, the system was only applicable at field sites equipped with a set of monitoring wells, and only microbial activity inside the wells, potentially influenced by oxygen access, could be assessed. In order to overcome these limitations, a new Direct-Push (DP) BACTRAP-probe was developed for application in DP devices, e.g. Geoprobe® equipment. With the help of this new probe, the approach can now be extended to field sites without pre-installed monitoring wells, and the microbial activity can even be assessed in the vadose zone above the aquifer.

[1] *For example at the BTEX-polluted megasite Zeitz (Germany), see also 8.7.2*

8.2 TYPICAL USE OF BACTRAPS

8.2.1 Investigation goals

The approach should generally be applied only after a thorough characterization of a polluted site, first by historical site use data and second by non-invasive and low-invasive geophysical and chemical methods like seismics, geoelectrics, tree core monitoring or the Direct Push membrane interface probe. At least, the type of contaminant and the groundwater flow direction should be known. For providing evidence of actually occurring microbial degradation and the potential for natural attenuation under in situ conditions at the sites, BACTRAPs can be incubated in the aquifer or the contaminated soil using Direct-Push techniques or, if available, using groundwater monitoring wells.

For evaluating enhanced natural attenuation (ENA) as a potential site remediation approach, BACTRAPs can be amended with terminal electron acceptors (TEA), e.g. sulfate, nitrate, or Fe(III). If significantly higher isotope enrichment occurs in the biomarkers on TEA-amended microcosms compared to unloaded ones and controls, one can conclude on the feasibility of a TEA supplementation approach.

To a certain extent, conclusions about the degradation pathways can be drawn by analyzing sorbed metabolites from the carbon surface of the BACTRAPs (Stelzer *et al.,* 2006; Kästner *et al.,* 2006). This information can additionally be used to support decisions about amendments to the soil or aquifer within the framework of ENA measures.

Combined with the approach of compound-specific stable-isotope analysis (CSIA, Stelzer *et al.* (2009), which should be conducted before the application of labeled compounds within the aquifer due to potential release of labeled compounds from the BACTRAPs to the respective wells), a general site model of the microbial contaminant degradation may be developed; this approach provides information about the degradation that has already taken place in the past and may enable estimations of future contaminant elimination rates.

8.2.2 Results

After exposition at the contaminated site, BACTRAPs are transferred to the laboratory for analysis. Extracting the activated carbon carrier material yields biomarkers such as fatty acids of bacterial cell wall lipids (see 8.5.4), proteins or DNA/RNA (for these analyses see references: Geyer *et al.*, 2005; Busch-Harris *et al.,* 2008; Miltner *et al.,* 2009; Bombach *et al.,* 2010b), which are analyzed for their isotopic composition in comparison to determined standards or non-labeled controls. Based on the isotopic

enrichment and patterns of biomarkers, conclusions can be drawn about the contaminant degradation occurring at the respective site.

Incorporating TEA in the BACTRAPs provides additional results for isotopically enriched biomarkers, based on stimulated microbial degradation under the respective electron-accepting process. However, this approach must be compared to unamended BACTRAPs or other TEA amendments in order to determine the most suitable ENA measures for the site.

Extraction of metabolites may show compounds enriched in isotopic label, which may be assigned to a specific degradation pathway. Based on this information, additional conclusions for implementing an ENA approach can be drawn.

8.2.3 Advantages and limitations

Contrary to commonly used ex situ microcosms operated in the laboratory, the proposed method provides measurements of potentials of microbial contaminant degradation under in situ conditions, which include the indigenous settling microbial community and the delivery rate of TEA to the microcosms. Thereby, soil microcosms and other laboratory microcosm studies can be complemented by a more rapid method and sampling bias due to sample handling, transport and lab microcosm setup can be largely excluded. Additionally, bias attributed to lab microcosms, based on sediment or water samples, being hampered by the very limited cultivability of microorganisms in the lab can be excluded. If DP BACTRAPs are used, the bias introduced by the artificial conditions inside groundwater monitoring wells (such as convection and thereby introduction of oxygen into anoxic systems) can also be minimized. DP-BACTRAPs can be easily included into a site characterization approach, whereby other DP measurements are used in order to identify formations which may contain contaminants (e.g. by using a Membrane-Interface-Probe; MIP). At points of interest, DP-BACTRAPs could be immediately incubated at the relevant depth.

However, the method is limited by i) the availability of the respective isotopically labeled contaminant, ii) contaminants that are used as carbon or nitrogen source by bacteria, and iii) the requirement for access to a GC-C-IRMS system (see 8.5.4). In addition, relatively long exposure times are needed in comparison to chemical or geophysical analyses.

8.2.4 Requirements

Site requirements

- 'Classical' BACTRAPs are only applicable in aquifers which are accessible by groundwater monitoring wells. DP-BACTRAPs are limited to field sites with unconsolidated materials, where DP can be applied. Probes have already been tested in soil with high gravel content.
- Generally no infrastructure is required for DP-BACTRAPs, except that the site should be accessible for DP equipment. For 'classical' BACTRAPs, however, conventional groundwater monitoring wells are obligatory.

Data/information necessary for reliable interpretation of results

- For a successful site survey, the groundwater level and flow direction has to be known in order to identify contaminant sources and plume zones for accurately mapping microbial activity.
- Main contaminants at the site have to be known so that the BACTRAPs can be loaded with the correct compounds.
- Information on electron acceptors is advantageous for estimating the main degradation pathways and for checking whether isotopic enrichment of biomarkers is likely.

8.2.5 Application range

(Ideal) operative range for applicability

• BACTRAPs are applicable at all sites which are accessible by DP machines and can be exposed in depths up to 60 m depending on the soil.

• A broad range of organic contaminants can be surveyed, such as BTEX, MTBE, MCB and other compounds which are available with stable-isotope labels (^{13}C, ^{15}N) and which are used as carbon or nitrogen source by bacteria.

• Analyses of the BACTRAPs after exposition should be performed within a few days, otherwise the samples should be stored frozen, preferably at -70°C for DNA/RNA analyses.

8.3 THEORY OF OPERATION

8.3.1 Introduction

The in situ microcosms are set up in the BACTRAP system (Fig. 8.1) as described elsewhere (Geyer *et al.,* 2005; Kästner *et al.,* 2006; Bombach *et al.,* 2010b). Briefly, the commonly used granulated activated carbon bead materials (AUF 540, Adako Aktivkohlen GmbH, Düsseldorf, Germany), or Bio-Sep® beads, or similar materials, are loaded with contaminants as a source of carbon and energy for microorganisms (Fig. 8.1 (1)).

The pellet or bead materials (size 2-4 mm) should provide a large internal surface area for colonization

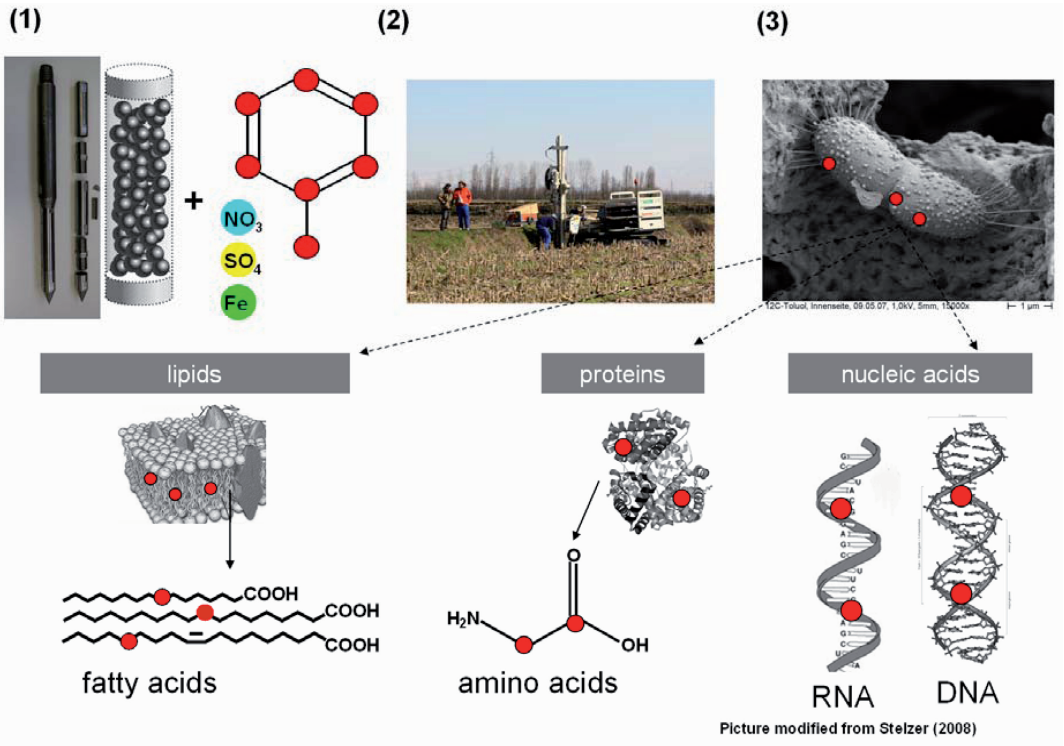

Fig. 8.1 - *General workflow for a BACTRAP experiment: (1) BACTRAPs or DP-BACTRAPs prototypes are loaded with ^{13}C enriched substrate (and terminal electron acceptors); (2) exposure inside the contaminated aquifer/soil; (3) During microbial degradation the ^{13}C label is integrated into degrading microbes, which can be identified by phospholipid fatty acid (PLFA), protein or DNA/RNA analysis for specific groups of microorganisms. Usually, ^{13}C-PLFA analysis is used for field applications*

or attached growth of bacteria (biofilm formation) and should provide sufficient sorption properties for the respective labeled target compound, in order to prevent cross-contamination and loss of labeled compound to the aquifer (Geyer *et al.*, 2005; Kästner *et al.,* 2006; Bombach *et al.,* 2010b). If the indigenous bacteria of the well or the soil next to the DP-probe colonize the beads and productively consume the contaminants under in situ conditions (Fig. 8.1(2)), the ^{13}C-labeled carbon or ^{15}N-labelled nitrogen will be incorporated into the growing cells and can be traced within biomarker molecules such as ^{13}C labeled fatty acids, amino acids and as ^{13}C or ^{15}N enriched nucleic acids (Fig. 8.1 (3)).

8.3.2 Preparation and exposure

The materials must be heated at 300°C for 4 hours in order to remove organic residues; 0.5-1 g of pellets per trap are then filled into perforated Teflon tubes and fixed with glass wool plugs at both ends. The filled microcosms are autoclaved for sterilization and hydration of the bead material and then air-dried. The microcosms are loaded with the substrates (for example with [$^{13}C_6$] labeled benzene, toluene, or chlorobenzene to a range of 30-40 mg/g) via gas phase under reduced pressure in a desiccator. In order to keep the beads anoxic, the vacuum is released by filling oxygen-free water into the glass container, and the beads are stored under anoxic conditions until their insertion into the monitoring wells or soil at the operating depth in single level wells. However, if multi-level wells are to be used, multi-level packer systems (MLPS) should be installed for depth-discrete sampling and exposure at the target depths in the wells of interest. A MLPS is a water-filled sock tube inserted into the well, which allows various sampling systems to be separated from each other at different depths. If permanent monitoring wells have been installed at the site for previous site characterization measures, BACTRAPs can also be exposed within these wells; however, care must be taken to avoid oxygen access to previously anoxic parts of the aquifer. Usually, MLPS are available for conventional groundwater monitoring wells; similar devices also exist for DP installations.

8.3.3 Extraction and analysis

For the lipid analysis, the beads from the exposed microcosms are extracted using a dichloromethane-methanol-water mixture as solvent, and the Bligh & Dyer protocol (Bligh & Dyer, 1959) with modifications according to White *et al.* (1979) and the procedure described by Bombach *et al.* (2010b) (see also 8.5.4). After phase separation, the dichloromethane phase containing the fatty acids (FA) is evaporated to dryness and derivated using a trimethylchlorosilane (TMCS) methanol mixture (1:8; v:v) as reactant for 2 h at 70 °C to obtain fatty-acid methyl esters (FAME). After evaporation to complete dryness, the FAME fraction is dissolved in n-hexane for subsequent analysis by means of gas chromatography - mass spectroscopy (GC-MS) and gas chromatography - combustion - isotope ratio mass spectrometry (GC-C-IRMS).

GC-MS is used for the determination of concentrations and the identification or structural characterization of FAME and/or metabolites. FAME are identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Supelco International) and quantified relative to an external standard mix or an internal standard (usually Henicosanoic acid, C21:0).

The metabolites are characterized by means of co-injection and comparison with mass spectra obtained for the authentic reference compounds.

The carbon-isotope compositions of the carboxylic acid fractions are analyzed with a GC-C-IRMS system, which usually consists of a gas chromatograph coupled via a specific interface (for oxidizing the carbon compounds and dewatering the resulting gas) to an isotope-ratio mass spectrometer. The methylation of carboxylic acids for gas-chromatographic analyses introduces an additional carbon into the structure of the analyte, which affects its isotopic composition. Therefore, the isotope signature of fatty acids ($\delta^{13}C_{FAME}$) must be corrected for the isotope effect due to derivatizing FAME with methanol.

8.4 TECHNICAL DESCRIPTION

8.4.1 'Classical' BACTRAPs

'Classical' BACTRAPs are constructed from a perforated PTFE-Tube, which is closed by glass-wool stoppers and cable-ties in order to keep the bead or pellet material within the microcosm (Fig. 8.2). For exposure of the microcosms inside the aquifer, they are mounted on a nylon rope for withdrawal and maintaining the proper depth position in a monitoring well. For obtaining appropriate results, statistical analysis is necessary, therefore, each experiment must be carried out in triplicate, i.e. with 3 unloaded microcosms (controls), 3 loaded with unlabeled contaminant, and 3 loaded with labeled contaminant for the calculation of the isotopic enrichment.

8.4.2 Direct Push BACTRAPs

For the design of DP BACTRAPs, several factors had to be taken into account. Firstly, the probe has to be integrated into the Geoprobe® DP equipment, thus it must be robust enough to withstand the up to 160kN pressure exerted by Geoprobe machines. The application with the DP approach also enables the exposition of the microcosms within the vadose zone above the aquifer. In order to reduce the sampling costs, three replicates each of ^{12}C, ^{13}C and blank BACTRAPs are included in each probe, thereby replacing 9 'classical' Teflon-caged BACTRAPs (Fig. 8.3).

Moreover, the probe can be adapted to varying sampling concepts: it is constructed in a modular fashion, which also allows easy replacement of worn-out parts. In order to prevent cross-contamination, mostly inert materials such as stainless steel are used.

DP-BACTRAP probes are constructed in a segmented fashion, whereby each segment represents 3 'classical' BACTRAPs. The chambers of each segment are filled with the same carrier materials containing the labeled substrates, so the replicates are exposed to similar conditions without cross-contamination between ^{12}C and ^{13}C. After exposure, chambers can be emptied separately via a specially constructed latch. Within the vadose zone, establishing the contact between the surrounding soil water and the activated carbon materials inside the microcosm is more difficult than within the aquifer. Therefore the design features an outer casing of the segments that provides high water conductivity inside the aquifer. For the vadose zone, the carrier material is additionally supplied with pre-dried silica gel in order to create sufficient suction potential for soil water at the beginning of the exposure.

As can be seen in Fig. 8.3, the segments are attached to each other via connectors with threads on both sides; corresponding threads are directly cut into the segments. The tubing of the segments is mechanically strong tubing having a lower diameter than the Geoprobe 1.25 inch tubing in order to prevent the probe from suffering cross-contamination and mechanical shear forces during retraction. The construction was further improved by providing force-locked segments. Taking mechanical shear forces into account, the tip of the probe was designed to be replaceable. Every other part of the probe is also easily replaceable, since no parts are welded together.

Fig. 8.2 - *"Classical"BACTRAP probe with activated carbon matrix*

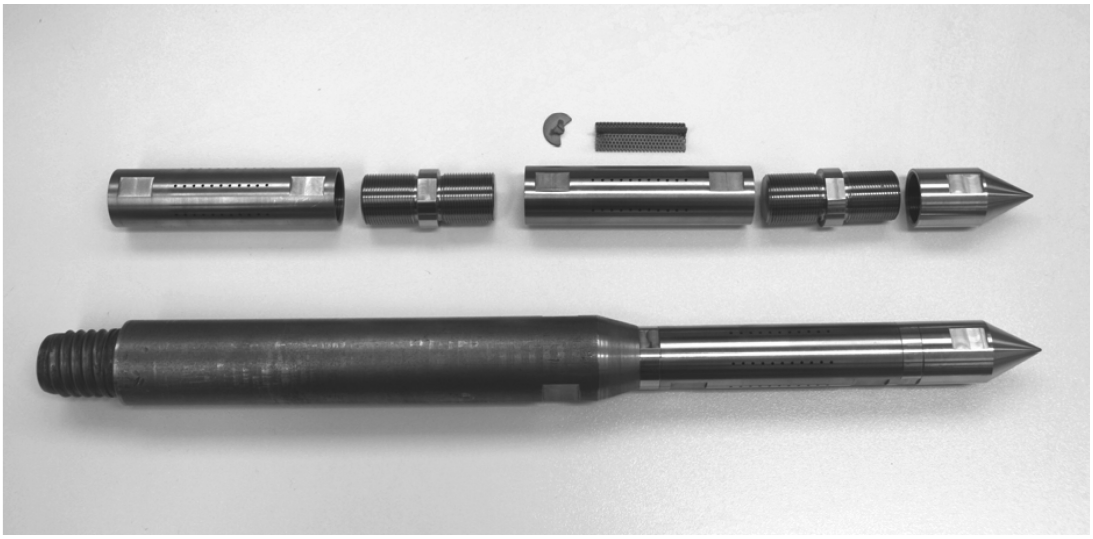

Fig. 8.3 - *Direct-Push BACTRAP® prototype with improved mechanical stability and adaptor to Geoprobe® 1.25 Inch tubing. Every part of the probe is replaceable, thus ensuring excellent maintainability*

8.5 PRACTICAL APPLICATION

8.5.1 Planning

During the planning phase of the site assessment, locations of interest for a BACTRAP survey should be selected according to the historical information as well as the geophysical and chemical data available. After selecting the appropriate contaminant based on site characterization, and taking into account the availability of stable-isotope labeled contaminants, the depth of installation and the availability of groundwater monitoring wells should be checked in order to decide whether to apply BACTRAPs or DP-BACTRAPs. Additionally, the timeframe of the exposure has to be chosen, usually 6 weeks have been shown to be enough even for degradation under anaerobic conditions (Geyer *et al.,* 2005; Kästner *et al.,* 2006).

If DP BACTRAPs are applied, the availability of a Geoprobe device must be checked and a drilling- and ordnance clearance must be requested from landowners and local authorities. The availability of intact pushing rods (with special focus on the stability of the screw joints) for the whole period of the survey should also be ensured.

If the pushing rods cannot be left at the side for the time of exposure, the microcosms have to be exposed within plastic liners remaining in the drilling hole. However, during field work, this was found to be more laborious, due to frequent breakage of the plastic liners during emplacement.

8.5.2 Preparatory work

After the number of sampling locations, the contaminant and the type of BACTRAPs have been established, the experiment must be prepared in the laboratory, whereby it is important to work under very clean conditions and to wear gloves at every step.

Firstly, 1 g activated carbon materials (typically in the range of 2 to 4 mm in diameter) per sample is to be weighed into a glass bottle and heated to over 300°C for at least 4 hours in order to remove any organic traces from the activated carbon surface. In parallel, the microcosm cages are to be rinsed and thereby cleaned with acetone (high purity) in order to avoid any contamination with biomarkers. After filling the clean activated carbon beads into the clean microcosms, the probes are to be closed either by a glass-wool stopper or by attaching the connecting nut (DP-BACTRAPs).The probes are then to be put into a beaker covered with a perforated aluminum foil and autoclaved (1 bar overpressure, 121°C) for sterilization and rehydration of the surface.

The method to be used for loading the BACTRAPs with the contaminants depends on the volatility of the compounds. For volatile compounds, such as BTEX, loading can be performed via the gas phase using a desiccator, as follows. Each set of replicates of either blank, ^{12}C or ^{13}C BACTRAPs are put into a separate container with a three-way valve attached to the container. The vessel is then evacuated and flushed with N_2 immediately afterwards. This procedure is carried out three times, and after the last flushing usually 100 mg of the contaminant per 1 g of activated carbon is added to the container in small GC-MS glass vials. The container is then evacuated for the last time, whereby care should be taken that the vial containing the contaminant remains in an upright position. In order to load the contaminant, the container is then kept under vacuum for at least 48 h at room temperature. For non-volatile compounds, the loading can be performed in a similar manner, the difference being that the contaminant is diluted in a suitable volatile solvent and the activated carbon is directly added to the solvent before it is placed into the desiccator.

In both cases, the next step is to prepare the samples for the field. BACTRAPs with similar loading are placed into a single bottle with sterile anaerobic water (= distilled water, autoclaved and flushed with N_2). The microcosms can be stored in the cold for at most 24 h before applying them at the field site.

8.5.3 Field application

At the field site, the correct positions for application of the BACTRAPs have to be found. The 'classical' BACTRAPs are each hung into the designated groundwater monitoring wells at the target depth via a nylon line with a stainless steel weight at the bottom. The line should be carefully fixed such that it does not get lost during 6 weeks of exposure.

For DP BACTRAPs, the designated sampling positions must be found with appropriate equipment. Rods with a solid tip are pushed to just above the target depth, then this tubing is retracted. Inside the hole of the first push, the tubing with the attached DP BACTRAP probe is pushed to the desired depth in order to provide the soil contact of the probe (with a smaller diameter). The top of the pushing rods is then secured and the samples are incubated for 6 weeks.

At the end of the incubation period, the BACTRAPs are retrieved from the sediment and placed into separate vials for cooled transport to the laboratory.

8.5.4 Sample analysis

After arrival at the laboratory, the samples should be stored frozen until further processing. Depending on the type of analysis required, various protocols can be applied. The protocol for extraction of PLFA is shown below.

Tab. 8.1 - *PLFA extraction protocol*

Day 1	**Start PLFA extraction:** • weigh 3*1g of carrier material into glass vials and add 5ml dichloromethane, then put the samples on the shaker in the climate chamber at 20°C overnight • schedule some measurement time with the GC-MS + GC-IRMS
Day 2	• take out the dichloromethane, put it into a new vial and dry it under a gentle nitrogen stream; • add 3ml phosphate buffer, 10ml methanol, and 5ml chloroform to the residual material in the vial and vortex it briefly, then put the samples onto the shaker in the chamber at 20°C • prepare silica gel columns in SPE columns with glass-wool stoppers and heat them to 110°C
Day 3	• take the samples from the shaker, add 5ml distilled water + 5ml chloroform, and vortex the mixture briefly • centrifuge the samples for 20min @2000 rpm and 4°C • take out just the lower phase (chloroform) with a Pasteur pipette and put it into the vial with the dried dichloromethane extracts • afterwards, dry the extracts with a gentle nitrogen stream
Day 4	• put 5ml 0.02M ammonium acetate in methanol solution onto the columns • activate the columns with 5ml acetone and 5ml chloroform (=> column becomes translucent) • resuspend the sample with 500µl chloroform and put it onto the columns with a Pasteur pipette • put 5ml chloroform onto the column (solution of neutral lipids) • put 5ml acetone onto the column (solution of glycolipids) • put a new vial underneath the column and put 10ml methanol onto the column to solute the PLFA • dry the PLFA under nitrogen
Day 5	• put 450µl methanol and 50ul chlorotrimethylsilane (TMCS) onto the dry PLFA and let them react for 2h at 60°C • dry the samples under nitrogen • add 200ul hexane to the dry samples and transfer them into GC-vials with inserts using a Pasteur pipette • evaporate the hexane under nitrogen and then add 100µl of 10µg/ml internal standard • put samples into the GC-MS

For the determination of concentrations and the identification or structural characterization of FAME and/or metabolites, use a GC-MS. For separation of the carboxylic acid fraction, e.g. a HP-5MS column (30 m*0.25 mm*0.25 µm; Agilent Technologies, Böblingen, Germany) can be used with a temperature program of 50°C for 1 min then 4 °C min^{-1} to 250°C, 20 °C min^{-1} to 300°C and hold for 10 min. FAME can be identified by co-injection of an authentic standard mix (bacterial acid methyl ester mix, Supelco International) and quantified relative to either an external standard mix or an internal standard (Henicosanoic acid, C21:0).

For the carbon-isotope composition of the carboxylic acids fractions, use a GC-C-IRMS. In the GC part, a BPX-5 column (50 m*0.32 mm*0.5; SGE, Darmstadt, Germany) may be used for chromatographic separation with helium as carrier gas at a flow rate of 1.5 mL min^{-1} and a temperature program with initial temperature of 60 °C for 2 min, heat at 20 °C min^{-1} to 120 °C, heat at 2 °C min^{-1} to 300 °C, and hold for 20 min. If the expected isotopic enrichment is higher than 10% ^{13}C, the samples can be analyzed on a normal GC-MS system by comparison of the isotopomer distribution (Bombach *et al.,* 2010b).

8.5.5 Quality control

In order to obtain appropriate results, statistical analysis is necessary, therefore, each experiment must be carried out in triplicate, i.e. with 3 unloaded microcosms (controls), 3 loaded with unlabeled contaminant, and 3

loaded with labeled contaminant for the calculation of the isotopic enrichment, as described in the technical description (see 8.4).

8.5.6 Occupational health and safety requirements

Since work is carried out using contaminants, the appropriate precautions for personal protection should be applied. During use of DP equipment, safety shoes, helmet and ear protection are obligatory according to the safety instructions of the supplier.

8.6 INTERPRETATION OF RESULTS

8.6.1 Main results

For analysis, biomarkers in the samples must be quantified by means of a suitable method, such as addition of an internal standard for quantification. After quantification, the carbon-isotope signature in ^{13}C samples is to be compared to other samples. If the isotope signature of biomarkers is significantly heavier in ^{13}C than in ^{12}C samples (which mean a shift in the δ units of > 100 δ‰), microbes have actively degraded the contaminant. The composition of biomarkers in the samples can then be analyzed and compared between the samples, thus allowing conclusions about the degrading microbial community to be drawn.

The next step is to compare the quantity of biomarkers between several parts of the contaminated zone to find out more about the relative size of the indigenous microbial community. This information is of interest when comparing the isotopic enrichment of these biomarkers in order to get insights whether microbes are more actively degrading contaminants, and are thereby showing a higher abundance, or are just settling on the BACTRAPs, without degrading contaminants.

Additionally, a data fusion of the BACTRAP derived data with groundwater flow data, data from compound specific isotope analysis and chemical data could yield a conclusive site model, where one could derive estimates on the behavior of the contaminant plume and, related to that, a risk assessment on the contaminated site.

8.6.2 Data accuracy

Depending on the level of care taken during preparation and field sampling, high accuracies can be reached. For isotopic measurements, the total uncertainty is usually around 0.5 δ‰. An incorporation of at least 0.12 atom % ^{13}C into PLFA is necessary for accurate assessment of the contaminant degradation potential in situ.

8.6.3 Examples

See 8.7.2

8.7 STATE OF DEVELOPMENT

8.7.1 Field applicability

Both types of BACTRAPs are fully applicable for field use; 'classical' BACTRAPs are even commercially available from Isodetect GmbH (www.isodect.de). A similar system called Bio-Trap is available from Microbial Insights (www.microbe.com).

8.7.2 Examples

'Classical' BACTRAPs have been successfully applied at the BTEX-contaminated mega-site Zeitz in Saxony-Anhalt (Germany). For these experiments, BACTRAPs were loaded with either benzene or toluene. After 32 days of exposure, the BACTRAPs were retrieved and analyzed for total lipid fatty acids (tFA) and phospholipid fatty acids (PLFA). Using a GC-IRMS system, isotopic enrichments ranging from +46 δ‰ to over +13000 δ‰ for tFA were measured both for benzene and toluene amended BACTRAPs (Geyer *et al.,* 2005). As a result of this study, monitored natural attenuation was accepted as a site remediation strategy for this mega-site by the local authorities of the federal state Saxony Anhalt in Germany.

In another experiment, 'classical' BACTRAPs were exposed inside an MCB-contaminated aquifer in Bitterfeld (Saxony-Anhalt, Germany). After 7 weeks of incubation, fatty acids were significantly enriched in ^{13}C; the enrichments ranged from +46 δ‰ to +579 δ‰ for PLFA (Kästner *et al.,* 2006), thereby demonstrating biodegradation under in situ conditions.

Classical and DP BACTRAPs have been applied in the BTEX-contaminated aquifer at the ModelPROBE reference site Zeitz (Germany). Both types of BACTRAPs were incubated in the center and at the fringe of the BTEX plume. During microbial degradation of the ^{13}C-labeled substrate, the ^{13}C label was incorporated into bacterial biomass; this was determined by PLFA analysis, and provided evidence for in situ natural attenuation. In addition, the bacterial communities on classical and DP microcosms were compared by analyzing PLFA patterns. The results of the BACTRAP approach documented the microbial degradation of BTEX compounds in this contaminated aquifer and led to the approval of a monitored natural attenuation measure at the site by the respective authorities.

In conclusion, classical BACTRAPs are a suitable technique to provide legally accepted evidence for microbial contaminant degradation activity under in situ conditions inside contaminated aquifers. In addition to that, DP-based BACTRAPs offer a promising cost-efficient means for monitoring natural attenuation or reme-

diation success at field sites currently inaccessible by the 'classical' BACTRAP approach (Schurig *et al.,* 2012) and inside the unsaturated zone of soil.

8.7.3 Expenditures

Expenditures for 'classical' BACTRAPs are in the range of 480-580 € per sample (for detailed price enquiries, please contact Isodetect GmbH; fischer@isodetect.de), assuming the availability of existing monitoring wells. Costs for DP BACTRAPs are higher, depending on the costs for the equipment. However, costs of DP BACTRAPs are much lower compared to drilling of conventional monitoring wells.

Specially trained staff is needed for operating the DP machinery and for carrying out the measurements and data analysis with the GC-MS or GC-C-IRMS systems.

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2.2 Characterisation of microbial activity in the framework of natural attenuation without groundwater monitoring wells?: a new Direct-Push probe

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Abstract:
The article describes the mechanical construction and testing of the newly developed DP-BACTRAP probe. Moreover, laboratory experiments indicated that the probe is applicable under vadose zone conditions down to a soil water holding capacity of 20% with the addition of silica gel to the activated carbon. Two field tests at the Zeitz (Germany) site revealed better performance of the DP-BACTRAPs compared to 'classical' BACTRAPs and better comparability of the microbial community colonising DP-BACTRAPs to the surrounding soil. Especially, the second experiment showed that the potential for natural attenuation at this site had diminished, due to an ongoing contaminant source remediation action, that led to the mobilisation of residual BTEX being trapped in the vadose zone prior to the start of the remediation action.[2]

Author contribution: 85%
The DP-BACTRAP probe and the experiments were designed by me and later discussed with Matthias Kästner and Anja Miltner. The laboratory experiments were carried out by Vinicio A. Melo under my supervision, and I carried out the field experiments and subsequent extraction and analysis of biomarkers by myself. The manuscript was written by me. Anja Miltner and Matthias Kästner assisted with the finalisation of the manuscript.

(signature of supervisor)

[2] Supporting information is provided in the appendix p. XL

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NEW APPROACHES FOR LOW-INVASIVE CONTAMINATED SITE CHARACTERIZATION, MONITORING AND MODELLING

Characterisation of microbial activity in the framework of natural attenuation without groundwater monitoring wells?: a new Direct-Push probe

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Abstract At many contaminated field sites in Europe, monitored natural attenuation is a feasible site remediation option. Natural attenuation includes several processes but only the microbial degradation leads to real contaminant removal and very few methods are accepted by the authorities providing real evidence of microbial contaminant degradation activity. One of those methods is the recently developed in situ microcosm approach (BACTRAP®). These in situ microcosms consist of perforated stainless steel cages or PTFE tubes filled with an activated carbon matrix that is amended with ^{13}C-labelled contaminants; the microcosms are then exposed within groundwater monitoring wells. Based on this approach, natural attenuation was accepted by authorities as a site remediation option for the BTEX-polluted site Zeitz in Germany. Currently, the in situ microcosms are restricted to the use inside groundwater monitoring wells at the level of the aquifer. The (classical) system therefore is only applicable on field sites with a network of monitoring wells, and only microbial activity inside the monitoring wells at the level of the aquifer can be assessed. In order to overcome these limitations, a new Direct-Push BACTRAP probe was developed on the basis of the Geoprobe® equipment. With respect to the mechanical boundary conditions of the DP technique, these new probes were constructed in a rugged and segmented manner and are adaptable to various sampling concepts. With this new probe, the approach can be extended to field sites without existing monitoring wells, and microbial activity was demonstrated to be measureable even under very dry conditions inside the vadose zone above the aquifer. In a field test, classical and Direct-Push BACTRAPs were applied in the BTEX-contaminated aquifer at the ModelPROBE reference site Zeitz (Germany). Both types of BACTRAPs were incubated in the centre and at the fringe of the BTEX plume. Analysis of phospholipid fatty acid (PLFA) patterns showed that the bacterial communities on DP-BACTRAPs were more similar to the soil than those found on classical BACTRAPs. During microbial degradation of the ^{13}C-labelled substrate on the carrier material of the microcosms, the label was only slightly incorporated into bacterial biomass, as determined by PLFA analysis. This provides clear indication for decreased in situ natural attenuation potential in comparison to earlier sampling campaigns, which is presumably caused by a large-scale source remediation measure in the meantime. In conclusion, Direct-Push-based BACTRAPs offer a promising way to monitor natural attenuation or remediation success at field sites which are currently inaccessible by the technique due to the lack of monitoring wells or due to a main contamination present within the vadose zone.

Keywords Natural attenuation · Microbial degradation activity · Vadose zone · Direct-Push · BACTRAPs · In situ microcosms · Toluene · Aquifer · Groundwater monitoring well

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Introduction

After centuries of industrial production, soil and groundwater contamination with hazardous organic substances is a large-scale and widespread problem of global importance.

Due to the large number of contaminated sites along with large volumes of affected soil and contaminant per site, conventional technologies such as pump-and-treat or soil excavation are not feasible everywhere. Nevertheless, possible risks for human health and the environment have to be minimized. Under these circumstances, monitored natural attenuation (USEPA 1999, UK-EA 2000, SYKE 2006) is an interesting remediation option for many sites, in particular when considering remediation effort and success (Bombach et al. 2010b). However, while the natural attenuation term includes processes like advection, dispersion, volatilisation and microbial degradation (Wiedemeier et al. 1999), only the microbial degradation leads to contaminant removal while the former processes just lead to lower overall contaminant concentrations, but higher affected volume of soil and groundwater. Until recently, a widespread application of this method was hampered by the small number of methods having been accepted as providing evidence of ongoing natural attenuation by the authorities, and by its uncertain legal status in some countries (Declercq et al. 2012). One of the governmentally accepted methods for monitoring microbial activity is the application of the recently developed in situ microcosms (BACTRAPs®; Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010a), which have been successfully applied in aquifers and are commercially available from various providers.

BACTRAPs consist of small perforated stainless steel cages or PTFE tubes filled with a carrier matrix material, e.g. granulated activated carbon (AC) or Biosep®-beads (Peacock et al. 2004). Subsequently, the microcosms are autoclaved and loaded with ^{13}C- or ^{15}N-labelled contaminant. Following an exposure within groundwater monitoring wells at the level of the aquifer for a few weeks, biomarkers are extracted from the microcosms; labelled biomarkers, such as phospholipid fatty acids (PLFA), provide evidence of ongoing natural attenuation at the site. In principle the method is applicable to a wide range of organic contaminants, that can be used as an electron donor by microbes, and has been proven to be successful for BTEX (Geyer et al. 2005, Stelzer et al. 2006, Bombach et al. 2010a, b), MCB (Nijenhuis et al. 2007, Stelzer et al. 2009) and MTBE (Busch-Harris et al. 2008). However, the method currently excludes monitoring the reductive dechlorination of contaminants such as chlorinated ethenes and ethanes.

Despite the success of the method, it requires exposure of the microcosms submersed in water. Its applicability thus remains limited to monitoring microbial activity inside groundwater monitoring wells at the level of the aquifer. Within these wells, the microbial community may be very distinct from the microbial community in the surrounding soil. One reason for this can be free convection inside the boreholes (Berthold & Boerner 2008) and a related transport of oxygen to formerly anoxic layers, which may result in the growth of aerobic microbes or a recharge of oxidised terminal electron acceptors such as iron(III), sulphate and nitrate. Furthermore, the microbial community inside the monitoring wells may overrepresent planktonic microorganisms in comparison to sessile microorganisms in the soil surrounding the well, and thus the water within the wells cannot be assumed to be representative of aquifer water without pumping (Chapelle 1993).

In order to overcome some of these limitations of BACTRAPs, the integration of this method into the Direct-Push (DP) technique was developed. The Direct-Push approach (Loxham et al. 1988, van Ree & Olie 1993, USEPA 2005) generally includes pushing probes for measuring physical and chemical soil parameters into soil by means of static pressure, hammering, or vibration. This technique allows fast and economical sampling of both water-saturated and water-unsaturated zones of soil along with DP-based logging methods, such as, for example, the membrane interface probe (MIP; Christy 1998, van Ree et al. 1999) or a probe applying laser-induced fluorescence (LIF; Grundl et al. 2003). MIP detects depth profiles of volatile organic compounds in soil via thermo-desorption and subsequent measurement with gas chromatography and photo ionization detectors or mobile mass spectrometers (Griffin & Watson 2002). The LIF probe detects polycyclic aromatic hydrocarbons by means of induced fluorescence from a pulsed excimer laser and evaluation of the spectra from a spectrometer (Grundl et al. 2003). Besides the speed of sampling and the cost efficiency, DP has the advantage of being a low-invasive sampling technology, meaning that sampling campaigns cause no or only little damage to the field sites and, in contrast to methods based on conventional drilling, no contaminated soil or water reaches the surface. However, although the method has many advantages, it remains limited to sampling on field sites with unconsolidated rock materials, and it typically reaches depths of 25–35 m, with a maximum of 50–60 m (Zschornack & Leven 2012). Combining the in situ microcosm approach with the DP technique broadens the applicability of BACTRAPs significantly. In particular, this combination allows monitoring sites without groundwater monitoring wells as well as the microbial activity in the vadose zone. Based on the direct contact of the soil to the microcosm, we also expect that the microbial community detected by the DP-BACTRAP approach reflects the actual soil microflora better than the microflora in a groundwater well.

Technical requirements for a successful DP-BACTRAP probe are different from those for 'classical' BACTRAPs. Considering the fact that this new type of BACTRAP probe is directly pushed into the soil by man power or by Geoprobe® machines, which can apply up to 16kN of push-and-pull force, a new rugged design was needed. Nevertheless, the probe must provide contact with the soil water in order to allow microbial colonisation (Madigan et al. 2012), which is even more critical under dry conditions in the vadose zone than under aquifer conditions. Moreover, sampling with this probe should be faster than

conventional sampling campaigns, which are based on drilling of groundwater monitoring wells, subsequent pumping, and sampling campaigns.

Therefore, the purpose of this study was: (1) to construct a Direct-Push probe allowing the introduction of BACTRAPs into soil at any site or depth, (2) to evaluate the performance of this new DP probe in terms of hydraulic equilibration with the soil surrounding the probe, (3) to compare it to 'classical' BACTRAPs in a field application test, (4) to discuss advantages and disadvantages with respect to some other commonly used site characterisation methods, and (5) to discuss the integration of this new approach into an innovative, low-invasive, DP-based site investigation approach (USEPA 2005, Kästner & Cassiani 2009).

Materials and methods

Field site

The field tests were carried out in direct proximity to the site of a former hydrogenation plant in Zeitz (Saxony-Anhalt, Germany). The site features quaternary sands and gravels as well as tertiary sands and lignite (detailed description: Schirmer et al. 2006) forming two aquifers, which are separated by a discontinuous tertiary silt and clay layer (Vieth et al. 2005). Previous studies (Gödeke et al. 2004, Vieth et al. 2005) revealed that the site is contaminated by BTEX compounds, with benzene and toluene being the most prominent contaminants. A contaminant plume starting from the source area has formed in the direction of the groundwater flow (northwards). Biodegradation of benzene and toluene inside the aquifers was demonstrated by compound-specific stable-isotope fractionation analysis (Fischer et al. 2004, 2007, 2009, Vieth et al. 2005) and BACTRAPs (Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010a), leading to the acceptance of monitored natural attenuation as a remediation strategy for the plume area of this site by the state of Saxony-Anhalt, Germany (LAF 2012).

In 2010 and 2011, the source area of the BTEX contamination was remediated by large-calibre borehole extraction of the contaminated soil down to 12 m below the surface (upper aquifer) and refilling with clean gravel material. According to the LAF (2012), the remediation action comprised about 3,600 m^2 of the source area, and 60,000 t of contaminated soil was removed.

Mechanical testing of DP-BACTRAP prototypes

In order to ensure functionality of the probes under aquifer- and vadose-zone conditions, probes were tested for mechanical stability and reusability. Mechanical stability and contact to the soil was tested under field conditions at the ModelPROBE reference site in Zeitz by use of several DP-BACTRAP prototypes, a Geoprobe 6610DT machine and 15 m of 1.25″ tubing (Geoprobe, Salina, KS, USA). The tests included pushing 1.25″ pushing rods equipped with the probes down to a depth of about 12 m by applying the full static pressure as well as the hammer system of the DP device, after which the probes were retracted by applying the full pull force of the DP device and the mechanical condition of the probe was evaluated. Although the probes could be pushed and retracted without damage, it was decided to perform a pre-sounding with pushing rods equipped with a solid tip down to a shallower depth than the depth of incubation for later field tests. This procedure prevents (a) microbial cross contamination, (b) failure to reach the target depth due to underground obstacles, such as large stones and boulders, and (c) loss of the probes due to mechanical failure of the pushing rods. The final DP-BACTRAP® probe can be purchased from Terra~direct GbR, Haigerloch, Germany.

Hydraulic characterisation of DP-BACTRAP prototypes

In order to ensure soil-microcosm contact, and thus sampling of the indigenous microflora, we tested the system under various soil moisture conditions. For establishing contact to the capillary fringe in the vadose zone, several properties were tested: firstly, the water conductivity of steel material with various mesh sizes (solid stainless steel material with 0.5 mm holes and 5 holes/cm^2, and a four-ply stainless steel meshwork with 0.25 mm pores) over time was tested by applying a fluorescent dye (uranine, Carl Roth, Karlsruhe, Germany) which was detected by means of 366 nm UV-light (Desaga HP-UVIS, Nümbrecht, Germany) (for a sketch of the measurement principle see Fig. S1). During this experiment, the flux of uranine dye from the bottom of metal sheets to the filter paper on the top was measured as a percentage of the total wetted area of filter paper with respect to time. The physical parameters influencing the results of this experiment are firstly the wettability of the stainless steel surface, that enables the uranine dye to reaching the material and wetting its surface, and secondly the abundance and diameter of the pores of the material, that account for conduction of the dye from the backside of the material to the front, where the filter paper is mounted. The results of this experiment therefore represent a relative measure for the wettability of the probe system along with the water conductivity through its pores.

After a reasonable compromise between stability and hydraulic conductivity was found, the probes were subjected to further testing for soil/water contact. Probes were filled with mixtures of silica gel (grain size 1–3 mm, Carl Roth, Karlsruhe, Germany) and activated carbon beads (AC; AUF 540, Adako Aktivkohlen GmbH, Düsseldorf, Germany) at the

following ratios: 100 % AC, 75 % AC/25 % silica gel and 50 % AC/50 % silica gel. The probes were immersed into a soil of known composition (for particle size distribution, see Fig. S2). The water content of the soil was adjusted to between 20 and 80 % of the water holding capacity (WHC), and filled into a closed container (volume 10 L) in order to prevent evaporation. For evaluating the gravimetric water uptake into the probes, pre-weighed probes were re-weighed after given periods of time (2, 4, 18, 25, 50 h) and the weight difference was attributed to water uptake by the silica gel–AC mixture.

Preparation of the DP-microcosms

Several preparation steps in the laboratory were necessary before applying 'classical' BACTRAPs and DP-BACTRAPs in the field.

In order to rid the granulated AC (AUF 540, Adako GmbH, Düsseldorf, Germany) of any biogenic organic carbon residues, it was heated to 310 °C for at least 4 h. For producing 'classical' BACTRAPs, 1 g of carrier material each was filled into a perforated PTFE tube (Peacock et al. 2004, Geyer et al. 2005). The same amount of AC beads was weighed into small vials for the DP-BACTRAPs. The beads were then autoclaved at 121 °C for rehydration and sterilization. After air-drying, the probes were distributed on three separate glass-desiccators for blank, ^{12}C and ^{13}C samples. Following exchange of air inside the desiccators against nitrogen gas (N_2), for ^{12}C- and ^{13}C-BACTRAPs a small vial containing ^{12}C-toluene (Sigma Aldrich, Taufkirchen, Germany) or ^{13}C-α-toluene (Sigma Aldrich, Taufkirchen, Germany), respectively, was put inside the desiccators and a 50 mbar vacuum was applied. For blank BACTRAPs, the same procedure was applied, but no vial was added to the respective desiccator. Subsequent to complete evaporation of the toluene and its sorption to the AC beads, 'classical' BACTRAPs were put into sterile anaerobic water, whereas the beads from the vials were filled into DP-BACTRAPs and put into sterile anaerobic water just prior to transport to the field.

Field work

Two wells in the plume area of the field site were chosen for comparing the performance of 'classical' and DP-BACTRAPs. While well SAF Zz57-03 was located more towards the centre of the plume, well SAF Zz52-03 (see Fig. 1) was chosen to represent the fringe of the BTEX plume according to previous investigations (Geyer et al. 2005, Kästner et al. 2006).

Triplicates of both types of probes were kept in the sterile anaerobic water until just prior to exposure in the upper aquifer at 9.3 m depth below ground surface. 'Classical' BACTRAPs were fixed on nylon ropes equipped with stainless steel weights and deployed directly in the groundwater monitoring wells. At a maximum distance of 1 m from the wells, a pre-sounding down to 8 m depth was performed using a Direct-Push machine (6610DT, Geoprobe, Salina, KS, USA) and 1.25″ pushing rods equipped with a solid tip. After retraction of those, the DP-BACTRAP probe was attached to the 1.25″ pushing rods instead of the solid tip, and pushed further down the open channel to a final depth of about 9.3 m.

Following a 6-week exposure inside the aquifer, the probes were retracted and continuous soil samples from 8.5 to 9.5 m depth were taken using the Geoprobe machine and a soil sampling system (DT22, Geoprobe, Salina, KS, USA). All samples were transported to the laboratory in sterile bags within cooled containers (~4 °C).

Fatty acid analysis

Upon arrival at the laboratory, samples were stored at −20 °C until further analysis. Within the next 48 h, the samples were further processed as follows. The AC beads were removed from the probes and transferred into acetone-rinsed vials. The phospholipids, as these are considered to represent the living biomass (Green & Scow 2000, Pinkart et al. 2002, Kaur et al. 2005), were extracted according to a modified the Bligh and Dyer (1959) approach as described by Schurig et al. (2012b). First, the samples were extracted with 5 ml dichloromethane overnight on a horizontal shaker at 30 °C. The dichloromethane was then transferred to new vials and evaporated under a gentle stream of N_2. After the solvent was removed, the samples were stored at −20 °C. The vials containing the activated carbon beads were then subjected to another extraction step with 5 ml chloroform, 3 ml phosphate buffer and 10 ml methanol overnight. Finally, 5 ml Micropure-water and 5 ml chloroform were added in order to achieve phase separation. The chloroform phase containing the lipids was transferred into the vials containing the dry dichloromethane extracts; the combined extracts were dried under a N_2 stream. When completely dry, the samples were stored at −20 °C until they were dissolved in 200 μl of chloroform and subjected to chromatography on preconditioned silica gel columns (transfer of silica gel with 0.02 M ammonium acetate in methanol into glass columns and subsequent conditioning with 5 ml acetone and 5 ml chloroform). Neutral lipids were eluted from the silica gel column by adding 5 ml of chloroform, followed by the elution of glyco-lipids with 5 ml of acetone and finally the elution of phospholipids with 10 ml of methanol. After drying the phospholipids with N_2, they were subjected to a derivatisation with tri-methyl-chlorosilane in methanol and the resulting fatty acid methyl esters (FAMEs) were subsequently dried by means of N_2.

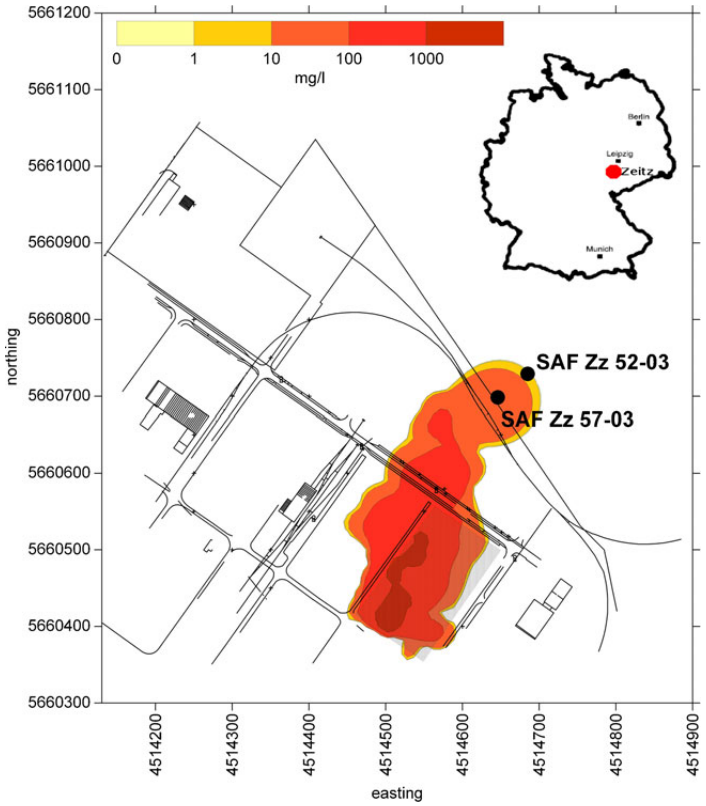

Fig. 1 Map of the Zeitz site: Remediated BTEX source area (LAF 2012) indicated by the hatched area; BTEX plume extension inside the upper aquifer before remediation action indicated by *coloured area*, with corresponding concentrations of BTEX given in milligram per liter. BACTRAPs were exposed inside wells SAF Zz 57-03 (represents centre of plume) and SAF Zz 52-03 (represents fringe of plume). DP-BACTRAPs were exposed at 1 m distance from the wells at the same depth. Soil samples were taken at a similar distance from the wells (figure modified from Gödeke et al. (2004) and Fischer et al. (2004))

After adding 10 μg of 21:0 FAME in hexane as an internal standard, FAMEs only from phospholipids were quantified by means of gas chromatography–mass spectrometry (GC-MS; Agilent GC 7890A and MS 5975C, Boeblingen, Germany) with the following parameters: MS source at 230 °C, MS quadrupole at 150 °C, injection in splitless mode at 280 °C, HP-5MS column (30 m × 0.25 mm × 0.25 μm), initial column oven temperature 50 °C for 1 min, then 4 °C min^{-1} to 250 °C, 20 °C min^{-1} to 300 °C for 5 min and finally a 10 min post-run at 300 °C. The FAMEs were identified by means of their retention times and a library of previously determined mass spectra of known fatty acids. Each FAME was quantified by comparison with the internal standard.

Determination of isotopic composition of PLFA

Evidence for natural attenuation at a site requires the detection of labelled FAMEs. The use of gas chromatography - combustion interface - isotope ratio mass spectrometry (GC-C-IRMS) allows very sensitive detection of isotopic enrichment. To this end, the previously quantified samples were analysed under the following conditions: GC: Agilent 6890 N (Boeblingen, Germany), combustion interface: Thermo-Fischer GC-Combustion 3 interface (Schwerte, Germany), IRMS: Thermo-Fischer MAT253 (Schwerte, Germany), parameters: injection in splitless mode at 250 °C, BPX-5 column (50 m × 0.32 m × 0.5 μm, SGE, Darmstadt, Germany) at 70 °C for 1 min, then 20 °C min^{-1} to 130 °C, 5 min hold, then 2 °C min^{-1} to 150 °C, 5 min hold, then 2 °C min^{-1} to 165 °C, hold 5 min, 2 °C min^{-1} to 230 °C, then 20 °C min^{-1} to 300 °C, 5 min hold.

This method is appropriate for detecting enrichments in a range from natural abundances to approximately 5 at%. However, relatively large amounts of sample are needed for correct determination of the isotopic enrichments of individual FAMEs. The isotopic enrichment of individual FAMEs was corrected for the introduction of one carbon atom with the methyl group during derivatisation, and is given in the internationally accepted δ notation:

$$\delta^{13}C = \left[\frac{\left(\frac{^{13}C_{sample}}{^{12}C_{sample}}\right)}{\left(\frac{^{13}C_{PDB}}{^{12}C_{PDB}}\right)} - 1 \right] \cdot 1,000 \quad (\text{in } ‰)$$

providing the determination of isotopic enrichment related to the Vienna Pee-Dee Belemnite (PDB) standard for carbon.

Results

The integration of the BACTRAP approach into the DP technique required the adaptation of both the probe design and the sampling concept. We therefore designed the probe to be reusable and generally applicable with various types of Direct-Push systems and in a wide variety of soil or sediment environments. The new DP probe is presented here together with the characterisation of its performance under water-unsaturated conditions in soils, and with data from a field test comparing the DP-BACTRAP system to the established classical BACTRAPs.

The construction of DP-BACTRAPs—evaluation of mechanical stability

Several prototypes were developed and vigorously tested at the Zeitz field site, which provides an ideal test site for ensuring mechanical stability and adaptability of the DP-BACTRAP probes because of the local geology and the high gravel content of the soils.

As a result of the above-mentioned considerations, the DP-BACTRAP probe was developed as a modular construction, whereby all parts were designed to be easily replaceable if worn out (see Fig. 2). This design has the advantage of allowing the probe to be adapted to various sampling concepts, e.g., with respect to the number of samples or replicates.

For a complete sampling campaign including unamended (blank), ^{12}C and ^{13}C microcosms, that are needed for the analysis of the isotopic enrichment, three segments (Fig. 2 part 5) are connected to each other by the connecting nuts and thereby replace nine conventional BACTRAPs with one Direct-Push probing. However, the modular design of the probe also keeps the system highly flexible and allows the system to be adapted for various experimental tasks, because the probe is applicable with various numbers of segments and with different Direct-Push systems by the use of individual segments and adaptors (Fig. 2 part 1).

In order to ensure complete recovery of the probes after the exposure time, the probe diameter was designed to be smaller than that of the pushing rods. The advantages of this design are better contact to soil during installation of the probe and very low mechanical shear forces during retraction of the probe.

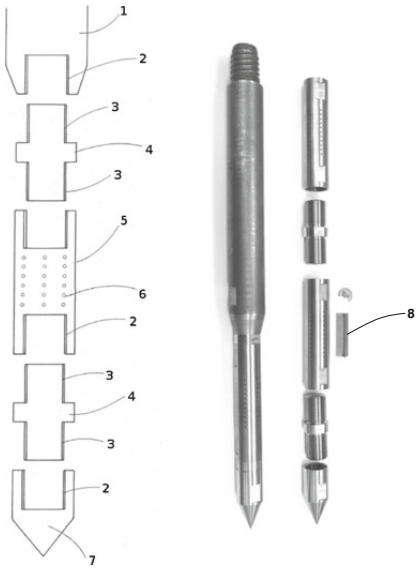

Fig. 2 DP-BACTRAP probe with following components: *1* adaptor to Geoprobe-tubing, *2* compatible thread to connection nut (*4*), *3+4* connection nut with threads on both sides and planes for spanner, *5* segment to be filled with 3×1 g activated carbon bead material (three replicates) and holes for ensuring contact to soil water (*6*), *7* replaceable solid tip, *8* segmentation for allowing three replicates inside (*5*) (figure modified from Schurig et al. 2012a)

Application of DP-BACTRAPs inside the vadose zone

In order to further broaden the applicability of the DP-BACTRAP system, the application under unsaturated moisture conditions in the vadose zone was developed. For ensuring microbial colonisation within the vadose zone, a hydraulic contact between the activated carbon beads within the DP-BACTRAPs and the soil pore water is required.

In order to evaluate whether fine stainless steel meshwork or a solid material with fewer, larger pores (pore size 0.8 mm) provides better water conductivity, a test based upon applying a fluorescent dye was performed. Figure 3 shows that the fluorescent dye passed through the solid material with few large pores in 3 minutes, whereas it needed 20 minutes to pass through the meshwork material. Therefore, it was decided to produce the probe with fewer, larger pores in the outer mantle, instead of using the less conductive and less robust meshwork material.

After choosing the appropriate material by taking both conductivity and stability into account, the application range of the probe inside the vadose zone was tested by exposing probes filled with AC into soils with various water contents and analysing the water uptake by the probes.

Figure 4 shows that the contact of the soil water to the AC is established under all tested soil moisture conditions

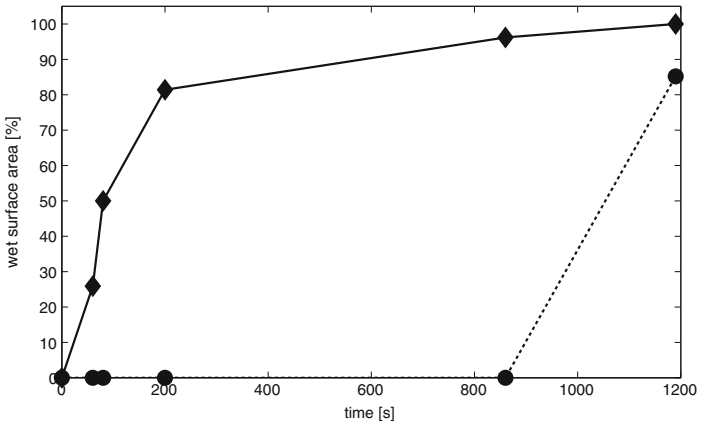

Fig. 3 Evaluation of meshwork (*filled circle*) against larger-pore-size solid steel material (*filled diamond*) with respect to water conductivity over time. For a detailed sketch of the measurement principle see Figure S1

(see Figure S2 for information on the texture of the tested soil). For assessment of the wetting of the metal material and conduction of the water into the probes needed to facilitate microbial colonisation, logistic fitting functions were applied in order to model the sorption behaviour of the water onto the AC. The data show that equilibrium is reached after less than two days of incubation under 80 to 40 % soil moisture conditions. For low soil moisture conditions (20 % of WHC), extended time was required in order to reach equilibrium (Fig. 4). In comparison to applying the probes under water-saturated conditions inside an aquifer, the incubation time of the DP probes within the vadose zone has to be extended due to the time-lag for equilibrating with soil moisture and for ensuring sufficient microbial colonisation of the AC.

In order to shorten this time delay, silica gel was added to the AC material for enhancing the water uptake. Figure 5 shows the water uptake of probes filled with various mixtures of silica gel and AC granules at various soil moisture contents (100 % AC, 75%AC/25 % silica gel, and 50 % AC/50 % silica gel, respectively).

The differences in time for reaching certain water uptake levels were calculated in order to ascertain the best-performing mixtures of AC and silica gel. The differences for 80 and 40 % are only in the range of a maximal decrease of lag time of about 5 h (~5 %). However, when working with drier soil conditions, such as 20 % WHC, the addition of 50 % silica gel decreases the lag time by about 12 h, which is 25 % better performance.

Field test at the Zeitz site

For evaluating the performance of DP-BACTRAPs in comparison to "classical" BACTRAPs, two separate field tests were carried out in the aquifer at the well-investigated

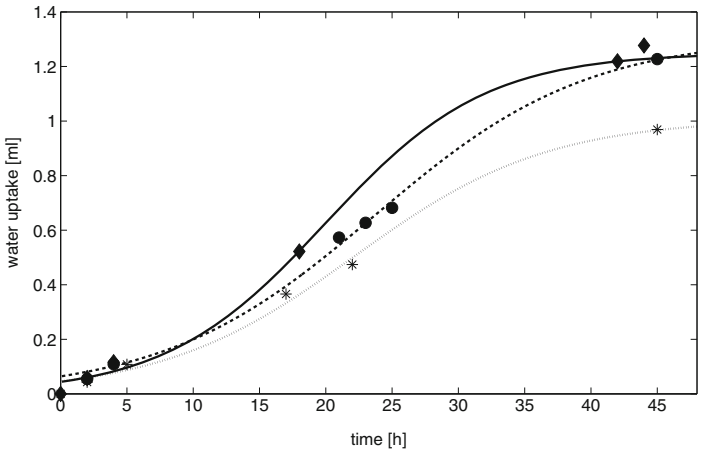

Fig. 4 Water uptake into DP-BACTRAPs over time under 80 % (*filled diamond*), 40 % (*filled circle*) and 20 % (*asterisk*) of soil water holding capacity (for details see "Materials and methods" section); *error bars* representing standard deviation are *hidden behind the symbols*. The *curves* represent fitted logistic functions: water uptake (80 %)=$1.2503/1+e^{-0.16581\times(t-19.9714)}$ (*solid line*) r^2=0.99; water uptake (40 %)=$1.3097/1+e^{-0.12515\times(t-23.7131)}$ (*dashed line*) r^2=0.99; water uptake (20 %)=$1.0083/1+e^{-0.13736\times(t-22.1553)}$ (*dotted line*) r^2=0.86

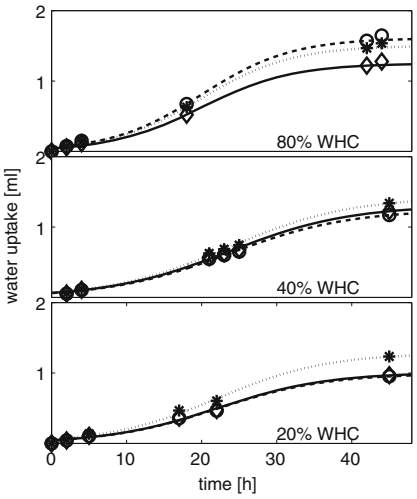

Fig. 5 The effect of addition of 0 % (*open diamond*), 25 % (*open circle*) or 50 % (*asterisk*) silica gel to the activated carbon (AC) on water uptake into DP-BACTRAPs under 80 % (*upper panel*), 40 % (*middle panel*), or 20 % WHC of the soil (*lower panel*), respectively (*error bars* representing standard deviation are *smaller than symbols*). The curves represent logistic fits: 80 %: (*solid line*) fitting curve for 100 % AC (see Fig. 4); water uptake (25 % silica gel)=$1.6115/1+e^{-0.16564\times(t-19.9701)}$ (*dashed line*) r^2=0.99; water uptake (50 % silica gel)=$1.5047/1+e^{-0.16577\times(t-19.9582)}$ (*dotted line*) r^2=0.99; 40 %:(*solid line*) fitting curve for 100 % AC (see Fig. 4); water uptake (25 % silica gel)=$1.2494/1+e^{-0.12533\times(t-23.6983)}$ (*dashed line*) r^2=0.99; water uptake (50 % silica gel)=$1.4292/1+e^{-0.12516\times(t-23.7108)}$ (*dotted line*) r^2=0.99, 20 %: (*dashed line*) fitting curve for 100 % AC (see Fig. 4); water uptake (25 % silica gel)=$0.99274/1+e^{-0.13738\times(t-22.173)}$ (*dashed line*) r^2=0.86; water uptake (50 % silica gel)=$1.2849/1-e^{-0.13742\times(t-22.1499)}$ (*dotted line*) r^2=0.86

BTEX-contaminated site Zeitz. In order to test whether the systems reflect the soil-indigenous microbial communities, soil samples were also directly taken from the depth of incubation inside the aquifer. The summarized concentrations of PLFA biomarkers in classical BACTRAPs, DP-BACTRAPs, and soil before and after the remediation measure are presented in Fig. 6. After the remediation, no significant differences of the summarized PLFA concentrations were observed between both BACTRAP systems and the soil. However, before the remediation action, higher concentrations of PLFA biomarkers were found in DP-BACTRAPs in comparison to BACTRAPs and soil samples; moreover, the concentrations of biomarkers in all samples were higher before than after the remediation action. Furthermore, concentrations of biomarkers were always higher in samples from inside the contaminant plume, as compared to samples from the fringe of the contaminant plume prior to the remediation action.

Detailed investigation of the concentration of individual PLFA (Fig. 7) reveals higher similarity of the PLFA spectra in soil and DP-BACTRAPs in comparison to conventional BACTRAPs. Therefore, the colonising microflora in the DP-BACTRAPs reflects the soil microflora more closely than conventional BACTRAPs.

Due to the lower concentration of biomarkers from samples from the fringe of the contaminant plume, it was not possible to obtain measurements of the isotopic enrichment of individual PLFA from these samples. Only a few individual PLFA in samples from the centre of the contaminant plume had concentrations above the detection limit of the GC-C-IRMS system. The isotopic ratios of the measureable PLFA are only slightly above the natural abundance of fatty acids stemming from toluene-degrading bacteria (about $-28.9\pm2.8\delta‰_{vs.VPDB}$, Pelz et al. (2001)), with values of −15.4 and −17.7 $\delta‰_{vs.VPDB}$ for 15:0 PLFA from BACTRAPs and DP-BACTRAPs, respectively, and −26.2 $\delta‰_{vs.VPDB}$ for 16:0 PLFA from both types of microcosms amended with ^{13}C toluene.

As mentioned above, the source area of the Zeitz site was remediated by large-calibre borehole extraction and refilling with clean gravel. This presumably led to a large-scale mobilisation of BTEX formerly trapped inside the vadose zone into the upper aquifer; as a result, the microbial community, including the toluene degraders, inside the sampled aquifer exhibited decreasing activities, and thus decreasing amounts of biomass with lower isotopic enrichment were detected consistently on DP-BACTRAPs and BACTRAPs. The results from both DP- and conventional BACTRAPs similarly reflect this situation before and after the remediation measure.

Discussion

In the present work, a new kind of DP probe is presented, that enables in situ measurements of soil microbial activity at field sites lacking groundwater monitoring wells in aquifers, but also, under certain conditions, in the vadose zone, both of which were previously not possible. In addition, the developed new measurement system allows for low-invasive sampling of soils contaminated with organic pollutants.

The construction of DP-BACTRAPs

A particular challenge for the construction of DP-BACTRAPs was to find a compromise between mechanical stability and hydraulic contact to the soil water surrounding the probe. Both goals were met by using a solid metal mantle with few, relatively large (0.8 mm) pores for the probe. This proved to be advantageous in terms of both mechanical stability and water conductivity. While pushing the probe into soil, soil enters the probe through these holes due to frictional forces and thereby comes in direct contact with the AC filling material. This leads to rapid colonisation of the AC with the indigenous microflora, whereby the rate

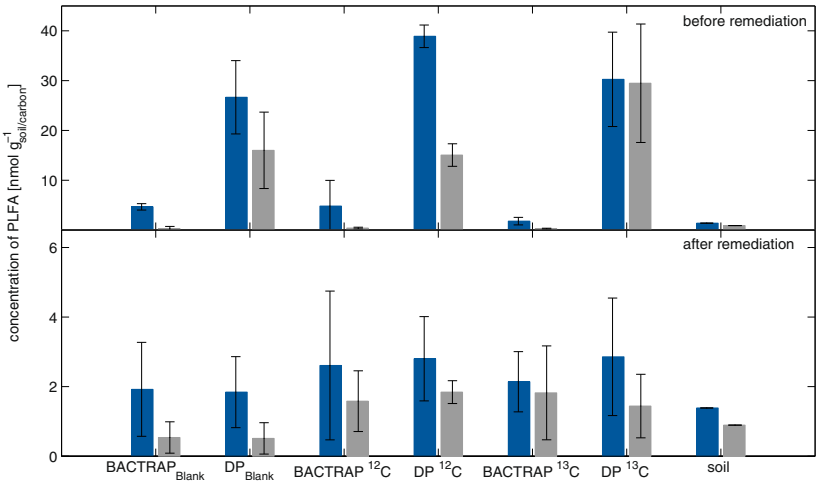

Fig. 6 Concentration of PLFA biomarkers inside DP-BACTRAPs, BACTRAPs and soil from the Zeitz site (9.3 m below ground surface) after 6 weeks incubation. *Blue bars* represent samples from inside the plume (well 57-03) and *grey samples* represent data from the fringe of the contaminant plume (well 52-03). *Error bars* represent standard deviation from triplicate extractions (except lower panel 52–03 DP_{blank}, where $n=2$, because one sample was selected as outlier by Grubbs-test $p=0.1$). *Upper panel* represents data from sampling campaign before the source remediation action and *lower panel* represents data from sampling campaign after the remediation action. Please note the different scales for the PLFA concentrations

of colonisation can be assumed to be limited by the growth of the microbes under anaerobic conditions rather than the rate of attachment (Robins & Switzenbaum 1990). However, under dry soil conditions microorganisms have to move actively towards the activated carbon surface (van Loosdrecht et al. 1990), and thereby a high degree of comparability between conditions inside the soil at the depth of incubation and the microcosms can be assumed. Contact of the AC material in the DP-BACTRAPs to the soil material or the capillary fringe is crucial under vadose conditions to enable microbial colonisation of the AC, which obviously limits the applicability of the method to soil materials with appropriate textures, i.e., the material needs substantial percentages of particles smaller than 0.8 mm (diameter of the holes in DP-BACTRAP probe). The applicability of the method was not yet tested for coarser materials such as gravel packs in the vadose zone. In terms of contaminant degradation inside the vadose zone of the soil, Liang et al. (2009) have shown that AC is rapidly colonised in soil and that the overall contaminant degradation potential was increased by the AC amendment. Similar results are to be expected with the DP-BACTRAPs, as shown by the equilibration time of 2 days between soil water content and water content inside the probe. Simultaneously with connection to soil pore water, active colonisation of the AC by microorganisms is possible. Therefore, microbial colonisation of DP-BACTRAP probes inside the vadose zone should be very similar to the colonisation of free AC in soil, and thus

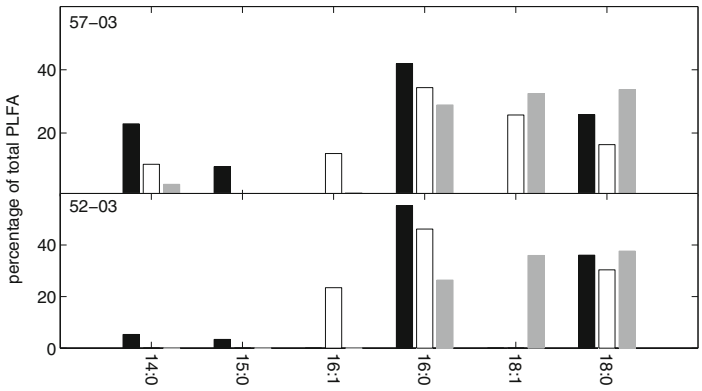

Fig. 7 Percentage of individual PLFA compared to the total amount of PLFA (see Fig. 6) of unamended samples extracted from, BACTRAPs, DP-BACTRAPs and soil: *black bars* BACTRAPs, *white bars* DP-BACTRAPs, *grey bars* soil. Samples from inside the contaminant plume (well 57-03) in the upper panel, samples from the fringe of the plume (well 52-03) in the lower panel. Data from experiment after the source remediation action

it can be expected that the probes are readily colonised with indigenous microbes within a short time frame, although colonisation within oxic aquifers might be faster, due to the water flow through the microcosms. However, when the time of exposition within the vadose zone is extended by about one week, this should ensure sufficient microbial colonisation of the AC.

The contamination at field sites usually consists of a mixture of contaminants (Rivett et al. 2011), where the behaviour of the mixture in terms of toxicity (Altenburger et al. 2004) and the potential for biodegradation may be very different in comparison to the evaluation of degradation of a single compound. In order to address this problem, the probe was designed to be applicable with a varying number of segments (Fig. 2(5)), thus allowing the degradation of more than one contaminant under in situ conditions to be evaluated simultaneously. However, for such experiments the vertical dimension of the contaminated zone has to be sufficiently large, since a complete probe with three segments is about 38 cm long and sampling of two compounds (five segments) will require about 75 cm of contaminated zone. In addition, the segmented design allows in principle the sampling of vertical profiles of microbial activity by mounting the required number of microcosm sets in preselected distances on the DP rods, though this has not been tested yet. In order to ensure sufficient height of contamination at the sampled spot, pre-investigations using MIP or LIF probes are highly recommended, because the distribution of contaminants at field sites cannot be regarded as uniform (Grundl et al. 2003). This can be assumed to be even more important when considering contaminants trapped inside the vadose zone, where effective transport processes such as advective flow with the groundwater may be negligible. In this case, distribution of contaminants via preferential flow paths along with sorption to soil constituents can be very important mechanisms (Rivett et al. 2011).

Comparison of DP-BACTRAPs with BACTRAPs in a field test

A field test at the BTEX-contaminated upper aquifer in Zeitz, Germany revealed that DP-BACTRAPs withstand the mechanical forces during installation and retraction and that the probes are readily colonised by indigenous microbes. Furthermore, a comparable performance in terms of quantity, abundance and isotopic enrichment of PLFA inside BACTRAPs, DP-BACTRAPs and soil was observed. Although some of the observed variability might arise from spatial variability (van Ree & Carlon 2003), this means that the DP-BACTRAPs are fully applicable for monitoring microbial contaminant degradation under in situ conditions within aquifers. This exemplifies that the need for groundwater monitoring wells for characterizing microbial activity at field sites can now be overcome by use of the DP technique.

In comparison to earlier investigations at this particular field site (Geyer et al. 2005, Kästner et al. 2006), we could show that the microbial contaminant degradation activity had diminished considerably after the source zone remediation measure. This is also reflected by the extremely low isotopic enrichment of 15:0 fatty acid of −15.4 for BACTRAPs and −17.7 $\delta‰_{vs.VPDB}$ for DP-BACTRAPs, in comparison to 7946 $\delta‰_{vs.VPDB}$ reported previously for the same site (Geyer et al. 2005, Kästner et al. 2006). Similarly, the lack of isotopic enrichment in the 16:0 fatty acid in contrast to earlier results (Bombach et al. 2010a) suggests no remaining degradation activity against labelled toluene. Analogous conclusions can be drawn from the decrease in quantity of PLFA from amended microcosms: values ranged from 1.44 nmol g^{-1}_{bead} (52-DP-^{13}C) to 2.85 nmol g^{-1}_{bead} (57-DP-^{13}C) in comparison with 8.48 to 8.87 nmol microcosm^{-1} from the Geyer et al. (2005) study and 5.6 to 8.4 nmol g^{-1}_{bead} as reported by Bombach et al. (2010a). The PLFA concentrations thus indicate a reduction of viable microbial cells by a factor of 3 in comparison to the conditions before the source remediation action.

These results correspond to decreasing natural isotopic ratios of the BTEX compounds inside the groundwater monitoring wells together with increasing concentrations of BTEX throughout the plume area (Schurig, unpublished data) in comparison to earlier studies (Fischer et al. 2004, Vieth et al. 2005, Kästner et al. 2006). Based on these data, along with the results of the current BACTRAP and DP-BACTRAP survey, we can conclude that after the remediation action (LAF 2012) BTEX was mobilised from around the source area and led to increasing BTEX concentrations and toxicity inside the plume area. This in turn led to diminished microbial activity, which is consistently shown by DP-BACTRAPs and BACTRAPs by the decreased amount and isotope enrichment of the PLFA, and an extension of the plume length along with increasing potential environmental risks.

The spectra of single fatty acids showed that DP-BACTRAPs provide better comparability to the conditions in the aquifer soil material, presumably because the probes provide direct contact to the soil. Conventional BACTRAPs exposed inside monitoring wells, however, may not be regarded as representative for the aquifer unless several well-volumes of water are pumped out of the well (Chapelle 1993); furthermore, the wells may form artificial paths introducing oxygen to formerly anoxic layers via heat-mediated convection (Berthold & Boerner 2008).

Comparison to other commonly used methods

Very few methods are commonly accepted for providing data related to ongoing microbial contaminant degradation in the

framework of natural attenuation at contaminated sites (for review see: Bombach et al. 2010b). When focussing on the analysis of isotopic ratios of contaminants, compound-specific isotope analysis (CSIA) of the residual contaminants in the groundwater is usually regarded as providing evidence on the contaminant degradation that already occurred at the field site (Meckenstock et al. 2004, Hunkeler et al. 2008). Increasingly heavier signatures prove natural attenuation, assuming that physical processes, such as volatilisation, do not lead to fractionation, and that the observed fractionation is solely due to microbial biodegradation activity (Bombach et al. 2010b, Braeckevelt et al. 2012). CSIA can be assumed to provide more representative results than the classical BACTRAP approach, because it is based upon representative water samples which are collected from a groundwater monitoring well after pumping (Chapelle 1993) and directly measures the contaminant degradation which has already occurred. This is in contrast to the classical BACTRAP approach, which measures degradation potentials in stagnant water within groundwater monitoring wells. This bias of the BACTRAP approach can now be overcome by the use of DP-BACTRAPs, which we showed to be more representative of the conditions inside the aquifer or by rigorous pumping during the exposure of conventional in situ microcosms within groundwater wells. Furthermore, the DP-BACTRAP method can be a valuable tool for the detection of changes in the microbial activity, as observed during the current field test. In this case, the application of the CSIA method consistently showed the decrease in microbial benzene degradation activity within the plume area after the remediation measure (Schurig, unpublished data).

However, since the microcosms still provide artificial conditions for microbial colonisation and activity with respect to degradation rates (Bombach et al. 2010b), the exact quantification of degradation rates will remain challenging. For example, AC has been shown to increase microbial degradation activity (Liang et al. 2009), although van Loosdrecht et al. (1990) reports that usually sessile microorganisms, not moving over large distances in soil, exhibit higher activities than planktonic microorganisms, which means that these opposing effects may result in averaged actual microbial contaminant degradation rates obtained by DP-BACTRAPs, thus providing an advantage in comparison to conventional BACTRAPs. Nevertheless, for a complete site assessment, CSIA and DP-BACTRAPs should be applied simultaneously in order to obtain information on past and present contaminant degradation.

While various methods were applied for analysing microbial activity within the aquifer zone either by direct detection of biomarkers (Tuxen et al. 2006) or by analysing proxies for microbial activity (Weiss & Cozzarelli 2008), like plume scale terminal electron acceptor balances (Thornton et al. 2001), in situ microcosms degradation studies of contaminants of interest (Bjerg et al. 1999) or injection and push–pull tests (Istok et al. 1997), these methods are usually not applicable in the vadose zone. Injection and push–pull tests exert higher disturbances to the aquifer in situ conditions than DP-BACTRAPs, due to the injection of high amounts of water together with tracers, nutrients and contaminants of interest. Regarding other methods for measuring vadose zone microbial activity, ex situ laboratory microcosm studies using water or soil samples are frequently used for determining microbial contaminant degradation activity (Strevett et al. 2002, Madsen 2005). Although they provide a valuable tool for elucidating degradation pathways and their associated isotopic fractionation factors, these studies are only able to provide information on potential rates of contaminant degradation rather than measurements of the actual in situ rates (Bombach et al. 2010b). In terms of quantification, some of these limitations can be overcome by the use of DP-BACTRAPs, which provide semi-quantitative rates of degradation under in situ conditions, even for samples from inside the vadose zone. DP-BACTRAPs cause fewer disturbances compared to taking soil cores for laboratory analysis (Strevett et al. 2002, Madsen 2005) or the insertion of previously prepared contaminant-spiked soils into the vadose zone (Höhener et al. 2006, Kaufmann et al. 2006).

Integration into an innovative site investigation approach

Since current site investigation approaches are regarded as too laborious, cost-intensive and not capable of supporting decisions at early project stages, new site investigation approaches were already requested in 2006 (USEPA 2006) and a modified approach was later proposed by Kästner and Cassiani (2009) and Kästner et al. (2012). The idea of this approach was to characterise a site with non- or low-invasive methods involving smart feedback loops, whereby non-invasive geophysical characterisation approaches (Cassiani et al. 2012) are followed by a low-invasive physical and chemical characterisation of the field site by means of DP (Dietrich & Leven 2005) or tree-core monitoring (Larsen et al. 2008). Only after this thorough characterisation should DP-wells or conventional monitoring wells be built for characterisation of the site by means of microbiological and ecotoxicological methods. This need for permanent installations can now be overcome with the help of the DP-BACTRAPs, whereby an identification of contaminants by means of MIP or LIF can be directly followed by the application of DP-BACTRAPs, which are providing complementary information regarding the microbial activity. Due to the limited sample preparation and analysis effort, the method provides clear results about the potential for

microbial contaminant degradation at a given site and thus allows a fast decision whether natural attenuation is a feasible option for this site. This approach may enhance cost- and time-efficiency, and moreover should provide better results in terms of comparability to true field conditions. With this new technique, microbiological methods within the ModelPROBE-approach (Kästner & Cassiani 2009, Kästner et al. 2012) can now also be applied to the vadose zone, where they were previously impractical.

Conclusions

A new probing system for monitoring microbial contaminant degradation activity in the framework of natural attenuation under in situ conditions was developed, which is applicable at sites without groundwater monitoring wells and was tested in the laboratory to demonstrate applicability also inside the vadose zone above aquifers as long as they are accessible by DP techniques and are not exclusively composed of materials with grain sizes larger than 0.8 mm. As a result of the off-road mobility of the DP-machinery, the probes can be applied at most field sites and the need to wait for drilling of an extensive network of groundwater monitoring wells can be overcome. The probes have been designed in a rugged manner, so that sampling is possible even under very harsh soil conditions featuring gravel and stones.

We have successfully shown that DP-BACTRAPs are applicable in non-water-saturated soils with water contents down to 20 % of the maximum WHC in a laboratory experiment. The efficiency of the contact to the soil water can additionally be increased for very dry soil by the addition of silica gel to the probes. Still, a field test with DP-BACTRAPs in the vadose zone should be done in the future to verify these results and to adapt the method to a wider range of contaminants, such as hydrophobic substances forming LNAPLs. A field experiment in an aquifer, however, revealed rapid and close contact with the soil, whereby the microbial community inside DP-BACTRAPs is better comparable to the indigenous community of the soil than is that of 'classical' BACTRAPs, which are exposed inside groundwater monitoring wells with limited representativeness.

A field test of DP-BACTRAPs and conventional submersed microcosms revealed comparable performance of both systems for the Zeitz field site with respect to quantity, distribution and isotopic enrichment of PLFA biomarkers. The presented field test proves that at least semi-quantitative conclusions regarding the activity of the indigenous microflora can be drawn by applying the general BACTRAP approach and comparing the results of the current study with those from earlier studies at the same field site (Geyer et al. 2005).

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2.3 Hexadecane and pristane degradation potential affected by varying redox conditions at the level of the aquifer – Evidence from sediment incubations and in-situ microcosms. *submitted*

Schurig, C., Miltner, A., Kaestner, M., 2013. Hexadecane and pristane degradation potential affected by varying redox conditions at the level of the aquifer – Evidence from sediment incubations and in-situ microcosms.
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Abstract:
The manuscript describes an experiment, which was carried out with soil material being obtained from the Trecate (Italy) field site, that was affected by an oil well blow out in 1994. Although, the top soil is presently being used for agriculture, the subsoil remains contaminated with LNAPLs floating on top of the groundwater. Sampling concentrated on soil samples from the aquifer and the highly contaminated vadose zone with the LNAPLs and permitted the conclusion that no significant differences regarding the microbial hexadecane degradation potential exist between both zones. Moreover, BACTRAPs were incubated in vadose zone soil and allowed similar conclusions regarding the contaminant degradation potential compared to the soil incubations, thus, demonstraing their general applicability for tracing microbial alkane degradation and for their application in the vadose zone. An incubation experiment with pristane, furthermore, revealed a high site inherent potential for alkane degradation, as this recalcitrant branched-chain alkane was also degraded by microbes.[3]

Author contribution: 90%
I sampled the field site, carried out the experiments, extracted and analysed the biomarkers. The experimental design was previously discussed with Matthias Kästner and Anja Miltner. The manuscript was written by me and finalised with the help of Anja Miltner and Matthias Kästner.

(signature of supervisor)

[3] Supporting information is provided in the appendix p. XLI

Hexadecane and pristane degradation potential affected by varying redox conditions at the level of the aquifer – evidence from sediment incubations and *in-situ* microcosms.

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Abstract
Monitored natural attenuation is widely accepted as a sustainable remediation method. However, methods providing proof of proceeding natural attenuation within the water-unsaturated (vadose) zone are still relying on proxies such as measurements of reactive and non-reactive gases, or sediment sampling and subsequent mineralisation assays under artificial conditions in the laboratory. In particular, at field sites contaminated with hydrophobic compounds, e.g. crude-oil spills, an *in situ* evaluation of natural attenuation is needed, because *in situ* methods are assumed to provide less bias than investigations applying either proxies for biodegradation or off-site microcosm experiments. In order to compare the current toolbox of methods with the recently developed *in situ* microcosms, incubations with Direct-Push-sampled sediments from the vadose and the aquifer zone of a site contaminated with crude oil were carried out in conventional microcosms and with *in situ* microcosms. The results demonstrate the applicability of the *in situ* microcosm approach outside water-saturated aquifer conditions. The sediment incubation experiments demonstrated relatively similar rates (vadose: 4.7 $mg_{hexadecane}/kg_{soil}$ per day; aquifer: 6.4 $mg_{hexadecane}/kg_{soil}$ per day) of hexadecane degradation in the vadose zone and the aquifer, although mediated by different microbial communities according to the fatty acid analysis. In addition, degradation potential for the hardly-degradable branched-chain alkane pristane was evident, although this compound is regarded to be recalcitrant and is thus used as a reference parameter for indexing the extent of biodegradation of crude oils. Altogether, the site had a high inherent potential for natural attenuation of crude oils both in the vadose zone and the aquifer.

Keywords: crude-oil-spill, alkanes, natural attenuation, sediment, ground water, microcosms, contaminant, bio-degradation, hexadecane, pristane.

Introduction
Soil and water contamination arising from accidental spills of crude oil or from leaking pipelines is a matter of global importance (MacLeod et al. 2001, Brassington et al. 2007, Towell et al. 2011) and is a result of intensified crude oil extraction measures including exploitation of oil wells or shale oil in various regions. While deteriorated sites in densely populated areas are usually remediated using fast, but also expensive measures, spills in remote areas are usually only insufficiently remediated due to financial constraints. Cost-efficient measures such as phytoremediation (e.g. Nicolotti & Egli 1998, Nie et al. 2011, Basumatary et al. 2012) only work for the topsoil or within the root zone, where the root exudates of certain plants have been shown to promote petroleum-degrading microbes (Miya & Firestone 2001). Consequently, oil entering deeper soil or sediment layers and ultimately reaching the aquifer cannot be efficiently remediated using cost-efficient and governmentally accepted methods. A particular challenge arises from the low solubility of most crude-oil components, for example alkanes (Eastcott et al. 1988), and leads to an enrichment of water-insoluble oil components on top of the groundwater table (light non aqueous phase liquids: LNAPL). Changing groundwater levels thereby lead to vertical spread of the LNAPLs, resulting in smearing effects within the vadose zone (Molins et al. 2010) that lead to the contamination of higher volumes of sediment, particularly on sites with

highly fluctuating groundwater tables, for example within the boreal zone during snow melt, within tidal systems or underneath irrigated agricultural fields or paddy fields. A solution for overcoming the lack of a cost-efficient remediation strategy may be provided by the monitored natural attenuation approach (US-EPA 1999, UK-EA 2000, SYKE 2006), which is already well-established for the remediation of contaminated groundwater and sediment systems, in particular within the US and the EU (Declercq et al. 2012).
However, monitoring the natural attenuation of crude-oil compounds within the unsaturated subsoil required considerable financial effort, due to the need for drilling of boreholes and subsequent preparation of sediment microcosms in the laboratory in order to evaluate the potential for natural attenuation. Moreover, these studies have been demonstrated to be constrained by artificial conditions in the laboratory (Strevett et al. 2002, Bombach et al. 2010b) and by the limitations of drilled wells being only barely representative for the conditions of the entire volume of contaminated sediment and aquifer. More detailed vertical and horizontal spatial resolution with feasible financial effort requires innovative methods, for example the Direct-Push method (US-EPA 2005). Using this method, probes for measuring geophysical parameters or for taking sediment- and groundwater samples from the vadose zone or the aquifer (US-EPA 2005) are pushed into the soil with the help of static pressure or a mechanical hammer system. Theoretically, soil samples can be obtained from any depth, and in contrast to samples obtained by conventional drilling, the samples do not come into direct contact with other sediment horizons while being transported to the surface. This prevents cross-contamination as a major source of bias, especially for investigations of natural attenuation. Sampling with this method is fast and cost-efficient; furthermore, the lighter machinery required is easier to bring to the site and causes less damage to the topsoil. Consequently, the method is generally accepted as a low-invasive monitoring and sampling method; soil and sediment materials obtained from this method are also less prone to bias caused by contact with other sediment horizons.
For the investigation of sites equipped with groundwater monitoring wells, the known *in situ* microcosm approach (Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010a, Kästner & Richnow 2010) successfully provides governmentally accepted measurements of the potential for natural attenuation under conditions inside the monitoring wells at the level of the aquifer. This approach applies initially sterile granulated activated carbon carrier material loaded with the contaminant of interest (stable isotopes labelled with ^{13}C or ^{15}N) for the respective field site. The microcosms are exposed for a few weeks in the groundwater monitoring wells and then analysed for biomarkers extracted from the carrier material. The presence of isotope-labelled biomarkers, e.g. phospho-lipid fatty acids (PLFA), provides ultimate evidence for contaminant degradation and the colonization by the indigenous microbes from the aquifer (Schurig & Kästner 2012). However, the method was limited to application within groundwater monitoring wells, which had been shown to be biased and unrepresentative of the microbial community within the aquifer (Chapelle 1993, Dejonghe et al. 2004). With the help of the newly developed Direct-Push-based probe for microcosms (DP-BACTRAP probe: Schurig & Kästner 2012, Schurig et al. 2013a), this bias could be minimized. Up to now, the assessment of the microbial activity with this probe has only been demonstrated for an aquifer under field conditions, whereby it showed superior performance in comparison to the BACTRAP system inserted in pre-installed wells (Schurig et al. 2013a). The general applicability of this new system within the vadose zone was tested in laboratory experiments, but field tests for measuring the natural attenuation potential under vadose zone conditions are still lacking. In particular, for contaminants with low water-solubility, these measurements would provide significant cost benefits because no drilling and well installation operations are needed, and thus measurements may not only be faster but can also be assumed to represent *in situ* conditions better.

Therefore, the purpose of this study was: I) to study the biodegradation of stable-isotope-labelled hexadecane, as a model compound for crude oil contamination, within sediments of the vadose zone in comparison to water-saturated aquifer samples, II) to elucidate the effect of different water saturation conditions on the biodegradation potential, III) to compare the microbial communities degrading hexadecane by means of phospho-lipid fatty acid biomarkers (PLFA), and IV) to test the applicability of BACTRAPs for assessing natural attenuation of hexadecane in vadose-zone sediment material under typical conditions regarding water holding capacity. In addition, the experiments were also designed to compare the results from BACTRAPs with the results from general sediment microcosm experiments regarding the active microbial community and the isotopic enrichment of biomarkers, and to check the site-inherent

potential for biodegradation of pristane as a model compound for hardly degradable, branched aliphatic petroleum components, which are commonly considered as recalcitrant; pristane is thus used as the reference compound for the assessment of the biodegradation of crude oils (Pirnik et al. 1974, Rojo 2009).

Methods and Materials

Field site

The field work was carried out at the crude-oil-contaminated site Trecate near Novara, Italy (Fig.1). The contamination with alkanes of various chain-lengths resulted from a major oil-well blowout of well Trecate-24 in 1994, causing the contamination of 1500 ha of arable land by a release of 12600 – 18000 m^3 crude oil (AGIP 1995, Nicolotti & Egli 1998, Brandt et al. 2002).

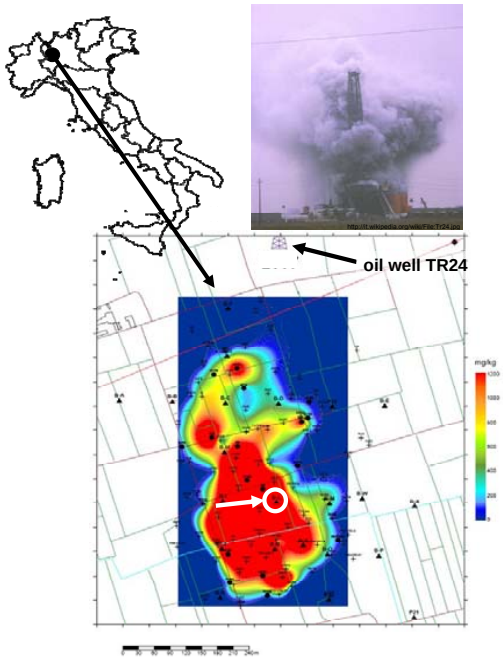

Fig.1: Map of the Trecate site, Italy, along with hydrocarbon concentrations at 10m below ground. The sampling location is marked by white arrow and circle (figure modified from: ENI 2008). Upper part presents location of the Trecate site in Italy along with a photograph of the oil-well blowout.

Prior to the contamination, the sediments of the area were mainly used for production of rice, and for that reason the area features an extensive network of channels for flooding and draining the rice fields. The ground water table of the site exhibits high seasonal fluctuations as a consequence of periodic flooding of the fields. In order to prevent fast infiltration of the water from the rice paddies into deeper sediment layers, the upper part of the sediment was sealed by a clay layer underneath, which luckily prevented most of the oil from reaching deeper sediment layers (Brandt et al. 2002).

Regarding the remediation of the contaminated soil, 60 cm of the topsoil was excavated and bioremediation was chosen for the soil below in order to recover the potential of the soil for agricultural production, as requested by the landowners and farmers of the area. According to Brandt et al. (2002), additional methods such as skimming of residual free-phase oil, soil farming and phytoremediation were applied and led to decreasing contaminant concentrations throughout the affected area in the upper soil-horizons.

However, due to de-watering wells, not all the crude oil could be prevented from infiltrating deeper sediment layers, ultimately reaching the groundwater, where it formed a highly contaminated plume. Combined with the fluctuating water table, LNAPLs floating on top of the water are distributed between the vadose and aquifer zones, forming varying redox conditions by microbial activity.

Field work

The field work was carried out on two consecutive days in March 2010. By means of a Direct-Push device (Geoprobe 6610DT, Kalina KS, USA) equipped with a Macro-Core sampling system (Geoprobe, Kalina KS, USA), continuous soil cores were taken from the vadose zone above the aquifer affected by ground water fluctuations (from 8 - 11 m below ground) and from the aquifer that remains permanently water-saturated (from 10.5 m to 13.5 m below ground). Sampling was carried out at a site representative of the highly contaminated area (see Fig.1). After the liners containing the sediment reached the surface, they were rapidly opened and the sediment was transferred into zip-loc bags, avoiding a gas-phase and extended air-contact. The samples were transported to the laboratory within gas-tight and cooled containers (~4°C). Upon arrival at the laboratory, the samples were stored under cool conditions (~4°C) until further analysis.

Preparation of sediment microcosms

In order to evaluate the potential for contaminant degradation, microcosms were prepared with 30 g (dw) material from the vadose zone or the aquifer sediment. Each microcosm consisted of a 250 ml bottle closed with gas-tight caps containing PTFE-lined septa. Ten mg unlabelled or $^{13}C_{16}$-Hexadecane (isotope purity 99 atom % ^{13}C; Sigma-Aldrich,

Taufkirchen, Germany), used as a model alkane compound, was diluted in pentane and mixed with a 3 g aliquot of the sediment of each microcosm. Following evaporation of the solvent at room temperature, the aliquot of the sediment containing the hexadecane was thoroughly mixed with the remaining 27 g of sediment and filled into the containers. This procedure prevented alteration of the sediment microbial community by contact with the solvent. In addition, unamended (blank) microcosms were prepared for comparison and calculation of background ^{13}C carbon. The weekly aeration procedure maintained sufficient oxygen levels within the microcosms by addition of 100 ml of fresh air. Experiments were carried out in triplicates and the microcosms were stored in the dark at 20°C. Microcosms from vadose zone sediment were prepared similarly for evaluating the sediment-inherent potential for degradation of unlabelled or ^{2}H-labelled pristane (2,6,10,14-Tetramethylpentadecane-$^{2}H_{40}$, isotope purity 98.9 atom % D; Campro Scientific, Berlin, Germany) at this field site. The hexadecane microcosms were destructively sampled at three elapsed times (28d, 47d and 61d), whereas the pristane-amended microcosms were continued for 180 days. After finishing the experiments, the sediment material was stored at -20°C until further analysis.

Comparison to in situ microcosms

For comparing the performance of *in situ* microcosms (BACTRAPs; Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010a) to sediment incubations in a laboratory experiment, BACTRAPs were prepared as described elsewhere (Schurig & Kästner 2012). Briefly, initially clean and sterile activated-carbon-bead carrier material was loaded with a ^{13}C or non-labelled ^{12}C hexadecane, inserted into a PTFE cage and subsequently exposed within the sediment material. After exposure, the phospho-lipid fatty acids (PLFA) biomarkers were extracted from the activated carbon beads. The presence of PLFA provides evidence for the colonization of the activated carbon beads by the indigenous microbes from the aquifer, whereas the identification of the stable isotope label in the biomarkers provides the ultimate evidence for biodegradation of the labelled contaminant at the field site. Similarly to the pure sediment incubations, 10 mg of unlabelled hexadecane or $^{13}C_{16}$-hexadecane, respectively, were diluted in pentane and loaded onto an activated-carbon surface (AUF540, Adako, Düsseldorf, Germany); the solvent was then evaporated. The unamended microcosm contained non-loaded carbon beads. After preparation, the microcosms were completely covered with 30 g of sediment from the vadose zone and incubated within 250 ml bottles closed with PTFE-lined screw caps. Weekly aeration ensured sufficient oxygen supply. After 61 d, the microcosms were retrieved from the sediment and stored at -20°C until further analysis.

Fatty acid analysis

For extraction of fatty acid biomarkers, a modified Bligh & Dyer (1959) approach was chosen. Phospho-lipid fatty acids (PLFA), considered to represent mainly living biomass (Green & Scow 2000, Pinkart et al. 2002, Kaur et al. 2005), were extracted and measured according to previously published protocols (Schurig & Kästner 2012, Schurig et al. 2013b).

Determination of isotopic enrichment

For determining the isotopic enrichment of the biomarkers, the extracts were injected into a gas chromatography - combustion/pyrolysis - isotope ratio mass spectrometer (GC-IRMS). Briefly, the following instrumentation and parameters were used: GC, Agilent 6890N (Boeblingen, Germany); for ^{13}C: combustion interface, Thermo-Fischer GC-Combustion 3 interface (Schwerte, Germany); for ^{2}H: Thermo-Electron GC-pyrolysis (Schwerte, Germany); IRMS, Thermo-Fischer MAT253 (Schwerte, Germany). Parameters: injection in splitless mode at 250°C, BPX-5 column (50m×0.32m×0.5µm, SGE, Darmstadt, Germany) at 70°C for 1 min, then 20 °C min^{-1} to 130°C, 5min hold, then 2°C min^{-1} to 150°C, 5min hold, then 2°C min^{-1} to 165°C, 5min hold, then 2°C min^{-1} to 230°C, then 20°C min^{-1} to 300°C, 5min hold.

The isotopic enrichments were referred to the international standards (^{13}C: Vienna Pee Dee Belemnite (VDPD); ^{2}H: Vienna Standard Mean Ocean Water (SMOW)) and are given in the internationally accepted δ‰-notation (IAEA 1995), for example for ^{13}C:

$$\delta^{13}C = \left[\frac{\left(\frac{^{13}C_{sample}}{^{12}C_{sample}} \right)}{\left(\frac{^{13}C_{PDB}}{^{12}C_{PDB}} \right)} - 1 \right] \bullet 1000 .$$

Results

Sediment samples (see fig. 1) were taken from both the aquifer (< 12 m) and the vadose zone (8 -11 m) in order to investigate the effect of the groundwater table variations on the microbial degradation potential in these sediments. Hexadecane was used as an

alkane model compound in the experiments. In addition, results from pure sediment incubations were compared with results obtained by the incubation of BACTRAPs in the vadose zone sediment for testing the applicability of the system in water-unsaturated sediments of vadose zones contaminated with alkanes. In order to evaluate the degradation of crude-oil components that are known to be highly recalcitrant, the branched-chain alkane pristane was tested in another sediment incubation experiment.

Quantities of biomarkers

Microbial PLFA were quantified as biomarkers in the samples and were found to increase over time for sediment incubations with unlabelled ^{12}C-hexadecane, ^{13}C-hexadecane and unamended microcosms (Fig. 2).

The amounts of PLFA in the ^{13}C-hexadecane-amended microcosms increased at higher rates in comparison to the unlabelled treatment, resulting in twice the quantity of PLFA biomarkers after 61 days of incubation.

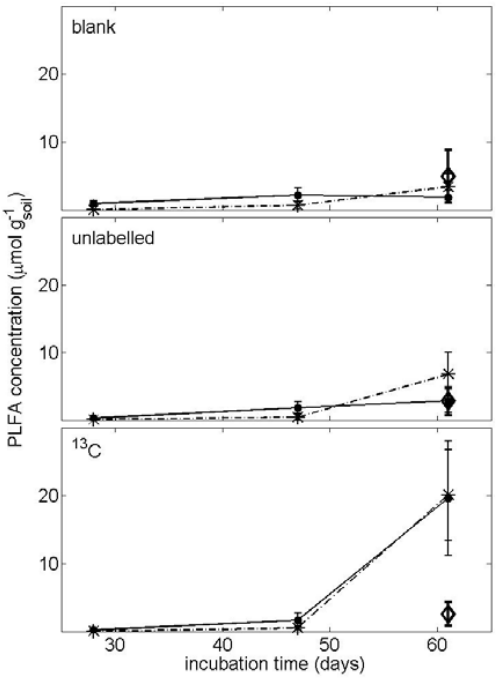

Fig.2: Concentrations of PLFA biomarkers within ^{12}C- and ^{13}C-hexadecane amended as well as unamended sediment microcosms containing material from the vadose (•) and aquifer (▪) zones and BACTRAP incubation (◊). Error-bars represent standard deviations for extractions from triplicate microcosms.

Only a slight increase of the PLFA concentrations was observed in the non-amended sediment incubation. Although not significant, a slightly higher average quantity of PLFA in sediments from the level of the aquifer was observed in comparison to the incubations with sediment from the vadose zone for ^{12}C- and unamended microcosms (Fig. 2). It was only in the ^{13}C amended microcosms that equal quantities of PLFA for both types of sediment were found after 61 days. Although differing quantities of PLFA biomarkers were found for ^{12}C and ^{13}C experiments, the differences are similar for aquifer and vadose sediments. Thus, they were most likely to be due to the supplied hexadecane stock solution applied for the preparation of the microcosms. Common to all microcosms is that only a slight increase in quantity of PLFA was observed during the first 47 days of incubation, and that the PLFA quantity in the sediment from the vadose zone was at a higher level than that in the sediment from the aquifer. Hence, the aquifer sediment exhibits a higher lag time for microbial activity in comparison to sediments from the vadose zone, but develops a higher microbial colonisation thereafter.

Isotopic enrichment of biomarkers

During biodegradation, the carbon from organic compounds is mineralised, integrated into biomass, or excreted within metabolites. In this framework, the detection of biomarkers with isotopic enrichment provides the ultimate evidence for on-going microbial degradation, thus indicating natural attenuation in the samples.

The enrichment of biomarkers accompanies microbial hexadecane degradation. Figure 3 (panel a) shows the extractable hexadecane concentrations as percentages of the total supplied hexadecane (300 µg g^{-1}) over time, indicating that hexadecane was degraded in all microcosms. However, the aquifer-sediment microcosms showed lower levels of hexadecane at the later stages of the experiment in comparison to the microcosms containing vadose-zone sediment, which may be due to a higher background level of crude oil in the vadose zone sediment (data not shown). With increasing time of incubation, the amount of biomass (based on summarized PLFA contents) was increasing along with decreasing contaminant concentrations in all experiments (Fig. 2). In order to investigate whether the biomass increase results from the activity of the specific hexadecane degrader community or from increasing activity of general crude-oil degraders, the amount of ^{13}C atoms derived from the ^{13}C-labelled hexadecane was analysed within single PLFA (Fig.3 panel b, Tab.1).

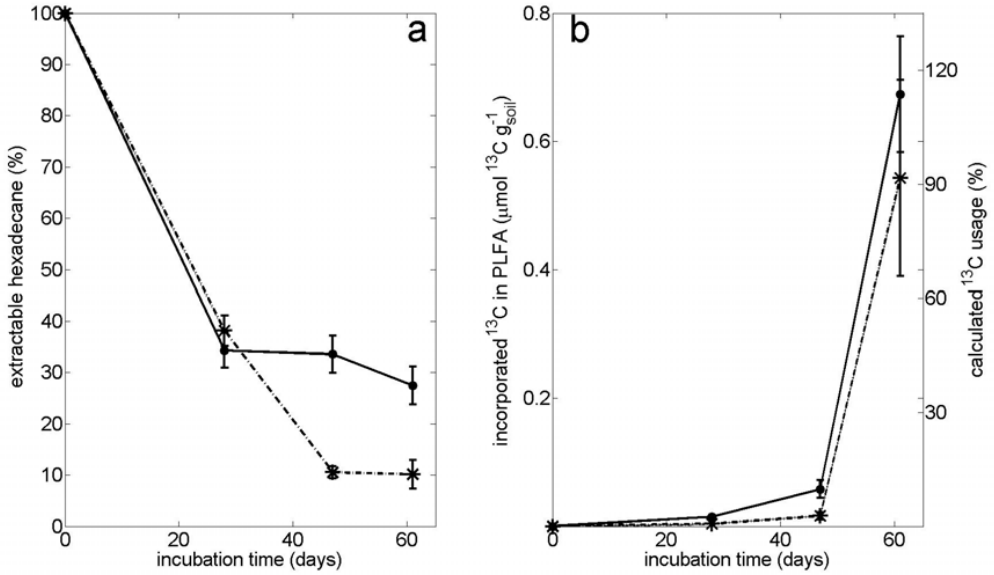

Fig.3: panel a: Extractable hexadecane from sediment material from the vadose zone (•) and the aquifer zone (·). Error-bars represent standard deviation for extractions from triplicate microcosms. Panel b left y-axis: Number of PLFA-integrated ^{13}C atoms derived from ^{13}C-hexadecane degradation over time of incubation in sediment microcosms with material from the vadose (•) and the aquifer zones (·). Right y-axis: Calculated usage of the supplied hexadecane-^{13}C label equivalents from the ^{13}C-PLFA data over incubation time. Assumptions: 30% biomass yield during hexadecane degradation (Geerdink et al. 1996), 10% of this biomass being PLFA (Madigan et al. 2012), and 70% mineralisation. Error-bars represent standard deviations for extractions from triplicate microcosms.

The results show that hexadecane-degrading bacteria are mostly active at later stages of the experiment, since the majority of ^{13}C from hexadecane was introduced into the PLFA at this time. However, when comparing the time course of the residual hexadecane concentrations with the incorporation of ^{13}C into PLFA, the delay between the concentration decrease and label increase points to the immobilisation of hexadecane carbon into intermediate products and later incorporation into PLFA. In addition, although the increase of the ^{13}C-PLFA label was similar for both types of sediment, the decrease of the overall hexadecane concentrations was not similar; this may be due to different degradation pathways or different residual crude-oil phases in the vadose zone material, resulting in a higher retention of hexadecane or a higher background of this compound from the original contamination. The latter hypothesis was supported by calculating the total biodegraded fraction of the ^{13}C-hexadecane (Fig. 3 b) with the assumptions of a biomass yield of 30% during hexadecane degradation resulting in 70 % CO_2 formation (Geerdink et al. 1996; in liquid culture) and around 10% of the biomass being represented by PLFA (Madigan et al. 2012). Thus, 90-110% of the supplied hexadecane was degraded towards the end of the experiment (Fig. 3 b).

Comparison of sediment incubations and BACTRAP

In order to compare the results of conventional sediment incubations with those of the recently introduced BACTRAP microcosms, a laboratory experiment with both test systems was conducted. Similarly to the other experiments, hexadecane was used as a model compound for the alkane pollution at the site. The amount of PLFA after 61 days of incubation showed no significant differences between the treatments (Fig. 2) except for the ^{13}C incubation, where the sediment microcosms show a 7-fold higher quantity of PLFA in comparison to the BACTRAPs. However, the BACTRAPs showed more homogeneous images regarding PLFA quantity in comparison to the pure sediment incubations.

Interpreting the summarized PLFA concentrations out of context would result in the conclusion that the BACTRAPs did not very accurately represent the microbial community of the sediment.

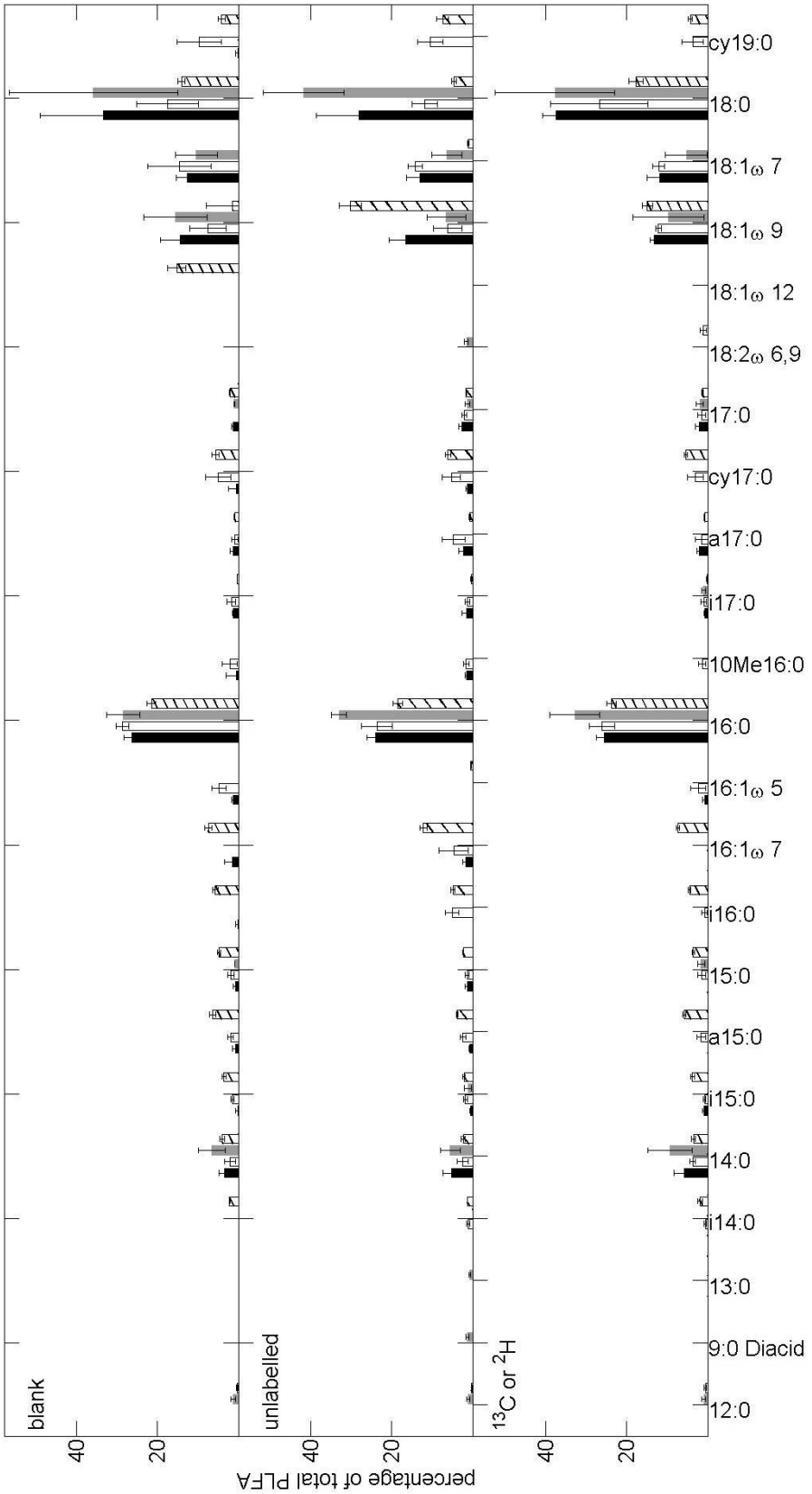

Fig.4: Relative abundance of PLFA related to the total amount of PLFA in samples from unamended (upper panel), unlabelled- (middle panel), and ^{13}C-hexadecane or ^{2}H-pristane (lower panel) amended microcosms after 61 or 180 days incubation, respectively. Black bars: vadose zone; white bars: aquifer zone; grey bars: BACTRAPs incubated within vadose zone sediment; white bars with hatches: pristane experiment. Error-bars represent standard deviations for triplicate experiments and triplicate extractions. Corresponding concentrations are shown in Table S1.

However, a detailed investigation of the relative abundances of individual PLFAs in each sample allows for a more accurate characterisation of the microbial communities in each incubation (Fig. 4, corresponding concentrations in Table S1).
The spectra of single PLFA (Fig. 4) are not very different between the unamended, ^{12}C-, and ^{13}C-hexadecane amended microcosms, whereas clear differences were seen between vadose-zone and aquifer sediment incubations. However, these differences are not consistently reflected by the ratios of certain groups of PLFA considered in the literature as being representative for specific groups of bacteria (Fig. S1). PLFA from the BACTRAPs are more similar to those of the vadose zone than to those of the aquifer sediment, and show a spectrum similar to those of the sediment incubation experiments. Some of the PLFA found in the sediment incubations are missing on BACTRAPs, which may result from a selective exclusion of some microbial species colonizing on BACTRAPs, or more likely to the lower overall quantity of PLFA on BACTRAPs, causing some PLFA to fall below the detection thresholds of the GC-MS and GC-IRMS systems. In the BACTRAPs, only a few PLFA could be analysed for their isotopic enrichment (Tab. 1), which mostly is as a result of the relatively low sensitivity of the GC-IRMS instrument. Nevertheless, similar conclusions regarding the natural attenuation at this field site can be drawn when comparing sediment incubations and BACTRAPs. Hence, BACTRAPs offer representative results regarding the composition of the sediment microbial community, at least on the basis of the relatively coarse phylogenetic resolution offered by the PLFA analysis approach.

Pristane biodegradation
Pristane is generally assumed to be hardly degradable by microorganisms, at least as long as other carbon sources are more readily available for microbial metabolism. Thus, indications for pristane biodegradation in the sediment of the Trecate site would clearly demonstrate a highly elevated potential for biodegradation of crude oils. Considering the expected much slower kinetics of pristane in comparison to hexadecane degradation, the incubation time was extended to 180 days.
After this incubation time, comparable amounts of PLFA biomarkers were extracted from the pristane-amended sediment incubations and the *in situ* microcosms containing hexadecane ($Pristane_{blank}$: 2.89 ± 0.69 µmol g_{soil}^{-1}; $Pristane_{1H}$: 11.99 ± 0.66 µmol g_{soil}^{-1}; $Pristane_{2H}$: 2.89 ± 0.28 µmol g_{soil}^{-1}). While the ^{13}C incubations in the hexadecane experiment showed the highest quantity of biomarkers, the unlabelled amendments showed the highest production of PLFA in the pristane experiment. However, the similar amounts of PLFA in the unamended and ^{2}H-pristane amended microcosms suggests no or only little degradation of the labelled pristane. Nevertheless, clear enrichments of single PLFA, with ^{2}H isotopic shifts of 68 - 580 δ‰ in comparison to the unlabelled amendments, were found by means of GC-IRMS measurements (Tab. 2). A detailed investigation of the PLFA enrichment reveals a specific distribution of the ^{2}H-label between the fatty acids. For example, the 16:0 and cy17:0 PLFAs show very high enrichments, while longer-chain PLFAs show lower enrichments in the range of 140 δ‰. These results provide evidence for the site-inherent potential for pristane degradation, which indicates that most alkanes arising from the oil-well blowout should be biodegradable by the indigenous microbial community.
The comparison of the relative abundance of single PLFA related to the total amount also shows a high similarity between the unamended and pristane-amended microcosms (Fig. 4). The microbial community in these microcosms appears to be clearly distinct from the one in the hexadecane-amended microcosms, although the same sediment material was used for producing the microcosms. This was presumably as a result of the extended incubation time or due to the different substrate.

Discussion
The present study provides data about the microbial degradation potential for crude oils and hexadecane in Direct Push sampled sediments from different depths (aquifer and vadose zone material) at an Italian field site impacted by a heavy crude-oil spill (AGIP 1995, Nicolotti & Egli 1998, Brandt et al. 2002). No significant differences between vadose-zone and aquifer sediments were found regarding accumulation and isotopic enrichment of PLFA-biomarkers with hexadecane or pristane as supplied substrate. Results differing between the two sediment types were found for the residual hexadecane concentration and for the composition of the microbial community as analysed by means of PLFA biomarkers. Furthermore, the vadose-zone sediment incubations were compared with *in situ* microcosms (Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010a) that have never previously been applied within the non-water-saturated vadose zone of sediments.

Tab 1: Enrichment of individual PLFA upon biodegradation of ^{13}C-hexadecane after 28, 47 and 61 days of incubation given in δ‰$_{vs.\ VPDB}$ and the associated standard deviation. Corresponding concentrations for 61d are shown in Table S1.

	28d		47d		61d		
fatty acid	**Vadose**	**Aquifer**	**Vadose**	**Aquifer**	**Vadose**	**Aquifer**	**BACTRAPs**
				[δ‰ vs. VPDB ± STD]			
14:0	n.d.	n.d.	n.d.	n.d.	n.d.	107.4 ± 1.2	n.d.
16:1	n.d.	148.9 ± 19.2	n.d.	254.93 ± 59.8	n.d.	2094 ± 69.5	n.d.
16:0	4424 ± 26.3	6636 ± 83.2	1531 ± 12.7	1134 ± 4.5	2250 ± 20.4	746.2± 1.4	327.5 ± 141.8
17:0	n.d.	n.d.	n.d.	n.d.	n.d.	130.57 ± 2.1	n.d.
18:1cis	n.d.	n.d.	n.d.	4.11 ± 0.4	n.d.	3.54 ± 0.2	n.d.
18:1trans	1722 ± 1.3	4249 ± 59.5	n.d.	428.5 ± 5.5	n.d.	212.1 ± 0.1	n.d.
18:0	261.6 ± 14.6	1501 ± 697.3	190.5 ± 18.5	550.9 ± 0.4	184.1 ± 7.9	179.9 ± 0.6	-22.06*
19:0cis	801.4 ± 52.4	n.d.	421.3 ± 304.8	n.d.	609.1 ± 17.2	n.d.	48.16 ± 23.4

*single measurement

Tab 2: Enrichment of individual PLFA upon biodegradation of pristane after 180 days of incubation of unamended (blank), unlabelled (^{1}H) and labelled (^{2}H) sediment stemming from the vadose zone of the Trecate field site.

PLFA	blank	STD_{blank}	^{1}H	STD_{1H}	^{2}H	STD_{2H}
			[δ‰ vs. SMOW]			
14:0	n.d.		148.45*		n.d.	
15:0	19.18	7.75	32.11	56.41	n.d.	
16:0	91.47	10.60	17.75	74.13	260.16	21.94
i-17:0	n.d.		15.71*		83.82	22.75
cy17:0	88.46	16.61	38.32	52.79	619.12	136.06
2-OH 16:0	45.56	16.32	37.34	25.72	n.d.	
18:1cis	0.54	9.22	22.76	19.63	170.33	66.06
18:0	-34.14	4.27	20.53	11.73	160.81	14.61

*single measurement

Similar PLFA contents were observed in the unamended and unlabelled experiments with hexadecane, but higher amounts were found in the ^{13}C-labelled experiments. The biomarkers extracted from BACTRAPs showed lower amounts in the ^{13}C experiment, but a similar composition of the microbial community. Therefore, the results from the BACTRAP microcosms lead to similar conclusions regarding the microbial contaminant degradation activity, and validate the applicability of the BACTRAP approach in the vadose zone of crude-oil-contaminated sediments. Combined with the recently developed Direct-Push-based BACTRAP probe (Schurig et al. 2013a), this will provide significant advantages in sampling time and reliability of results for the assessment of the *in situ* field conditions. The present study also shows that contaminated sites with a high potential for crude-oil biodegradation also show microbial degradation potential for pristane, a hardly degradable, branched aliphatic petroleum component, which is commonly considered as recalcitrant and thus used as a reference compound for the assessment of the biodegradation of crude oils (Pirnik et al. 1974, Rojo 2009).

Hexadecane biodegradation

Crude-oil or petroleum spills usually comprise a mixture of different compounds; therefore, hexadecane is frequently used as a model compound in soil and sediment microcosm studies that aim to elucidate the inherent potential for natural attenuation of crude oil (Towell et al. 2011, Adetutu et al. 2012, Aislabie et al. 2012, Zyakun et al. 2012). In contrast to the present study, Towell et al. (2011), Adetutu et al. (2012) and Aislabie et al. (2012) used radio-labelled ^{14}C-hexadecane to assess the rate of mineralisation by detection of $^{14}CO_2$, which provides the advantage of fast and accurate detection of the fraction of the supplied contaminant that becomes mineralised. However, this technique does not allow conclusions to be drawn regarding the identity of the degrading microbes. Towell et al. (2011) and Aislabie (2012) therefore estimated the number of colony-forming units by plating techniques and correlated it to contaminant degradation rates determined by the $^{14}CO_2$ measurements. This method is limited by the cultivability of microbes on agar plates (Amann et al. 1995), in contrast to the determination of living active biomass by PLFA extraction. Increasing microbe abundances along with increasing concentrations of petroleum hydrocarbons, analysed by PLFA quantification in a contaminated oil profile, have been documented previously by Ringelberg et al. (2008). Consequently, the higher rates of mineralisation found by Towell et al. (2011) and similar rates found by Aislabie et al. (2012) at lower incubation temperature cannot be directly compared to the increasing PLFA quantity, isotopic enrichment and residual contaminant concentrations reported in this study. However, only the PLFA measurements or RNA-/DNA-extractions (Schurig & Kästner 2012) are applicable under *in situ* conditions, thereby enabling a later comparison with the BACTRAP approach. The data of the present study suggest that the sediments from Trecate show lower microbial activity in comparison to the topsoils used in other studies (Towell et al. 2011, Adetutu et al. 2012, Zyakun et al. 2012). In addition, most of the supplied hexadecane was degraded after 61 days in the present study and the 30% biomass yields (Towell et al. 2011, Aislabie et al. 2012) taken to estimate the mineralisation are also reasonable for the sediments used.

In general, sediments have been shown to contain lower bacterial numbers than topsoils (Bone & Balkwill 1988, Bekins et al. 1999) and

thus exhibit lower microbial activity, which is accompanied by increased lag times until microbes proliferate and the rate of degradation of the supplied hexadecane increases. Another explanation is that the crude oil within the Trecate sediment is aged and thus the bioavailable, easily degradable hexadecane has already been consumed. This is in accordance with the lag of microbial degradation activity. The incubation temperature may also have major influence on the enzymatic activity and on the degradation rates, which explains why Aislabie et al. (2012) reported similar times needed for hexadecane degradation although they used topsoil in their study.

Similar amounts of PLFA were found after 61d in both sediments, from the vadose zone and from the aquifer. However, upon the first two sampling dates the vadose zone sediment contained not only a higher amount of PLFA but also a higher residual hexadecane concentration, which may be due to priming effects or better electron acceptor (oxygen) supply providing the microbial communities in the vadose zone with higher degradation capabilities resulting from the direct contact to hydrocarbons arising from the crude-oil spill (MacLeod et al. 2001, Towell et al. 2011). This may explain why microbes within the aquifer zone were initially less abundant and required more time for proliferation. The presented results are in good accordance with the potential for microbial crude-oil degradation documented in other studies showing natural attenuation (ENI 2008) at the Trecate site and with the finding that the aquifer sediment appeared optically and olfactorily unremarkable, in contrast to the vadose zone sediment.

Extrapolating the results to other field sites that exhibit a high fluctuation of the water-table (e.g. sediments in aquifers and soils within the boreal zone or tidal systems) shows that crude-oil compounds within the soils or sediments accumulate in the smear zone near the water-table with increasing microbial activity. Biodegradation of crude-oil contaminants requires a certain lag time for the microbes to adapt to the substrates; thereafter, microbial contaminant degradation proceeds as long as the supply of suitable terminal electron acceptors is safeguarded. However, the oxygen supply within the aquifer will presumably be lower, resulting in lowered redox potentials (Bekins et al. 1999, Molins et al. 2010), and thus the vadose zone may develop a higher amount of crude-oil-degrading microbial biomass.

Redox zonation is actually observed in the Trecate aquifer and the vadose zones, in particular with oxic and anoxic microsites in the vadose zone material. During sampling, sediment aggregates were only mildly ruptured, and the sediment was not sieved prior to the experiments in order to conserve these unique properties of both sediment horizons. The PLFA profiles of vadose- and aquifer-zone sediments are different at the end of the experiment, which indicates that different microbial communities with presumably different pathways for hexadecane degradation or at least different carbon fluxes prevailed. Different degrader communities were also found by Zhang et al. (2012) when comparing crude-oil-contaminated upland- with paddy-field-soil from Asian sites. Overall, Gram-negative bacteria dominated both types of sediments in the present study, which corroborates the finding that many hydrocarbonoclastic bacteria are Gram-negative in marine environments (Yakimov et al. 1998, Golyshin et al. 2002, Yakimov et al. 2003, Yakimov et al. 2004) and that the microbial community resulting from crude-oil contamination might be comparable for terrestrial and marine ecosystems. Markers for fungi were detected at the first sampling in the vadose zone samples (Fig. S1 B); however, similar to the results of Adetutu et al. (2012), the influence of fungi on hexadecane degradation is negligible, as no PLFA-biomarkers for fungi were detected during the later sampling times and consequently no isotopic enrichment was found. These results confirm that the abundance of fungi within deeper layers of sediments and aquifers can be assumed to be much smaller than in topsoil. Streptomycetes have been shown to degrade hexadecane (Alvarez 2003, Pizzul et al. 2006, Gallo et al. 2012); PLFA biomarkers (10Me 16:0 & 10Me 18:0) for this group were increasing relative to the bacterial ones in both vadose zone and aquifer zone sediments (Fig. S1).

In situ microcosms

The applied *in situ* microcosms are intended to provide evidence for microbial contaminant degradation under *in situ* conditions by detection of biomarkers isotopically enriched from degradation of the supplied ^{13}C-labelled substrate. This approach complements indirect methods such as the detection of concentration and isotopic enrichment of reactive (O_2, CO_2 and CH_4) and non-reactive gases (Ar and N_2) (Mailloux & Fuller 2003, Molins et al. 2010, Zyakun et al. 2012). Much more importantly, this approach can replace sediment incubation studies measuring the mineralisation of radiolabelled contaminants via the detection of $^{14}CO_2$ or $^{14}CH_4$ (Strevett et al. 2002, Madsen 2005). The limited comparability of laboratory-scale incubation

studies, in particular those conducted with vadose zone material, to true field conditions (Bombach et al. 2010b) may be explained by the fluctuations of the groundwater table that periodically leads to changes in redox conditions, the destruction of the network of 'anaerobic microsites' during sampling of the sediment samples, and the temperature of incubation, which may lead to an overestimation of degradation rates. Moreover, these changes exert effects on the composition of the microbial communities in the artificial setups. Another drawback of laboratory-scale incubations are the associated costs due to the need for a thorough pre-investigation of the site in order to locate contamination hot spots and later sediment sampling by means of drilling or (more cost-efficient) DP techniques (Dietrich & Leven 2005, Schurig & Kästner 2012).

In order to overcome the limitations of laboratory-scale incubations, a new DP-based BACTRAP probe has been developed recently (Schurig & Kästner 2012, Schurig et al. 2013a), which can be applied directly in aquifer sediments as well as in the vadose zone by Direct Push devices. While the hydraulic equilibration with soil water was demonstrated for vadose zone conditions by means of laboratory experiments (Schurig et al. 2013a), no published data exists regarding the applicability of the BACTRAP approach for providing evidence on microbial activity in vadose zone material. In order to enable the method to be used during site characterisation within an innovative approach (Kästner et al. 2012), a comparison experiment between BACTRAPs incubated in contaminated vadose-zone sediment and conventional sediment incubations was performed. The data show lower quantities of PLFA biomarkers in the BACTRAPs in comparison to conventional sediment microcosms; however, both the fatty acid spectra and the isotopic enrichment are similar to those from the laboratory-scale incubations of sediments, thus indicating ongoing natural attenuation processes at the Trecate site. This demonstrates the general applicability of BACTRAPs within the vadose zone of oil-contaminated field sites. The difference in PLFA quantity is a result of the selective enrichment of degrader microbes on the BACTRAPs due to the supply of hexadecane as sole carbon source on the activated carbon material. The differences in surface area available for colonization on the activated carbon in comparison to sediment may also influence the results. This could be accompanied by a slower nutrient supply in comparison to the sediment, which may slow down the hexadecane degradation (Towell et al. (2011). Furthermore, sessile microbes have been shown to exhibit higher activity than motile bacteria within groundwater ecosystems (van Loosdrecht et al. 1990), which may result in underestimating the degradation of hexadecane in BACTRAPs exposed to sediments. However, in true field tests the systems may be different, since other authors report increasing abundances of motile microbes within contaminated zones of aquifers (Harvey et al. 1984, Godsy et al. 1992, Bekins et al. 1999). The presented results are in accordance with the previous results from the field application of DP-BACTRAPs within an aquifer sediment (Schurig et al. 2013a), where the microbial community on DP-BACTRAPs has been demonstrated to be highly similar to the surrounding sediment and clearly different to the one on BACTRAPs incubated in groundwater monitoring wells. Having now demonstrated the general applicability of the BACTRAP approach under vadose-zone conditions, the next step is the field application test with DP-BACTRAPs in the vadose zone at a contaminated site.

Pristane biodegradation

Branched-chain alkanes, in particular pristane and phytane, have been demonstrated to be hardly degradable in comparison to the easily degradable straight-chain alkanes (Pirnik et al. 1974). Consequently, the kinetics of pristane degradation are slower and therefore the decreasing ratio of hexadecane to pristane is often used as an indication for proceeding alkane biodegradation at field sites (Ringelberg et al. 2008). However, some bacterial strains have been shown to degrade pristane and phytane along with other alkanes (Hara et al. 2003), which was considered to give these microbes a competitive advantage. Therefore, the hexadecane to pristane ratio may not be an accurate proxy on field sites with high degradation potentials for crude oils. The present study showed that the ^{2}H label of pristane was transferred to PLFA, which are markers for active biomass (Green & Scow 2000, Pinkart et al. 2002), thereby providing the ultimate evidence of microbiologically mediated natural attenuation of pristane on the Trecate field site. Furthermore, these results demonstrate the applicability of deuterium-labelled alkanes for degradation studies using sediment microcosms and BACTRAPs, which have until now only rarely been applied for liquid pure cultures (Alexandrino et al. 2001, Friedrich & Lipski 2010). However, as a result of the use of much more easily degradable compounds, both Alexandrino et al. (2001) and Friedrich & Lipski (2010) found higher isotopic labelling in their PLFAs.

As pristane degradation requires specialised microbes (Pirnik et al. 1974, Hara et al. 2003, Rojo 2009), the PLFA spectrum is clearly different compared to the microbial spectrum in the hexadecane microcosms and the BACTRAPs. These differences result from a small group of bacteria rising in abundance due to the competitive advantage of suitable metabolic degradation pathways and enzymes needed for the degradation of branched-chain alkanes (Watkinson & Morgan 1990, Nhi-Cong et al. 2009, Rojo 2009).

Conclusions

Applying sediment incubations combined with *in situ* microcosms of Direct Push sampled materials we could provide evidence for a high natural attenuation potential for alkanes at the terrestrial-crude-oil contaminated Trecate site in Italy. The degradation of hexadecane was proven to occur at similar rates in sediments stemming from both the unsaturated crude-oil impacted vadose zone and from the permanently water-saturated aquifer zone, although the microbial communities in the sediments were shown to be distinct. The main degraders in these ecosystems are bacteria; fungi do not seem to play a role in these degradation processes. In addition, we provided evidence for pristane biodegradation, which indicates a very high crude-oil degradation potential at this site.

By comparing the sediment microcosm incubations with the *in situ* microcosms (BACTRAPs), we demonstrated the full applicability of the BACTRAP approach for the evaluation of the *in situ* microbial degradation potential under oxic conditions even in the contaminated vadose zone, which has not previously been demonstrated. This is particularly relevant for field sites that experienced spillage of hydrophobic compounds (crude oils or tar oils), which do not dissolve in groundwater, but instead form a smear zone on top of the aquifer. Together with the newly developed Direct-Push-BACTRAP-probe (Schurig et al. 2013a), showing that these probes are much more representative for the microbial *in situ* activity than probes exposed in groundwater wells, the provided results offer significant improvements for site assessment and monitoring, in particular by extending the applicability of the technique to contaminated vadose-zone sediment without the need for groundwater monitoring wells and fully water-saturated conditions. Using this new method could provide field site managers with significant savings in cost and time, thus allowing site investigations for example where the contamination has not reached the groundwater, which in turn allows earlier implementation of effective countermeasures. This may also provide benefits regarding the contaminated volume of sediments and groundwater. In addition, the application of *in situ* microcosms offers the option for avoiding the bias often introduced by laboratory-scale microcosms.

Finally, we demonstrated the applicability of ^{2}H labelled contaminants not only within sediment microcosms but also for the BACTRAP-approach. This allows the use of labelled hydrocarbon contaminants which are not available with a ^{13}C stable-isotope label or which are less expensive with a ^{2}H label.

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Abstract:
The manuscript describes two field experiments, where DP-BACTRAPs were exposed in the Zeitz (Germany) aquifer and in the rhizosphere of a constructed wetland at the Leuna (Germany) site. After exposition, the activated carbon samples from Zeitz were embedded into resin and polished for visualisation on a NanoSIMS instrument. This data visualised for the first time microbial contaminant degradation on DP-BACTRAPs and gave reason for the conclusion that in this ecosystem most of the microbes were actively involved in contaminant degradation. However, with the preparation technique used for these samples the 3 dimensional structure of the microbial community was lost. In order to avoid this, the samples from Leuna were sectioned and analysed unembedded. This permitted analysis on the fluorescence microscope, the scanning electron microscope and the NanoSIMS. The data showed a biofilm like structure on the mantle of the AC and less dense colonisation at the inner surfaces of the AC, but only few hotspots of ^{13}C were identified and the active fraction of microbes was comparable between the mantle and the inner surfaces. Moreover, the application of a CARD-FISH approach (HISH-SIMS; Musat et al., 2008) proved to be as succesful in the NanoSIMS, as identification of active microbes by means of their shape and their phosphorous content.[4]

Author contribution: 85%
The experiments were designed and carried out by myself. Henrike Beck assisted with the field work in Leuna. Carsten Mueller and Carmen Höschen assisted with the NanoSIMS analysis, especially of the first sample, and Andrea Prager obtained the SEM images. I wrote the manuscript and Carsten Mueller, Carmen Höschen, Erika Kothe, Anja Miltner and Matthias Kästner helped finalising it.

(signature of supervisor)

[4] Supporting information is provided in the appendix p. XLIII

Various methods for visualising microbial toluene and benzene degrader activity in in situ microcosms

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Abstract
Investigating the in situ microbial contaminant degradation activity in soils and sediments remains challenging, due to the scarcity of methods providing in situ microbial activity measurements. Methodologies like the in situ microcosm approach (DP-BACTRAP) are relying on the insertion of a carrier material with a high surface area amended with isotopically labelled compounds into the soil or sediment. The extraction of biomarkers from the carrier material after a certain incubation period and the subsequent detection of an isotopic enrichment in the extracted biomarkers provides the proof of in situ microbial activity. Other methods, like fluorescence in situ hybridisation (FISH), are directly visualising microorganisms from liquid samples. The FISH method provides phylogenetic information on the labelled biomass by attaching fluorophores via gene probes to nucleic acid sequences of certain microorganisms. However, the FISH method fails to provide data on the activity of the visualised microorganisms and is not applicable on samples with a high surface area. In order to enable a link between microbial activity and phylogeny of contaminant degraders in contaminated aquifers and constructed wetlands under in situ conditions, we combined the recently improved in situ microcosm approach (DP-BACTRAP) with nano-scale secondary ion mass spectrometry (NanoSIMS) analysis.

A stepwise approach using different kinds of activated carbon material was, therefore, tested. In order to test the feasibility of the general method, DP-BACTRAPs containing commercially available activated carbon beads were loaded with ^{13}C-toluene and exposed in the BTEX-plume of an anoxic aquifer at the site of a former hydrogenation plant at Zeitz (Germany). These samples were embedded into resin and polished, in order to prevent contamination of the NanoSIMS instrument with activated carbon dust. Most of the microbial biomass was moderately enriched with respect to the background. However, the preparation technique prevented direct visualisation of single cells.
With the general applicability of the approach demonstrated, the second step was to use a special kind of commercially unavailable activated carbon, which is embedded into Nomex and, therefore, was reasoned to be measureable at the NanoSIMS unembedded without dust formation. Samples for this experiment were loaded with ^{13}C-benzene and incubated in the gravel bed of an aerated constructed wetland-system in Leuna (Germany) treating groundwater contaminated with benzene. Moreover, the samples from the wetland were subjected to a FISH protocol, which led to deposition of fluorine-containing dyes (HISH-SIMS; Musat et al. 2008). The microbes were visualised by both fluorescence microscopy and scanning electron microscopy. Later analysis by NanoSIMS showed that, contrary to the aquifer, only a small fraction of

microbes was highly enriched in ^{13}C, and therefore was actively degrading the benzene in the wetlands. Moreover, we could identify and localise bacterial biomass in 3 dimensional structures and based on elevated ^{31}P and ^{19}F signals for the biomass.
In conclusion, we successfully visualised toluene and benzene degrading microbes on materials with high surface area under in situ conditions using DP-BACTRAPs together with NanoSIMS. Moreover, we could show, by application of special activated carbon beads, that NanoSIMS analysis of unembedded samples with high topography is feasible.

Keywords: NanoSIMS, ^{13}C, natural attenuation, in situ microcosms, DP-BACTRAPs, benzene, toluene

Introduction

In situ assessment of microbial activity in contaminated environmental compartments, such as aquifer and constructed wetland ecosystems, faces challenges arising from the limited access for taking representative samples and the high spatial variability coupled to the pronounced heterogeneity of these systems. With respect to representative sampling of the active microbial community, only in situ methods avoid the bias of the very limited cultivability of microbes in the laboratory (Amann et al. 1995, White et al. 1998). In principle, the common in situ methods rely either on direct extraction and analysis of microbial biomarkers from environmental samples to conclude on the identity of the indigenous microbes (e.g. MacCormack & Fraile 1997, Entcheva et al. 2001, Gomes et al. 2010), or in situ incubation with isotope-labelled tracer substances and subsequent extraction and analysis of labelled biomass for resolving the active fraction of the microbial community. The latter is performed within the recently introduced in situ microcosm approach in groundwater samples (BACTRAP; Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010a, Schurig & Kästner 2012), which has been modified to be applicable also with the Direct-Push approach. Therefore, it is now also applicable to soil samples from the vadose zone (DP-BACTRAPs; Schurig et al. 2013a). Depending on the method used for analysis these approaches provide adequate phylogenetic resolution down to the species level, while all information regarding the spatial arrangement of the microbes is lost upon biomarker extraction. The lost spatial arrangement of active microbes, however, aggravates easy elucidation of microbial contaminant degrading consortia. Moreover, if the fraction of active microbes or microbial consortia is small compared to the total microbial community, their label gets attenuated in the biomarkers from the unlabelled biomass. This attenuation may prohibit accurate conclusions regarding the microbial metabolic activity when methods with high phylogenetic resolution, like RNA or DNA stable isotope probing (SIP; e.g. Manefield et al. 2002, Kasai et al. 2006), are applied, because these methods require a high isotopic enrichment in the microbial biomass. Less enrichment is needed e.g. for PLFA analysis, but the phylogenetic resolution of this method is rather coarse (Bombach et al. 2010a). Additionally, none of the methods applied so far is able to visualise both the degrading activity and the phylogenetic information for individual cells on in situ microcosms.
Microbes can be visualised by means of the fluorescence in situ hybridisation method (FISH; Amann et al. 1995). This technique is based on artificial fluorophore labelled nucleic acid probes, which can be tailored to identify microorganisms on various phylogenetic levels. However, the method requires a priori knowledge on the available microbes in the system of interest and thus prohibits a random walk approach. Contaminated aquifers and wetland sediments are opaque systems and have been shown to exhibit a high degree of auto-fluorescence (Tischer et al. 2012), which impedes the application of the FISH method. In order to increase the sensitivity of FISH in such systems, Tischer et al. (2012) developed a catalysed reporter deposition-FISH (CARD-FISH) approach for aquifer sediment samples. This approach, however, still requires cell detachment from the sediment prior to the fluorescence measurements, resulting in loss of information on the spatial arrangement of the microbial cells in the original sample. Finer scaled images with similar phylogenetic resolution and without loss of the spatial assembly can be obtained using antibodies or nucleic acid probes with attached gold-labels (GOLD-FISH; Schmidt et al. 2012) in combination with scanning electron microscopy (SEM). The drawback of both methods is that no information on the microbial contaminant degradation activity can be visualised. The use of radiolabelled contaminants together with a micro-autoradiography approach could be an option, whereby radiolabelled biomarkers are formed during biodegradation and represent the nucleus for deposition of elemental silver, which can later be detected by light

microscopy or by SEM. Besides being a valuable method for laboratory experiments, an in situ application would require the release of radioactive labelled contaminants to the environment, which definitely should be avoided.
For linking information on the morphology, the spatial arrangement and the activity of single microbial cells, nano-scale secondary ion mass spectrometry (NanoSIMS) offers significant advantages (Watrous & Dorrestein 2011). While studies already employed NanoSIMS to elucidate the identity of microbes and establish a link to their activity in water samples by means of a FISH approach with halogenated dyes (HISH-SIMS; Musat et al. 2008, Morono et al. 2011, Alonso et al. 2012), the potential of the instrument for studies in porous media such as soils or aquifers is currently just explored (e.g. Herrmann et al. 2007a, Herrmann et al. 2007b, Behrens et al. 2012, Mueller et al. 2012). This is, in part, due to the limitations of the NanoSIMS method, which prohibit analysing samples with a pronounced topography (Winterholler et al. 2008). Usually this issue is circumvented either by extraction and subsequent deposition of microbial cells onto filters (e.g. Musat et al. 2008) or by resin embedding approaches (e.g. Herrmann et al. 2007a, Mueller et al. 2012). However, both methods either destroy the spatial setting of microbes (filters) or prevent ancillary visualisation of the same samples by means of SEM (embedding).
Currently, to our best knowledge, no methods exist that can visualise the incorporation of an isotope label from a tracer compound together with the phylogenetic affiliation of individual cells in under in situ conditions in solid samples with a high degree of topography. In order to provide such an approach, we linked the recently improved in situ microcosm method (DP-BACTRAP; Schurig et al. 2013a) with NanoSIMS analysis and SEM. We chose a stepwise approach, which in the first step should provide the general feasibility of the chosen approach, by using a commercially available activated carbon material together with resin embedding. In the second step we preserved the three-dimensional structure of the sample by analysing sections of a special kind of commercially unavailable consolidated activated carbon carrier material, which rendered the resin embedding unnecessary. With these approaches we aimed at: I) providing data on microbial contaminant degradation activity in an aquifer and a wetland sediment, II) linking the activity of microbial cells to their spatial arrangement on activated carbon samples with high topography, III) providing a proof of principle for the linkage of the identity of microbes to their microbial activity by cultivating the indigenous microbial community of an aquifer and a constructed wetland by means of the HISH-SIMS method together with SEM, IV) optimising the sample preparation strategy for analysing DP-BACTRAPs for the spatial distribution of the microbes and their activity by a combination of stable isotope labelling, HISH-SIMS and SEM.

Methods and Materials

Field sites, The experiments were carried out at two well characterised field sites that feature extensive groundwater contamination with hazardous organic substances.
The first experiment was carried out in the BTEX contaminated upper aquifer at the field site Zeitz (Saxony-Anhalt, Germany). For a detailed site description see Gödeke et al. (2004), Vieth et al. (2005) and Schirmer et al. (2006). Briefly, the site shows extensive BTEX contamination of the subsurface, which originates from a former hydrogenation plant. Dissolution of the contaminants in the groundwater and advective transport led to the formation of contaminant plumes in two aquifers of the site. Our experiment was conducted in the upper aquifer, at the centre of the contaminant plume. Microbial mediated natural attenuation was earlier accepted as a site remediation strategy by the state of Saxony-Anhalt (LAF 2012) based on a proof of principle provided by means of in situ microcosms (BACTRAPs; Geyer et al. 2005). However, recent results suggest that microbial contaminant degradation is not effective anymore, because of a mobilisation of contaminants caused by an invasive source remediation action (Schurig et al. 2013a).
The second experiment was carried out at a site in Leuna (Saxony-Anhalt, Germany), where the groundwater is contaminated with fuel constituents like methyl-*tert*-butyl-ester (MTBE) and benzene due to decades of petroleum refining activities (for details: Seeger et al. 2011). From 2007 the contaminated water has been treated downstream of the contaminant plume by pilot scale constructed wetlands (Seeger et al. 2011). For our experiment, we selected a planted horizontal flow gravel wetland.

Preparation of the in situ microcosms, Several preparation steps in the laboratory were necessary before exposing the in situ

microcosms (BACTRAPs) in the wetland or the aquifer.
Two different carrier materials based on activated carbon were used for the experiments: For the Zeitz experiment a commercially available granulated activated carbon *(AC)* was used (AUF 540, Adako GmbH, Düsseldorf, Germany) in order to test, if the approach is feasible. For the Leuna experiment a special kind of commercially unavailable activated carbon beads (*Biosep*® beads; Peacock et al. 2004, manufactured by Kerry Sublette, University of Tulsa, USA) were used. *Biosep* beads consist of activated carbon dust embedded into a polymerised Nomex matrix, which was assumed to lead to no dust formation during NanoSIMS analysis and, thus, allows for analysis of unembedded sections. Biosep-beads, however, contain both carbon along with nitrogen, which possibly leads to a higher $^{12}C^{14}N$ background, which was accounted for by the analysis scheme.
The preparation procedure followed the one described by Schurig et al. (2013a): Prior to the use of the activated carbon materials, they were rid from any biogenic organic carbon residues by means of heating to 310°C for at least 4 h. 50 beads of the carrier material were filled into an acetone-rinsed vial (15 ml capacity). The beads were then autoclaved at 121°C for rehydration and sterilisation. After air-drying, the beads were put into a glass desiccator. The air inside the desiccator was exchanged against nitrogen gas (N_2), a small vial containing 50 µl of ^{13}C-α-toluene or $^{13}C_6$-benzene (Sigma Aldrich, Taufkirchen, Germany) was placed in the desiccator and a 50 mbar vacuum was applied. After complete evaporation of the substance and its sorption to the activated carbon (AC) beads, the BACTRAPs were transferred into sterile anaerobic water just prior to transport to the field.

Exposure of in situ microcosms, The field work at the Zeitz site was carried out as described previously (Schurig et al. 2013a). Briefly, DP-BACTRAPs were exposed at 10 m depth within the upper BTEX contaminated aquifer by means of a Direct-Push device (6610DT, Geoprobe, Salina, KS, USA) in the centre of the contaminant plume. After a six-week exposure, the probes were retracted by means of the same device.
For the experiment in Leuna, the beads were transferred into spades equipped with chambers upon arrival at the field site. These spades were exposed for four weeks in the wetland at 30 cm depth and 1.5 m away from the inlet. After exposure the spades were retrieved.
After both experiments the beads for NanoSIMS analysis were directly transferred to glass vials containing 2.5% glutaraldehyde fixation solution in water (w/w) and transported to the laboratory in cooled (~4°C), opaque containers. The fixation solution was renewed upon arrival to the lab and samples were stored overnight at 4°C. Within 24 h the fixation solution was exchanged against distilled water and samples were stored in an opaque glass container at 4°C until further processing.

Fluorescence in situ hybridisation (FISH), For the samples from Leuna, a modified catalysed reporter deposition – fluorescence in situ hybridisation approach (CARD-FISH; Pernthaler et al. 2004) with a fluorinated dye (Oregon Green 488, Invitrogen, Darmstadt, Germany) was performed to permit localisation of labelled cells by both epi-fluorescence microscopy, via the fluorescence of the dye, and by NanoSIMS, via the detection of the deposited fluorine (Musat et al. 2008), on the carbonaceous matrix material. The general CARD-FISH approach followed the procedure described by Pernthaler et al. (2004). Briefly, a horse-radish peroxidase (HRP)-labelled FISH-probe (biomers.net, Ulm, Germany) capable of detecting most bacteria (sequence 5' to 3': GCTGCCTCCCGTAGGAGT; Amann et al. 1990) was hybridised with the samples. In the next step Oregon Green labelled, fluorine containing tyramides, synthesised according to the procedure of Musat et al. (2008), were deposited at the HRP labelled spots.

Sample preparation, Analysing with NanoSIMS or SEM requires complete drying of the sample. For this purpose the water inside the samples was exchanged against acetone with a graded acetone series followed by critical point drying of the samples as described previously (Schurig et al. 2013b).
The following preparation procedure was different for the two sampling campaigns. The samples from Zeitz were completely embedded into a two component epoxy resin (Araldite 502, Electron Microscopy Sciences, Hatfield, PA, USA). After curing of the resin, the samples were sectioned and polished according to Herrmann et al. (2007a) and Heister et al. (2012).
The samples from Leuna were not embedded into resin, in order to avoid possible carbon detection interferences with the resin, and to test if the embedding step can be omitted. The

samples were mounted onto aluminium stubs using adhesive carbon tape (Plano, Wetzlar, Germany). Subsequently, loose particles were blown off by means of N_2 gas at 7 bar.
After finishing the preparation procedures, the samples were stored at room temperature in an opaque container within a glass desiccator over dry silica gel until further analysis.

Fluorescence and Scanning Electron Microscopy, The dry samples were analysed by means of fluorescence microscopy (Axioskop with HBO 50 lamp and AxioCam MRc, Carl Zeiss Microscopy, Jena, Germany).
In order to avoid any charging of the samples while analysing with NanoSIMS and SEM the surface of the samples were coated with a 30-nm layer of gold by means of a sputter coater (SCD 050, Balzers, Liechtenstein). The same sampling spots that were analysed by fluorescence microscopy were visualised by means of a field emission-scanning electron microscope (Ultra 55, Carl Zeiss SMT, Oberkochen, Germany) with the following parameters: working distance: 7.3-7.4 mm, excitation voltage: 2 kV, magnification: 1000-50000x.

NanoSIMS analysis, Nano-scale secondary ion mass spectrometry (Cameca, Gennevilliers, France) was used to visualize the spatial distribution of the ^{13}C label together with the organic matter and the F derived from the HISH treatment. Fields of view sizes ranged from 30*30 µm (256*256 pixels) for overview images to 6*6 µm (128*128 pixels) for detailed images and dwell times of 5-100 ms pixel^{-1} (5 ms pixel^{-1} for images providing a relatively fast overview and 100 ms pixel^{-1} for small detail images) were chosen. The aperture was adjusted to prevent saturation of the mass spectrometer detectors, especially with ^{12}C. Prior to each measurement the sample surface was implanted with Cs^+ primary ions until stable count rates were reached. This procedure enhances the yield of secondary ions for analysis. For the samples from Zeitz: $^{12}C^-$, $^{13}C^-$, $^{16}O^-$, $^{12}C^{14}N^-$ and $^{13}C^{14}N^-$; and the samples from Leuna: $^{12}C^-$, $^{13}C^-$, $^{16}O^-$, $^{19}F^-$, $^{12}C^{14}N^-$, $^{31}P^-$ and $^{32}S^-$ were detected. Prior to the measurements the mass accuracy of the instrument was checked by means of reference compounds, such as CaF_2 minerals for ^{19}F. No charge compensation was necessary, as the AC sample matrix together with the gold coating provided sufficient conductance to avoid excessive charging of the samples.

Data analysis, The NanoSIMS data were processed with the help of the Matlab-based (Matlab R2012b, The Mathworks, Natick, MA, USA) software package Look@NanoSIMS (Polerecky et al. 2012). For detailed comparison, ROIs assumed to be specific for pristine carrier material (class a), ^{13}C labelled biomass (class b), or unlabelled or moderately labelled biomass (class c) were selected and analysed. On every analysed image of the sample, 5 ROIs of each class were laid out manually and were analysed for differences in $^{13}C/^{12}C$ and C/F/P-ratios. Subsequently the images were grouped according to whether they represented the exterior of the AC beads or their interior, because previous SEM classification has shown a higher abundance of microbial biomass on the outside of the AC beads after exposure in groundwater.

Results & Discussion
The assessment of microbial activity under in situ conditions requires advanced techniques, especially for microbial communities that live and proliferate in opaque systems, like aquifer sediments or wetland gravel beds. In this study active microbial degradation of toluene and benzene was traced by metabolic stable isotope labelling in the recently improved in situ microcosm approach (DP-BACTRAP; Schurig et al. 2013a) This approach was combined with NanoSIMS, SEM and FISH analyses for visualisation and identification of the microbial cells and the incorporation of the labelled carbon into these cells. A stepwise approach, using two kinds of activated carbon carrier materials, was chosen in order to, first test the general applicability, and to, afterwards, test the approach with high topography samples.

Visualisation of microbial toluene degradation activity
Incubation of microcosms (DP-BACTRAPs) loaded with toluene in situ in the anoxic BTEX contaminated aquifer in Zeitz (Germany) led to colonisation of the supplied AC carrier material by indigenous microbes. Figure 1 demonstrates that the previously clean AC material is covered with intact microbial cells, but also with cells in various states of decay.
Together with the quantification of the extractable phospholipid fatty acid (PLFA) biomarkers presented by Schurig et al. (2013a), this provides evidence of microbial activity and colonisation of the AC.

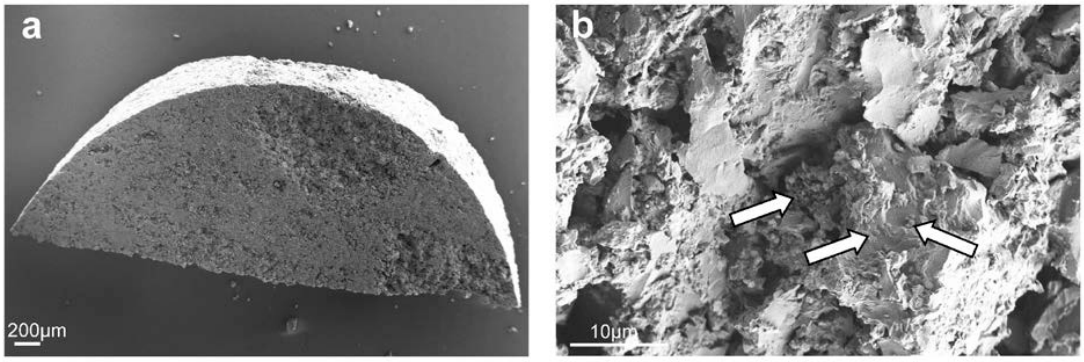

Fig.1: Scanning electron micrographs of the activated carbon material (AC) used in the first experiment in the Zeitz aquifer: panel a: overview of a sectioned part of the AUF-AC; panel b: detailed scanning electron micrograph shows coverage of the surface with intact bacterial cells (white arrows)

However, detailed optical inspection reveals that the microbial community appears to differ from the one previously found on conventional BACTRAPs exposed within groundwater monitoring wells at the same site, at the same spots and in the same depth (Miltner et al. 2012). In particular, no signs of microbial extracellular polymeric substance (EPS) are visible and the surface seems to carry a significantly lower amount of bacteria. Presumably, diminished microbial activity along with a remediation measure at the site, as shown previously (Schurig et al. 2013a), could be the reason for this finding. Besides this, Schurig et al. (2013a) already demonstrated by means of PLFA analysis, that the microbial community on DP-BACTRAPs is more similar to the one found in adjacent sediment than the one found on conventional BACTRAPs exposed in groundwater monitoring wells, such as the ones used by Miltner et al. (2012). Therefore, the different appearance of the microbial community could also be a result of the direct exposition of DP-BACTRAPs in the aquifer sediment. The lower number of microbes and the composition of the microbial community also seem to compare better to the microbial community commonly found in soil and groundwater sediment samples (e.g. Heijnen et al. 1993, Fakih et al. 2008, Lindedam et al. 2009, Cuadros et al. 2010), particularly when the lower microbial abundance commonly found in deeper soil layers is considered (Bone & Balkwill 1988, Bekins et al. 1999).

The mere presence of microorganisms, however, does not prove that these microbes show any particular activity, e.g. contaminant degradation. In earlier studies at this field site, evidence of in situ microbial contaminant degradation activity was provided by the detection of biomarkers enriched in the supplied stable isotope tracer from the BACTRAP carrier material (Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010b). Schurig et al. (2013a) showed that the bulk extraction of PLFA from the AC yielded lower amounts with almost no isotopic enrichment of the biomarkers compared to the previous results, presumably caused by a remediation measure. The SEM analysis, however, does not allow obtaining information on the isotopic enrichment, and thus the activity, of the visualised cells. Moreover, only the morphology of the visualised cells can be evaluated, but exact phylogenetic information requires further taxonomic analyses by molecular biology methods. Phylogenetic information can only be obtained by parallel extraction of biomarkers and subsequent comparison of the bulk extractions to the images. However, biomarkers are usually extracted from 1 g of AC. This weight corresponds to a surface area of 300 to 600 m^2 of the carrier material, which cannot completely be inspected by SEM. Even if the surface was representatively imaged by SEM, it is not possible to assign phylogenetic information to specific cells because the information on the spatial distribution of ^{13}C incorporation into the cells is lost during biomarker extraction. The GOLD-FISH method (Schmidt et al. 2012) can provide this phylogenetic information by hybridising gold-labelled RNA probes to particular cells of a selected taxonomic group. However, some data on the metabolic activity of microbes can be inferred from this method by means of the rRNA content of the cells (Bulgarelli et al. 2012). Information on whether these cells are actively degrading contaminants cannot be provided. The visualisation of active bacteria by means of SEM would require a microautoradiography approach, such as the one used by Lolas et al.

(2012), which, however, was only combined with optical microscopy for enrichment cultures in the Lolas et al. (2012) study. Furthermore, such an approach would either require loading the BACTRAPs with radiolabelled contaminants prior to exposition or incubating colonised microcosms with radiolabelled contaminants in the laboratory subsequent to exposition. The potential release of radioactive isotopes to environmental compartments prohibits the application of the first approach. The second approach, however, is flawed by a potential bias arising from the laboratory incubation, which possibly leads to preferential growth of degrader microbes or even does not allow for cultivation of all microbes (Amann et al. 1995). In order to avoid these potential sources of bias, in the present study stable isotope labelled compounds and a visualisation approach based on NanoSIMS analysis of ^{13}C labelled biomass were used.

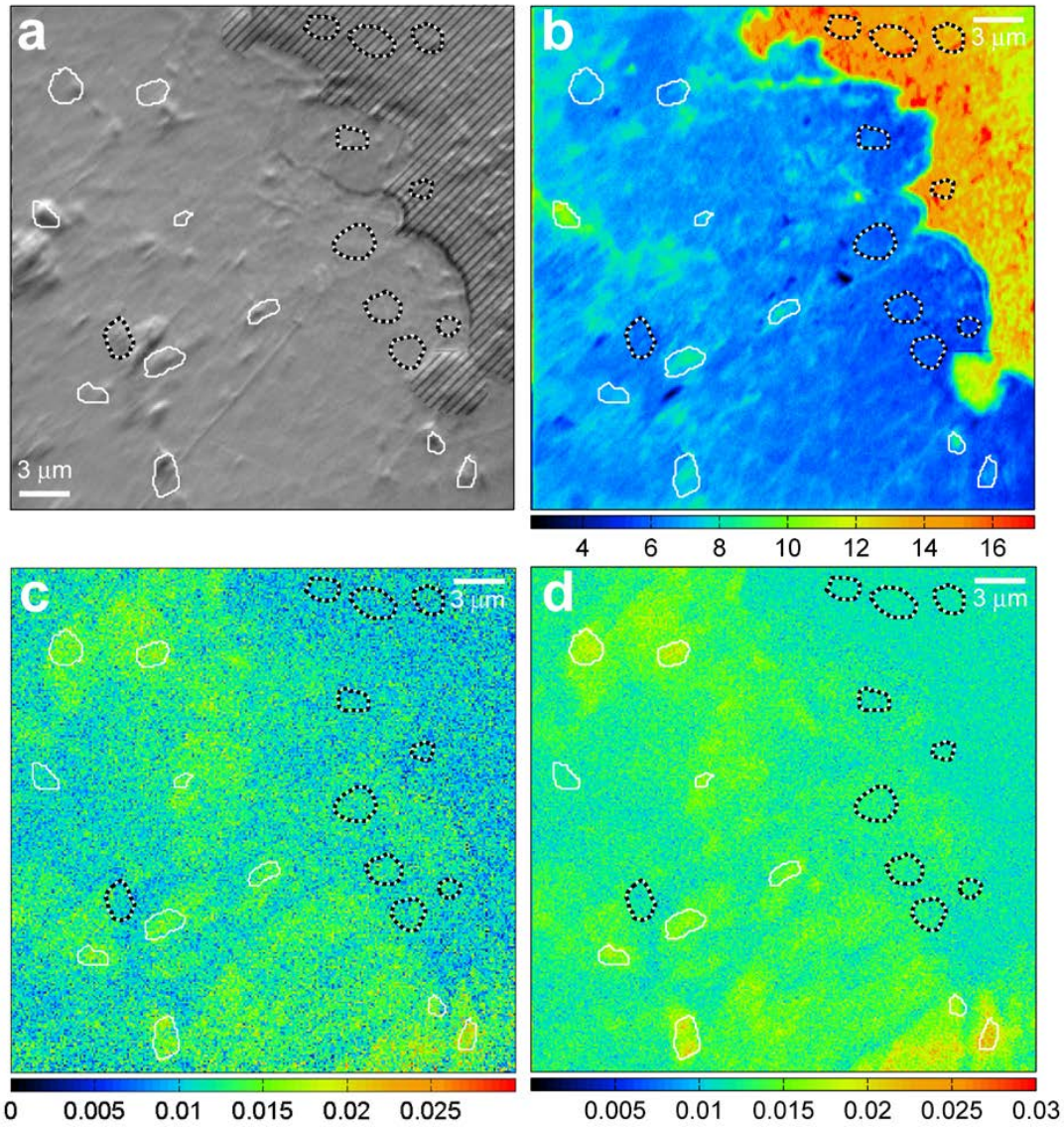

*Fig. 2: NanoSIMS images (30*30 µm): panel a: secondary electron image (SE) of the samples surface with embedding (hatched area), and selected regions of interest (ROIs) indicated by circles (white: biomass; black: background); panel b: cyanide ($^{13}C^{14}N$) image for indication of enriched biomass; panel c: $^{13}C/^{12}C$ mass ratio distribution; panel d: $^{13}C^{14}N/^{12}C^{14}N$ ratio mass distribution as biomass indicator.*

Figure 2 shows the images recorded by NanoSIMS. The secondary electron (SE) image (figure 2a) demonstrates a low topography of the resin ebedded and subsequently polished sample surface. On the one hand, this improves the quality of the NanoSIMS analysis by minimising topographic effects (Winterholler et al. 2008, Mueller et al. 2012). On the other hand, microbes are not only being present at the cross section which was exposed during sample preparation. Therefore, a substantial fraction of the information regarding the spatial arrangement of the microbes, in particular their spatial variability in the z dimension, is lost due to the embedding procedure, as the AC material has a very large and tortuous surface area. The morphology and cell shape of microbes, thus, cannot be identified due to the difficulty to detect their cellular shape in the embedded samples.

We were incubating AC carrier material in the aquifer, thus a high C background (including ^{13}C background at natural isotopic abundance) can be expected, which complicates the identification of microbial biomass. The background is further increased for both C and N by the embedding procedure, again for both elements at natural isotopic abundance. Nevertheless, we could show by analysis of $^{12}C^{14}N$ ions (m/z 26, figure 2b), which can be used to discriminate biomass from other carbonaceous material in NanoSIMS studies (Lechene et al. 2006, Herrmann et al. 2007b, Mueller et al. 2012), that this problem can be overcome. However, as figure 2b shows, the embedding medium leads to higher counts for this ion species than the AC surface that harbours the microbes. This is due to the N content of the DMP-30 hardener used for the embedding resin. Yet, the calculation of the C/N ratio shows very low values for the resin and higher values throughout the AC surface (figure S1) and is therefore indicating that the embedding procedure is not adversely affecting the detection of microbial biomass on the interior surfaces of the AC beads. Areas enriched in this ion species are clearly visible in the interior of the AC beads, and hence two classes of regions of interest (ROI) were chosen, also with help of the ^{13}C and $^{13}C^{14}N$ data, to represent either biomass or background referring both to the AC and the embedding resin.

In order to determine the contaminant degrading activity of the microbial biomass, the isotopic enrichment of the detected cells was calculated relative to the background ratio. The data for the $^{13}C/^{12}C$ ratio is presented in figure 2c, while the ratio of $^{13}C^{14}N/^{12}C^{14}N$ is presented in figure 2d. No distinct differences could be found when comparing both images. This indicates that most of the ^{13}C-labelled toluene, which was loaded onto the AC, was converted to biomass, because otherwise regions with higher ^{13}C content, and thus higher $^{13}C/^{12}C$ ratio, would not be reflected in the $^{13}C^{14}N/^{12}C^{14}N$ ratio, because toluene contains no nitrogen whereas biomass does. Moreover, the ^{13}C labelled biomass is not equally distributed over the AC. This arises from both a patchy pattern of the colonising microbial community and from analysing just the few microbial cells located in the intersecting plane of the highly porous material. The patchy distribution of microbes is similarly shown by the SEM images (figure 1) and can also be seen in the images presented in the earlier study of Miltner et al. (2012). Patchy distribution pattern of microbes is also known from soil systems (Franklin & Mills 2003, Becker et al. 2006, Lehmann et al. 2008); however, up to now, the presence and the activity of the microbes could not be linked under in situ conditions.

Detailed comparison of the ROIs by means of Kruskal-Wallis tests (Kruskal & Wallis 1952) reveals that the two selected groups of ROIs are significantly different ($p=0.0005$) for both ion ratios, but only a small enrichment of the labelled biomass ROIs of 1.45 % ± 0.18 for $^{13}C/^{12}C$ and 1.6 % ± 0.22 for the $^{13}C^{14}N/^{12}C^{14}N$ ratio was found, which, nevertheless, shows an approximate labelling efficiency of 10at%, because the supplied ^{13}C-α-toluene is only labelled at one position and thus contains only 14at% ^{13}C. Moreover, the natural isotopic ratio of $^{13}C/^{12}C$ of 1.07 % is adequately reflected for both ion species, with values for the background ROIs of 1.10 % ± 0.05 for $^{13}C/^{12}C$ and 1.18 % ± 0.03 for the $^{13}C^{14}N/^{12}C^{14}N$ ion ratio. The results demonstrate that the embedding effect can be overcome, besides having higher carbon and nitrogen content than the biomass on the AC. However, the resin embedding has resulted in a dilution of the biomass label, which cannot be quantified. The significant enrichment of the labelled biomass ROIs demonstrates that microbes in the upper aquifer of the Zeitz (Germany) site are still actively degrading toluene after the remediation action. However, the isotopic enrichment found in the microbes colonising the AC beads is still considerably lower than the one found in earlier studies at this particular field site (Geyer et al. 2005, Kästner et al. 2006, Bombach et al. 2010a). Nevertheless, the NanoSIMS visualisation

approach using resin embedding, seems to be more sensitive than the PLFA-SIP approach used by Schurig et al. (2013a), which showed very low isotopic enrichments of the PLFA-biomarkers of about -17$\delta‰_{vs.\ VPDB}$. The present study, thus, supports the conclusion of Schurig et al. (2013a), that the microbial contaminant degradation activity at this site decreased recently, most likely caused by the source remediation action.

The comparison of the ROIs reveals that almost all biomass shows ^{13}C enrichment of equal magnitude. This demonstrates that most of the detected biomass inside the AC originates from toluene degraders, or stems from microbes that have been cross-feeding on labelled degrader microbes. The low isotopic enrichment of 1.45% suggests that these organisms also use other C sources in addition to toluene. While the cross-feeding effect can currently not be quantified, Ringelberg et al. (2008) demonstrated higher cell numbers of microbes in contaminated zones relative to uncontaminated zones of soil in equal depth. Other researchers demonstrated a higher fraction of motile degrader microbes within contaminated zones (Harvey et al. 1984, Godsy et al. 1992, Bekins et al. 1999). Both facts reasonably support the conclusion that a high fraction of the colonising microbes are actively degrading toluene, and that the DP-BACTRAPs, in this framework, lead to a selective enrichment of degrader microbes. However, these conclusions are burdened by some uncertainty resulting from the resin embedding; therefore the resin was omitted in the second approach for visualisation.

Microbial benzene attenuation in a constructed wetland

In order to test the applicability of the approach in a system with much higher microbial activity, like the rhizosphere, which is harbouring orders of magnitude more microbes (Bone & Balkwill 1988, Tringe et al. 2005). We incubated benzene loaded BACTRAPs in the aerobic rhizosphere of a constructed wetland loaded with benzene contaminated groundwater. After 4 weeks of exposure, the initially clean Biosep-bead surface was completely covered with several layers of a diverse microbial community (figure 3a + b). In contrast to the AC exposed in the groundwater, no colony formation was observed, because all microbes seem to be embedded into a biofilm like structure, which also contained extracellular polymeric substances (EPS). This suggests that, compared to the microcosms incubated in the aquifer, more microbes have been colonising the Biosep-beads in shorter time. The observed biofilm is a common feature of constructed wetlands (e.g. Weber & Legge 2011, Wu et al. 2011, Faulwetter et al. 2013) and the observable fungal growth (figure 3b: highlighted by arrows), supported by the aerobic nature of the system, highlights that streptomycetes and fungi can be an integral part of these systems (Fester 2013). While streptomycetes are known to be capable of degrading benzene (Thumar & Singh 2009); Fester (2013) reported rapid fungal colonisation of wetlands with arbuscular mycorrhiza within a few years. Our data, however, suggest that hyphae are an integral part of the newly established biofilm on the BACTRAPs even after 4 weeks.

Another feature evident from figure 3b (insert) is the presence of extracellular polymeric substance (EPS), which filamentous, spiderweb-like shape in the micrographs is caused by the critical point drying procedure (Miltner et al. 2012). These substances are excreted by microbes along with biofilm formation in order to aid adhesion to surfaces and cell-to-cell communication (Madigan et al. 2012). They are therefore a sign of an active biofilm (Miltner et al. 2012). Although many microorganisms in soil are capable of producing EPS (Chenu 1993), dense EPS networks, as found in figure 3b, are rarely observed in natural soils and sediments with the exception of biocrusts (Fischer et al. 2010) and soils amended with microorganisms (Maqubela et al. 2009). Consequently, their observation on the Biosep-beads indicates that the BACTRAPs are more densely colonised by microbes in comparison to the wetland sediments, although EPS along with hyphae and bacterial cells were also found on sand grains from these wetland systems (E. Seeger, unpublished results).

For analysing the spatial colonisation visible without embedding, we applied the HISH-SIMS approach according to Musat et al. (2008) for labelling bacteria. This is a special CARD-FISH approach attaching a halogenated fluorescent dye to a nucleic acid probe that hybridises to bacterial rRNA. This approach enables detection of bacteria by means of epi-fluorescence-microscopy via the fluorescent dye and by NanoSIMS via the detection of the deposited halogens (Musat et al. 2008).

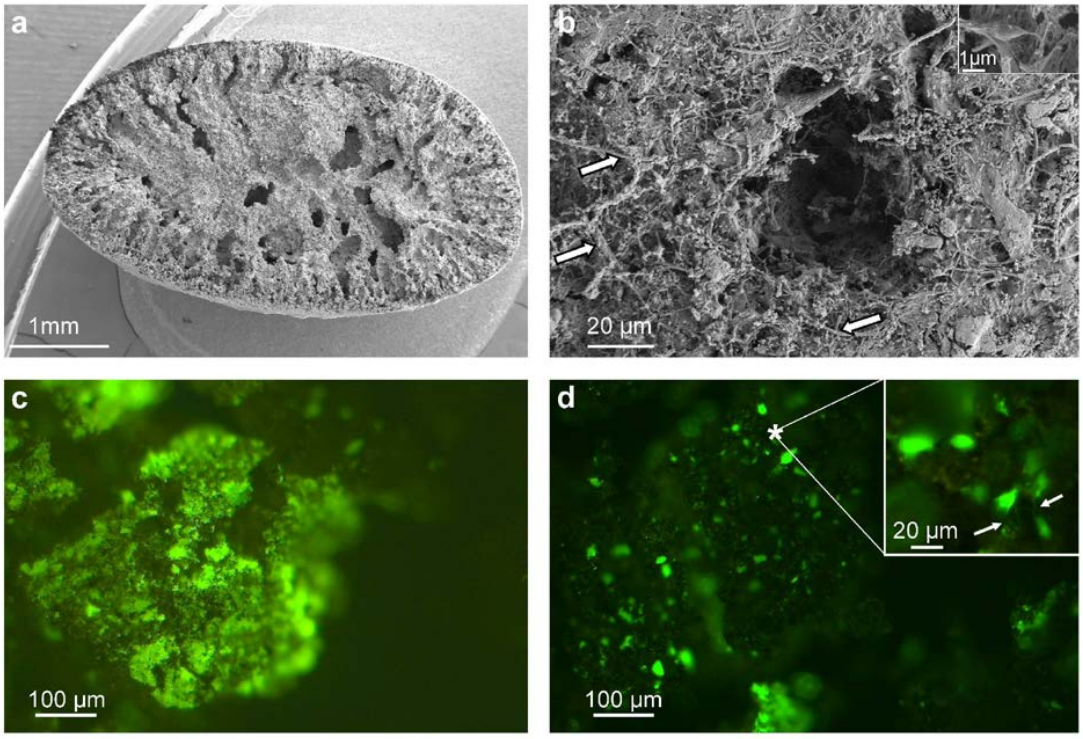

Fig.3: SEM and fluorescence images of the Biosep beads used in the Leuna experiment: panel a: overview image reveals high internal surface area of the clean Biosep-beads prior to exposure; panel b: The Biosep-beads after 4 weeks long exposure, arrows highlight hyphae. Insert (*) presents microbial cells along with EPS; panel c: fluorescence image of the outer surface after the HISH-SIMS method; panel d: fluorescence image of the inner surfaces. Insert (*) demonstrates labelled EPS strands (white arrow).

The fluorescence images are presented in figure 3c & d for parts of the sample representing the outer surface of the Biosep-beads (figure 3c) and the interior of the sample (figure 3d). In agreement with the SEM results, the outer surface of the Biosep-beads is almost fully stained, indicating that it is almost completely covered with active bacterial cells or cell residues. Moreover, a part of the EPS is also labelled with the fluorescent dye, which suggests that, embedded into the EPS, extracellular RNA exists and is labelled in this biofilm (figure 3d: insert). Although extracellular nucleic acids were previously assumed to mainly consist of DNA (Lorenz & Wackernagel 1994), recent results, however, suggest that a significant part of these nucleic acids is RNA (Ando et al. 2006), which is assumed to influence both biofilm proliferation and intercellular communication within biofilms.
Another explanation for extracellular staining could be that some of the intracellular RNA has been leaking out of the permeabilised cell membranes during the CARD-FISH procedure (Eickhorst & Tippkötter 2008), although this would not explain the strand like appearance of the stained regions in the images. Bombach et al. (2010a) performed confocal-laser-scanning microscopy on stained (SYTO 60) samples of Biosep-beads, which were previously incubated in laboratory microcosms containing an enrichment-culture from the Zeitz site. While Bombach et al. (2010a) detected mainly rod-shaped bacteria, our results from the SEM (figure 1 + 3) and the FISH approach (figure 3) suggest that a different, more diverse, microbial community was inhabiting both the aquifer samples and the wetland rhizosphere. While the result from the rhizosphere could be due to the completely different microbial community inhabiting the wetland, the result from the aquifer (see Zeitz experiment), together with the data of Miltner et al. (2012), suggests that the microbial community of Bombach et al. (2010a) was already affected by the laboratory cultivation.
Another feature of the fluorescence data is the clear difference in fluorescence intensity of the exterior and the interior of the Biosep-beads. This suggests that most of the active bacteria

are situated at the outside of the Biosep-beads, where they could not be detected with the resin embedding approach used for the aquifer samples.
While the use of Biosep-beads enabled us to examine the samples in parallel by fluorescence microscopy, SEM and NanoSIMS, the high degree of topography as already observed in figure 3 (a+b) affected the SIMS measurements (Herrmann et al. 2007b, Mueller et al. 2012). In order to normalise for topographic and matrix effects we use ratios of different ions for further analysis, assuming that all ion species are equally affected to the same extent.
Figure 4a presents the results of the NanoSIMS analysis for the $^{12}C^{14}N$ ion of a part of the mantle biofilm. The recorded distribution of $^{12}C^{14}N^-$ supported our assumption of a multi-layered biofilm, as already suggested from the SEM images as well as from the fluorescence data. Cellular shapes are now clearly identifiable (white arrows, figure 4a).

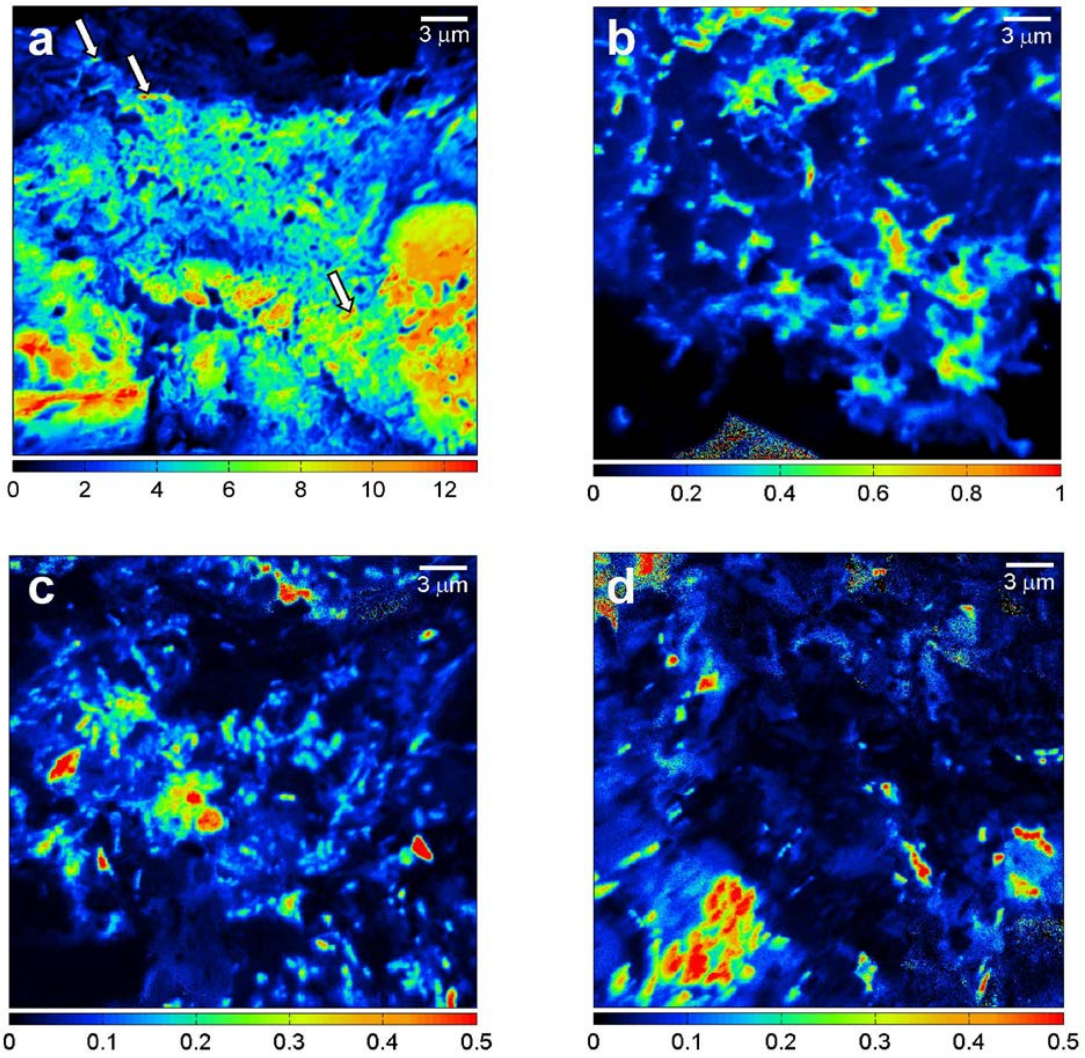

*Fig.4: NanoSIMS images (30*30 µm) from the Leuna experiment: panel a: cyanide ($^{12}C^{14}N$) image indicates coverage of the mantle area of the Biosep-beads by biomass; panel b: cyanide to ^{12}C ratio ($^{12}C^{14}N/^{12}C$) from an inner part of the sample reveals negligible influence of the nitrogen stemming from the Biosep-beads and less coverage of the surface with biomass compare to panel a; panel c: $^{13}C/^{12}C$ ratio of mantle area indicate only few highly ^{13}C- labelled spots of microbial phenotype; panel d: $^{13}C/^{12}C^{14}N$ image of inner surface reveals similar performance of detection of active microbes caused by the Nomex. (for more images see Fig. S2)*

For further analysis the NanoSIMS images were divided into 3 spots representing the mantle area (figure 4c & figure S2 a+c) and 2 spots representing inner surfaces (figure S2 b&d) of the Biosep-beads to enable a comparison regarding the quantity of the active microbes by means of their $^{13}C/^{12}C$ ratios. From the abundant biofilm formation on the outer mantle area of the Biosep-beads (figure 3) we expected relatively more active microbes on this part of the sample. However, as figure 4 and figure S2 demonstrate, areas with a relatively high ^{13}C enrichment (>10 at%) are distributed in a patchy manner, with no clear difference between the exterior and the interior of the Biosep-beads. For example, figure 4c and figure S2b both represent a high amount of highly enriched areas, although they are representing the exterior and the interior of the Biosep-beads, respectively. In contrast, figure S2c represents a part of the outside which shows, similar to figure S2d (interior), only a very small amount of highly enriched areas, which, nevertheless, could clearly be attributed to cellular shapes in both cases. Altogether, these data suggest a selective enrichment of compound specific degraders on the inner surfaces of the Biosep-beads, because benzene substrate availability or sheltering from predators within these microniches influences their distribution. Moreover, these results are in contrast to the previous results from the toluene degradation experiment in the aquifer (see Zeitz experiment), where most biomass was identified as toluene degraders by its ^{13}C label. However, it has to be kept in mind that the outer surfaces of the AC could not be analysed in the aquifer experiment. Higher or similar degradation potential caused by microniches was demonstrated by Barragan-Huerta et al. (2007), who supplied DDT contaminated cultures with coffee beans and reported rapid colonisation of the inner surfaces of the beans with degrader microbes. The relatively patchy distribution of the microbial biomass on the inside of the Biosep-beads more closely represents the results of Bombach et al. (2010a), who reported formation of micro-colonies containing rod-shaped bacteria upon toluene degradation. Gomes et al. (2010) extracted and enriched petroleum hydrocarbon degrading microbial consortia from the contaminated rhizosphere of mangrove trees and found the petroleum hydrocarbon related genes and plasmids to be below their detection limits in environmental samples. Our data might provide reason for the data of Gomes et al. (2010), as it shows a very low fraction of active degrader microbes embedded in a biofilm-like formation in the rhizosphere of a constructed wetland. This low fraction means that bulk extractions of biomarkers from the rhizosphere can lead to underestimation of microbial degrader activity of particular cells on the single cell level, because their labelled biomarkers get attenuated in the larger amount of unlabelled biomarkers from non-degrader microbes. However, Braeckevelt et al. (2007) investigated the monochlorobenzene degradation in a similar constructed wetland system as the one used in our study and were still able to detect highly enriched fatty acid biomarkers with in situ microcosms (BACTRAPs).

While the previous results were just focussing on the identification of active microbial cells by means of their morphology, the HISH-SIMS approach (Musat et al. 2008) labels the target organisms with F (m/z 19) and thus can be applied to additionally discriminate ^{13}C-labelled bacteria from residual ^{13}C-labelled benzene via the localisation of areas enriched in fluorine. The same ROIs used for identification of the isotopic label were also used for analysis the $^{13}C/^{19}F$ ratio. The approach provided clear advantages and results for our samples, as the $^{13}C/^{19}F$ ratios were significantly different between the biomass ROI classes (classes *b* and *c*; see Methods) and the background ROI class (class *a*; Table 1). However, identification of single cells remained challenging due to the homogeneously distributed F label in the images. Reasons for this can be the relatively high background of fluorine stemming from the adhesive carbon tape (figure S3) or fluorine resulting from the exposure in the wetland. Moreover, as already evident from figure 3 (c&d), the Biosep-beads are showing a´n almost complete fluorescence on every visualised image and, thus, fluorine is widely distributed. Only the use of a more specific FISH-probe for benzene degraders could have produced more significant labelling of ^{13}C-labelled spots, but would not have stained unrelated bacteria. Consequently, for future applications of the Musat et al. (2008) approach in soil or samples with high topography, care should be taken to avoid potential fluorine contamination arising from the samples matrix or from the environmental incubation and, moreover, the intensity of the HISH-SIMS labelling should be increased in order to have higher yields of ^{19}F counts in the biomass.

*Table 1: Selected ratios of ion-species compared for ROI classes (a=background; b=highly ^{13}C enriched biomass; c= moderately enriched or unlabelled biomass). Several spots with more than 5 ROIs per class were analysed (outside n=3; interior n=2). *represents significant differences to other ROI classes*

position on AC	ROI class	13C/12C	STD	13C/19F	STD	13C/31P	STD
exterior	**a**	0.02*	0.00	3.16*	1.17	173.80*	79.60
	b	0.15*	0.05	18.40	7.01	33.17	16.83
	c	0.07*	0.02	9.07	2.67	29.57	14.86
interior	**a**	0.01*	0.00	4.73*	1.65	129.65*	45.15
	b	0.22	0.08	47.00	18.31	39.70	15.05
	c	0.10	0.04	24.15	17.88	42.70	20.30

Schmidt et al. (2012) proposed the deposition of gold-labels via a CARD-FISH approach to identify certain microbes in soil samples by means of SEM. While the original Schmidt et al. (2012) method is clearly not applicable for monitoring the activity of the labelled microbes, because the isotopic enrichment cannot be detected by means of SEM, a combined approach of cell identification by means of SEM and later activity identification by means of NanoSIMS would maybe aid the phylogenetic identification of certain active microbes. However, with using this approach, a thorough pre-characterisation of the sample by SEM is mandatory, since the spots analysed by NanoSIMS are damaged to some extent by the primary Cs^+ beam (figure S4). Also, for this combination, gold cannot be used as a conductive coating for the samples during sample preparation, because it would impair the detection of the gold deposited during the CARD-FISH procedure.

In order to localise biomass, in spite of the unfavourable conditions for the detection of enrichments in ^{19}F, we included ^{31}P into the NanoSIMS analysis. Table 1 shows that the $^{13}C/^{31}P$ ratio is significantly different for most of the ROIs representing ^{13}C-labelled biomass and moderately labelled biomass compared to ROIs representing background material. These data demonstrate that the ROIs with high $^{13}C/^{12}C$ ratio are actually representing active microbial cells and not just areas with residual ^{13}C-benzene-substrate, because phosphorous is a significant part of the microbial biomass in the form of PLFA, nucleic acids and other biomolecules (Madigan et al. 2012). Thus, together with the nucleic acid staining by the CARD-FISH procedure (Pernthaler et al. 2004, Musat et al. 2008), the $^{19}F/^{12}C$ (figure 5b) and $^{31}P/^{12}C$ (figure 5c) ratios should mirror bacterial cells, but while the ^{31}P signal quite accurately follows the microbial cellular shapes (figure 5c), the ^{19}F counts seem to be more equally distributed over the entire image (figure 5b). Hence, the ratio of $^{19}F/^{31}P$ (figure S5) is above 10 at areas (red) without microbial cells and lower directly at the microbial cells, because very little or no ^{31}P was detected outside the cells (figure S5).

Consequently, the detection of the ^{31}P signal together with the isotopic enrichment of the biomass is an equally accurate proxy for microbial biomass in our experiment compared to the ^{19}F deposited by the HISH-SIMS approach (Musat et al. 2008) and, moreover, helps identifying biomass by means of the ^{19}F proxy in parts of the sample with high background. Only low phosphorus enrichment could be found in the unlabelled biomass, which indicates that it either consists of dead microbial biomass fragments (Miltner et al. 2012, Schurig et al. 2013b) or of dead plant material. This dead material can be assumed to be depleted in ^{31}P since phosphorus-containing biomolecules, such as PLFA and RNA, are rapidly degraded after cell death and can hence be used as markers for living biomass (Green & Scow 2000, Pinkart et al. 2002, Kaur et al. 2005).

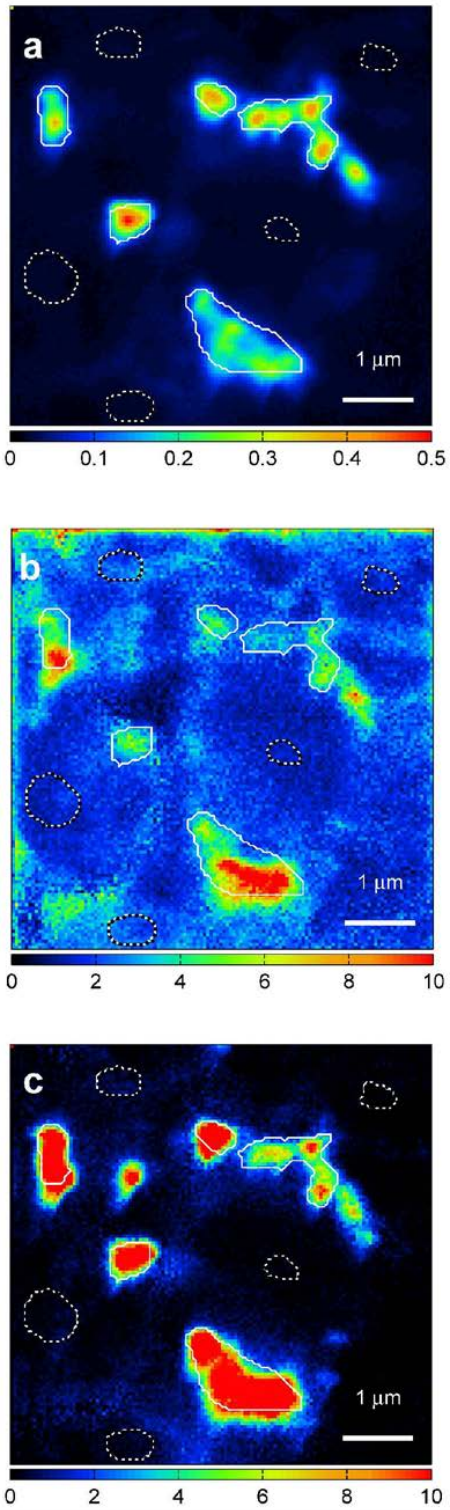

*Fig. 5: Detailed NanoSIMS images (6*6µm) of highly ^{13}C enriched microbial cells, ROIs: white line labelled biomass, dotted line background: panel a: $^{13}C/^{12}C$ ratio; panel b $^{19}F/^{12}C$ ratio (legend= ratio*1000); panel c: $^{31}P/^{12}C$ ratio (legend= ratio*1000).*

Altogether we have demonstrated the general applicability of the HISH-SIMS approach for this kind of samples; together with the analysis of ^{31}P enrichment this allows for easy identification of active microbial biomass in samples with a high background and topography. However, detailed conclusions on the taxonomic affiliation of the visualised microorganisms would require the application of a more specific RNA-probe. This can be achieved by further development of the HISH-SIMS approach in order to enable measurements with two or more halogens with degrader-specific and unspecific RNA-probes. The study showed the potential of NanoSIMS analysis together with an in situ isotope labelling approach to investigate the three-dimensional distribution of microbial contaminant degradation under in situ conditions. Therefore, the approach combining fluorescence microscopy, SEM and NanoSIMS will be a powerful tool to link taxonomy of microorganisms with their activity on the single cell level even in opaque and highly spatially heterogenic soil, sediment or activated carbon materials with high surface areas.

Conclusions

Using a combination of the recently improved in situ microcosm system (Schurig et al. 2013a) and NanoSIMS analysis enabled us to visualise the actively toluene or benzene degrading microbial community in an aquifer and a wetland under in situ conditions, while preserving its three-dimensional formation. The analysis revealed that most of the microbial community colonising the exposed AC and Biosep-beads was ^{13}C-labelled and thus was either involved in direct toluene or benzene degradation or was cross-feeding on degrader microbes. In the aquifer, similar images for the $^{13}C/^{12}C$ and the $^{13}C^{14}N/^{12}C^{14}N$ ratios allowed the conclusion that most of the supplied ^{13}C-label was transferred from the labelled toluene to the biomass, thereby indicating microbial contaminant degradation potential. In order to deal with the high degree of topography these samples can be resin embedded prior to NanoSIMS analysis. While this procedure permits separate analysis of the single ion species, it prohibits correct identification of the morphology of single microbial cells and the investigation of microbial cells attached to the outside of the AC beads.

Unembedded sections of more consolidated Biosep-beads were used for analysis in a wetland, together with a FISH approach with fluorescence microscopy, SEM and

NanoSIMS. This approach enabled us to correctly identify microbial cells and to test the applicability of this approach for later studies using more specific nucleic acid probes. The fluorine labelling approach (HISH-SIMS) in the NanoSIMS was inaccurate for our samples due to the high background resulting from the almost complete fluorescence labelling. We could clearly identify highly ^{13}C-labelled, and thus highly metabolically active, microbial cells, both by their $^{13}C/^{12}C$ ratio, and by their phosphorous content. Detailed analysis, moreover, revealed that, besides the observation of a dense biofilm formation at the outer side of the Biosep-beads by means of SEM, the quantity of microbes involved in benzene degradation was similar to the one in the interior of the Biosep-beads. Thus, similarly to the Musat et al. (2008) study, only a small fraction of the microbial community is highly active in this environment.

We have demonstrated the potential of the combination of two advanced techniques for tracing in situ microbial contaminant degradation, which can become very helpful for future studies to elucidate trophic dependencies of microbial communities upon contaminant degradation. Moreover, bias due to cultivation of microbes in the laboratory can be avoided by means of these techniques.

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2.5 Microbial cell-envelope fragments and the formation of soil organic matter - a case study from a glacier forefield

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Abstract:
The contribution of microbial cell-envelope fragments, which have previously been reasoned to be the nucleus for SOM formation (Miltner et al., 2012), to SOM was traced in SEM images of soil samples obtained from a chronosequence of soil formation in a glacier forefield in Switzerland. This data was related to C and N contents, the active microbial community analysed by PLFA extraction, and to the water contact angles on macro- and microscales. The results demonstrated increasing number of cell-envelope fragments in accord with increasing C/N ratios, contact angles, PLFA quantity and a changing microbial community. Thereby, the Miltner et al. (2012) theory was proven in the field.[5]

Author contribution: 60%
The samples were obtained by Matthias Kästner, Rienk Smittenberg and me. The SEM images were obtained by myself on the instrument of Jürgen Berger and I developed the image analysis scheme. The PLFA extractions were done by Fabio Kraft under my and Hermann Heipiepers supervision. Susanne Woche and Marc-Oliver Göbel did the contact angle and ESEM measurements. I collected the data and prepared the manuscript together with Matthias Kästner and Anja Miltner.

(signature of supervisor)

[5] Supporting information is provided in the appendix p. XLVIII

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Microbial cell-envelope fragments and the formation of soil organic matter: a case study from a glacier forefield

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Abstract Genesis of soil organic matter (SOM) during pedogenesis is still a matter of controversy in soil science. Recently, it was hypothesized that microbial cell-envelope fragments contribute significantly to SOM formation. We tested the relevance of this process during pedogenesis by evaluating the development of SOM along a chronosequence of a glacier forefield (Damma glacier). Samples of increasing soil age collected along the forefield were analyzed for C and N contents, phospholipid and total fatty acids (PLFA and tFA), water contact angle, micro-hydrophobicity and surface coverage by microbial cell-envelope residues. The surface coverage was visualized and quantified by analysis of representative, equally-scaled scanning electron micrographs (SEM). Increasing SOM contents were accompanied by increasing coverage and overall abundance of microbial cell-envelope fragments as evaluated on the basis of scanning electron microscopy; this is also reflected in the amounts of tFA and PLFA, the trend of C/N ratios, and the increasing hydrophobicity and water contact angles of the soil samples. Using SEM and the image analysis approach, we can provide a process-based description of the development of SOM in the newly developing ecosystem of the glacier forefield. The majority of small-sized SOM visible with scanning electron microscopy appears to consist of bacterial cell envelope fragments that remain stable after cell death, such that their shape does not change with soil age. Our results show the importance of microbial processing of SOM, and highlight the existence of microbial necromass as a significant part of the fine-particulate SOM even in later stages of soil development.

Keywords SOM formation · Microbial cell-envelope residues · PLFA · FA · C/N ratio · Contact angle · Hydrophobicity · Chronosequence · Glacier forefield

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Introduction

Beside fossil carbon, non-living soil organic matter (SOM) is the second dominant pool of organic carbon within the C cycle on earth and is important for soil fertility (Schmidt et al. 2011). SOM is generally assumed to be mostly inert (Schulze and Freibauer 2005), but significant losses of SOM have been found in several countries within the last few decades (Sleutel et al. 2003; Bellamy et al. 2005). In general, the organic matter contents in soils represent steady-state concentrations controlled by the input from litter and dead organisms on the one hand and microbial degradation on the other hand. The degradation of the particulate organic materials leads to mineralization products including CO_2, as well as the formation of dissolved organic matter (DOM) in the soil pore water. In addition to losses by erosion, losses of SOM are thus caused either by diminished inputs (e.g. by land-use changes such as increased cultivation of energy crops) or by increased microbial metabolization (Bellamy et al. 2005). In the Bellamy study, estimated annual losses by extrapolation for the UK ($\sim$13 million tons of C) are considered to be of the order of a decade's reduction in greenhouse gas emissions (Schulze and Freibauer 2005). This documents the climate relevance of carbon dioxide emissions from SOM degradation. In addition, appropriate management of SOM is of paramount importance for soil quality, particularly because soil resources for food production are endangered in many areas of Southern Europe and around the world (Lal 2004).

In order to ensure that SOM remains a reliable sink for atmospheric CO_2, concepts for sustainable sequestration of C in SOM are needed. However, this is difficult with only incomplete mechanistic understanding of the genesis of SOM. The stabilization and formation of SOM depends on various processes (von Lützow et al. 2008) as well as on the origin of the material, its chemical composition, the local soil system, and the microbial transformation of the plant input material, but the stabilization processes and the chemical composition of SOM are not yet fully understood (Kelleher and Simpson 2006). Schmidt et al. (2011) summarized recent analytical and experimental advances demonstrating that molecular structure alone does not control SOM stability: instead, environmental and biological controls predominate. In general, plant material dominantly transformed by microbes is regarded as the primary source for SOM formation (Kögel-Knabner 2002), but the details of the transformation to SOM and the molecular structures are still subjects of discussion. Recent results (Kindler et al. 2006; Kindler et al. 2009; Miltner et al. 2009) indicated that the degradable plant organic matter is first processed by microbes for growth and formation of microbial biomass; this bacterial biomass is then transformed into non-living SOM and may provide the molecular structures of a major part of the SOM material. Additional observations by means of scanning electron microscopy showed that particulate cell debris, specifically fragments of cell-envelopes of bacterial and fungal cells, is the most abundant material visible on mineral surfaces in soil (Miltner et al. in press).

Studies on ecosystem development, including pedogenesis, are ideally conducted by comparing locations of different age but developed from the same parent material (Walker et al. 2010). Glacier forefield chronosequences are thus ideal objects for analyzing the development of soil systems. In the forefield of a receding glacier, newly exposed mineral surfaces with little or no organic coating exist near the glacier, while aged surfaces showing more extensive organo–mineral interactions with SOM are present more distant from the glacier (Dümig et al. 2011). Many studies dealing with the succession of microorganisms related to soil development have been conducted at alpine glacier forefields like at the Damma Glacier in Switzerland (Bernasconi et al. 2011). The results generally show increasing contents of total organic C with increasing soil age, which is accompanied by increasing abundance of living microbes as indicated by phospholipid fatty acid (PLFA) analyses (Sigler and Zeyer 2002; Hämmerli et al. 2007; Miniaci et al. 2007; Bernasconi et al. 2011; Zumsteg et al. 2011).

According to the hypothesis of mineral surfaces in soils being progressively covered by SOM, in particular by flat microscopically-visible fragments of microbial cell envelopes (Miltner et al. in press), the aim of our study was to evaluate the relevance of these biomass residues as the macromolecular source for SOM during initial phases of pedogenesis, on the basis of samples from the chronosequence at the glacier forefield of the Damma glacier in Switzerland (Bernasconi et al. 2008). Particular goals of the work were: I) to analyze the abundance and coverage of the mineral surfaces by these residues, using scanning electron microscopy; II) to relate their abundance to the development of SOM

(C and N contents) during early pedogenesis, with increasing input of plant litter and the formation of complex microbial communities (as indicated by phospholipid fatty acids: PLFA); and III) to test the relevance of increasing abundance of microbial residues for the development of soil properties, with particular focus on wettability (as determined by the contact angle of the soil samples)—this property determines physical, chemical, and biological pedogenetic processes and thus will have a pronounced impact on the further development of the soils.

Materials and methods

Site description

Samples were obtained from the Damma glacier forefield located in Switzerland (46°38′ N, 8°27' E; ~2000 m above sea level). Similar to many glaciers worldwide, the glaciers in the Alps have mostly retreated during the last 150 years. In particular, the Damma glacier has retreated more than 1000 m (data from the Swiss Glacier Monitoring Network; http://glaciology.ethz.ch/ swiss-glaciers/glaciers/damma.html) and thus the glacier forefield was chosen to be developed as a major research object within the BigLink project. Details of the project and the conditions at the Damma glacier have been described previously (Bernasconi et al. 2008; Bernasconi et al. 2011; Dümig et al. 2011).

Historical records report a recession of the glacier since 1850, with an average retreat rate of about 10 m yr^{-1} that was interrupted by two re-advances between ca. 1920 and 1928, and between 1970 and 1992. These re-advances resulted in small end-moraines in the forefield. Therefore, the forefield is divided into three classes of soil age (for details, see Fig. 1, Table 1). At present, the mean annual air temperature at the forefield is in the range of 0–5 °C and mean annual precipitation is about 2400 mm (Spreafico et al. 1992). The bedrock material of the forefield is granite and is highly similar throughout the chronosequence (Bernasconi et al. 2008; Bernasconi et al. 2011). The development of organic matter in the glacier forefield has been described recently (Dümig et al., 2011). The vegetation consists of various mosses, grasses (e.g. *Poa alpina, Agrostis rupestris*), herbaceous plants (e.g. *Leucanthemopsis alpine, Lotus alpinus*; (Miniaci et al. 2007; Duc et al. 2009; Schmalenberger and Noll 2010; Töwe et al. 2010; Bernasconi et al. 2011), fern, woody shrubs and trees (*Salix sp.*; Töwe et al. (2010)).

Soil sampling

Soil samples were randomly taken in August 2009 at 8 sampling sites, in direct proximity to the BigLink project sites (Fig. 1) in order to permit meaningful comparison with other studies. The soil sampling procedure was similar to the method described by Sigler and Zeyer (2002) and Sigler et al. (2002). Briefly, at each sampling site ten subsamples were taken according to a grid within a 5 m radius around the sampling sites (0 – 18, see Table 1). Samples were obtained from depth of 5-20 cm below ground underneath the root layer of the surface vegetation in order to avoid the effects of the rhizosphere on microbial abundances which were previously described (Tscherko et al. 2004; Töwe et al. 2010). Subsamples were pooled in order to obtain a mixed sample for each site and to average small-scale heterogeneities observed at these recently exposed sites. The soil samples were sieved immediately by passing through a 2 mm sieve. All samples were kept on ice during transport to the laboratory. Some earlier studies (Sigler et al. 2002; Sigler and Zeyer 2002; Edwards et al. 2006) used other sampling points in the forefield, which nevertheless represent the same soil age classes. Approximate soil age spans from 0 to 120 years (Table 1, for details on sample site selection and age determination, see Bernasconi et al., 2011) and the sampling sites were chosen according to defined gradients in physicochemical parameters representing increasing ages (Smittenberg, personal communication). Due to the variation of soil age, the distance from the glacier was chosen as a definite scale.

Preparation of samples for scanning electron microscopy

Samples for scanning electron microscopy were fixed with 2.5 % glutaraldehyde solution (w/w) immediately after sampling and were stored in the dark. Directly after arrival of the samples at the laboratory, the fixation solution was renewed. After 12 h, the solution was removed and the samples were washed three times with phosphate-buffered saline (8 g NaCl,

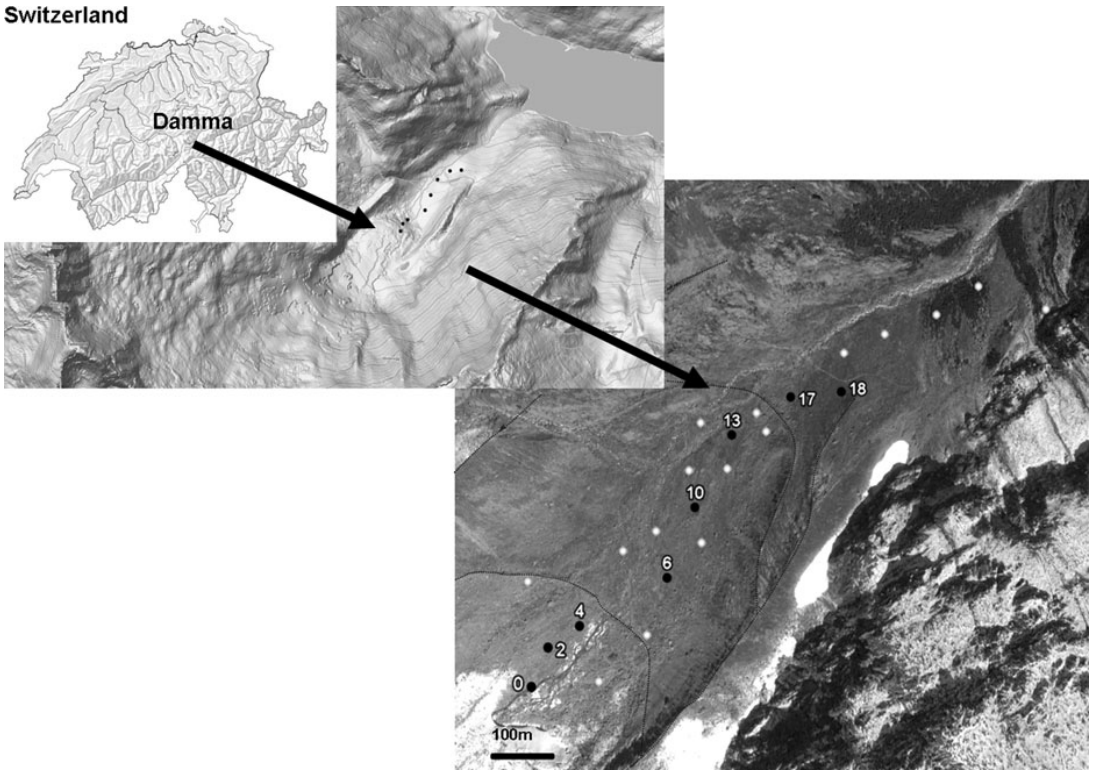

Fig. 1 Soil sampling sites (*empty circle*) of the BigLink project in the forefield of the Damma glacier, Switzerland. Sampled sites in this study (*filled circle*) are indicated with numbers (Table 1). Figure adapted from (Göransson et al. 2011). The *black lines* indicate the location of the moraines resulting from glacier advances and retreats, which enable a rough estimation of soil ages

0.2 g KCl, 1.42 g Na_2HPO_4, 0.27 g KH_2PO_4, 1L H_2O). The water in the samples was then exchanged by acetone solutions of increasing concentrations (25, 30, 40, 50, 60, 70, 80, 90, 100 %) by soaking the samples in each solution for 30 min, after which the samples were immediately transferred into a pre-cooled (9 °C) critical-point-drying device (Polaron E3000, Ashford, UK), where the pure acetone was exchanged by liquid carbon dioxide. Following complete exchange of the acetone, the device was heated to above the critical point of carbon dioxide (31 °C, 73.8 Bar) and the pressure was slowly released after disappearance of the liquid phase. This procedure enables drying of the samples without deformation by the water/gas interface, and retains the spatial structure and organization, in particular that of the microbial biomass (Miltner et al. in press). The dry samples were stored in a desiccator over dry silica gel until they were mounted on aluminum stubs using conductive silver. Before analysis, all samples were coated with a 20 nm gold/palladium layer using a sputter coater (SCD050, Balzers, Liechtenstein). Finally, the samples were visualized on a field-emission scanning electron microscope (FE-SEM, Hitachi S800, Krefeld, Germany), and the elemental composition at selected locations was studied using energy-dispersive X-ray analysis (SEM-EDX, LEO 1450VP with an Oxford INCA Energy 200 Premium Si(Li) SATW-detector, Cambridge, UK).

Evaluation of bacterial cell-envelope fragments

The samples were intensively screened for the constitution of the material, and areas representing the majority of the views were recorded for image analysis. Representative areas with the targeted

Table 1 Soil sampling sites at the Damma glacier forefield, representing a chronosequence of soil organic matter development after retreat of the glacier ice. Distance to moraine/present glacier front related to soil age (for details see "Soil sampling" section)

Sampling site	Distance from glacier front (m)	Age range according to intermediate glacial advances	Approximate time since deglaciation[a](yr)
0	5	Less than 17 years	0
2	55		7
4	110		15
6	255	58–81 years	65
10	375		70
13	515		80
17	620	109–150 years	110
18	710		120

[a] Approximate age since deglaciation as described in Bernasconi et al. (2011), based on glacier movement data (Swiss Glacier Monitoring Network; http://glaciology.ethz.ch/swiss-glaciers/glaciers/damma.html)

round-shaped bacterial cell-envelope fragments, as described in detail elsewhere (Miltner et al. in press), are highlighted by arrows and circles in Fig. 2. Fragments of the cell-envelopes were quantified by means of image analysis as follows. With the aid of the MATLAB software package (R2009a, The MathWorks Inc., Natick, MA, USA), 10 by 10 grids were laid over 4 to 8 representative scanning electron micrographs (SEM) from each sampling point (with the exception of site 17, where only one SEM was available). All images had the same scale, with a pixel size of 0.0322 μm × 0.0322 μm. Ten fields of analysis were selected randomly from the grids on each micrograph (Fig. 2) and the presumed cell-envelope fragments in these fields were manually color-tagged by three individuals independently (Fig. 2). Finally, the number of tagged pixels in the pictures was related to the total number of pixels in the grid, resulting in an averaged surface coverage by cell-envelope fragments for each sample. When analysing the SEMs, we found that, particularly in the medium aged soils, the variability within a particular SEM was quite large, whereas the overall impression of the SEMs from each site was more homogeneous. The size of the error bars include the variation i) within a particular SEM, ii) between the SEMs of a given site, and iii) between the three persons who counted the fragments. This also indicates that we found a good compromise between covering sample heterogeneity and feasibility of the analysis, since the visual inspection of the SEMs was very time-consuming.

Water contact angle measurements

Contact angles were determined with the sessile drop method (Bachmann et al. 2000) using a CCD-equipped horizontally-oriented contact angle microscope (OCA 11, DataPhysics, Filderstadt, Germany). For the measurements, native soil samples were dried in a desiccator above calcium chloride for 40 days until constant weight was reached (usually after 20 days). Dry samples were subsequently fixed to glass microscope slides by means of adhesive tape, while trying to achieve a fixed monolayer of soil particles. Contact angles were determined directly after slide preparation by placing six drops of 3 μL at different locations on the surface. The initial contact angle (directly after placing the drop and after mechanical perturbations ended) and contact angles after 1000 ms were evaluated at both intersections (left and right) of the drop contour line with the solid surface line (baseline) by means of an automated drop shape analysis using the SCA20 software (DataPhysics, Filderstadt, Germany). Contact angles are given as the arithmetic mean and standard deviation of six independent measurements at both sides of the droplet. The samples contained a considerable amount of coarse particles that could not be fixed on the tape. Consequently, the contact angles measured mainly refer to the fine material.

Environmental scanning electron microscopy

Environmental scanning electron microscopy (ESEM) (Quanta 200, FEI Company, Eindhoven, The Netherlands) was used to visualize the wetting properties on the microscale. The measurements were carried out in a gaseous environment at pressures ranging from 550 to 810 Pa. The water vapor in the measurement chamber provided extended charge reduction. For the measurement, a small amount of soil material was placed on a Peltier cooling stage, allowing adjustment of the sample temperature. At constant temperature (5 °C) the relative humidity was controlled by the chamber pressure. For the wetting experiments

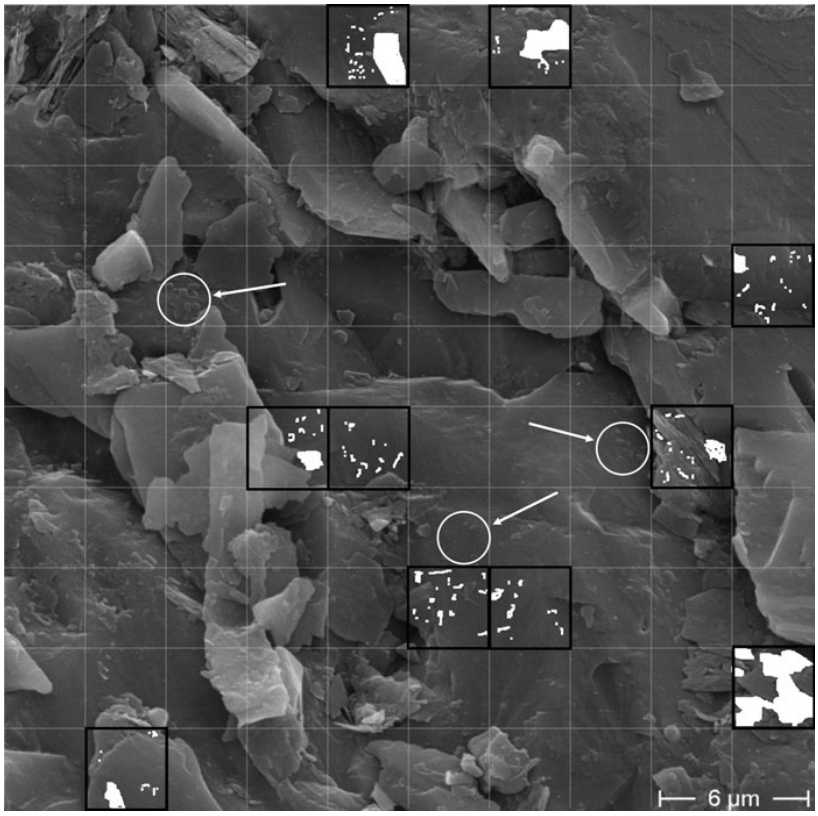

Fig. 2 Example of scanning electron micrograph (SEM) analysis of the soil samples from the Damma glacier forefield (age: 64 years; site 6). The *encircled areas* indicated by the *arrows* contain target fragments. The surface coverage of soil particles by fragments of bacterial cell-envelopes is estimated by laying a 10 × 10 grid over equally scaled SEM. Out of this grid 10 fields are selected randomly for analysis (for details see text); these fields are analyzed for bacterial cell-envelope fragments (*marked in white*). See Supplement A3 for higher resolution

(Goebel et al. 2007), the pressure was increased until condensation of water on the particle surfaces took place.

Carbon and nitrogen contents

C and N contents from finely powdered soil samples were analyzed using a Flash EA 1112 elemental analyzer, as described previously (Bernasconi et al. 2011).

Fatty acid analysis

For fatty acid analysis, triplicates of 1 g (dry weight) of frozen soil sample were extracted according to the Bligh and Dyer protocol (Bligh and Dyer 1959) as modified by White et al. (1979), (Frostegård et al. 1993; Frostegård and Bååth 1996). Phospholipid fatty acids (PLFA) were separated from the other lipid fractions by means of column chromatography: The sample was added to a silica gel column and eluted stepwise with chloroform to yield the neutral lipids, acetone to yield the glyco-lipids and methanol to yield the PLFA (King et al. 1977; Frostegård et al. 1991). PLFA are commonly considered to represent living biomass (Pinkart et al. 2002), although their turnover rate in soil might vary (Frostegård et al. 2011). Before measurement, the fatty acids were methylated with methanol/trimethylchlorosilane (9:1) at 60 °C for 2 h. The reagent was removed by evaporation under a stream of N_2, and subsequently 10 µg of 21:0 fatty-acid methyl ester in hexane was added as an internal standard for quantification. Total fatty acids (tFA), also representing also dead microbial biomass residues and cell debris, were extracted from triplicate soil samples by means of direct methylation with methanol/trimethylchlorosilane (9:1) for 2 h at 70 °C (Miltner et al. 2004). After purification over a silica gel column, 10 µg of 21:0 fatty-acid methyl ester in hexane was added for quantification.

Table 2 Specific marker PLFA for various groups of organisms (Federle 1986; Frostegård et al. 1993; Bardgett et al. 1996; Zelles 1997; Zelles 1999; Heipieper 2010; Ruess and Chamberlain 2010)

Parameter	Characteristic fatty acids
Bacteria	14:0, *i*15:0, *a*15:0, *i*16:0, 16:1ω7*cis*, 16:1ω7*trans*, *i*17:0, *a*17:0, cy17:0, 17:0, 18:1ω7*cis*, cy19:0
Gram-positive bacteria	14:0, *i*15:0, *a*15:0, 15:0, *i*16:0, *i*17:0, *a*17:0
Gram-negative bacteria	16:1ω7*cis*, 16:1ω7*trans*, cy17:0, 18:1ω7*cis*, 18:1ω7*trans*, cy19:0, 20:1ω9
Fungi	18:2ω9,12
Actinomycetes	10Me 18:0
Stress and starvation in Gram-negative bacteria	$\frac{\sum(\text{cy17:0,cy19:0})}{\sum(\text{16:1}\omega\text{7}cis\text{,18:1}\omega\text{7}cis)}$

The samples were analyzed by means of gas chromatography—mass spectrometry (Agilent GC7890A and MS5975C, Boeblingen, Germany) with the following parameters: MS-source at 230 °C, MS-quadrupole at 150 °C, inlet with splitless mode at 280 °C, HP-5MS column (30 m * 0.25 mm* 0.25 µm) at 50 °C for 1 min then 4 °C min^{-1} to 250 °C, 20 °C min^{-1} to 300 °C for 5 min and finally a 10 min post-run at 300 °C. The mass spectra were evaluated using a library of previously determined mass spectra of known fatty acids.

The total amounts of PLFA and tFA are related to the whole microbial community and its residues in soil, respectively. For assessment of the relative contributions from different groups of organisms, the ratios of specific marker fatty acids were quantified and compared according to previously suggested analysis schemes (Federle 1986; Frostegård et al. 1993; Bardgett et al. 1996; Zelles 1997; Zelles 1999; Heipieper 2010; Ruess and Chamberlain 2010) (Table 2).

Results

The soil samples obtained from the chronosequence of the Damma glacier forefield are unaltered by land-use history, thus providing potentially unbiased samples for the analysis of SOM formation and stabilization on initially barren mineral surfaces. According to the hypothesis that during SOM genesis in soils, mineral surfaces are covered by round-shaped microbial cell-envelope fragments of 100-500 nm size (as described in detail elsewhere: Miltner et al. (in press), we analyzed soil samples from the glacier forefield, having continuously increasing SOM contents and SOM age (Bernasconi et al. 2011, Dümig et al. 2011). Here, we characterize the development of SOM on the basis of SEM as well as in relation to soil C and N contents, contents of lipids as direct and residue biomarkers, and water repellency as a potential stabilization factor.

Scanning electron microscopy

Figure 3 compares a selection of representative SEM of all soil samples at the same scale. In the samples close to the glacier (Fig. 3a–c), bare quartz surfaces (identified by EDX, data not shown) dominate the micrographs. Even in the youngest sample (Fig. 3a) a substantial number of rounded patches can be identified, which we consider to be bacterial cell-envelope fragments (see methods for details of the image analyses). They are clearly distinguishable from the sharp-edged mineral particles. Unfortunately, elemental analysis of the fragments by means of EDX is not possible because the small size and thickness of the fragments in comparison to the beam size and vertical interaction volume of EDX results in a substantial contribution of surrounding and underlying material to the EDX spectrum. The fragments could therefore only be identified optically, on the basis of their appearance. With proceeding pedogenesis, the quartz surfaces become more and more eroded by weathering, and the number of the bacterial cell envelope fragments increases, whereas the number of sharp-edged clay mineral particles decreases. In parallel, the quartz surfaces also become more and more coated by amorphous organic material. Finally, on site 13, 17 and 18, almost all visible particle surfaces are completely coated by amorphous organic material and by multilayers of cell envelope fragments. On none of the images could intact bacterial cells be seen, because the bacterial colonization was low in comparison to the large soil particle surface. Chenu and

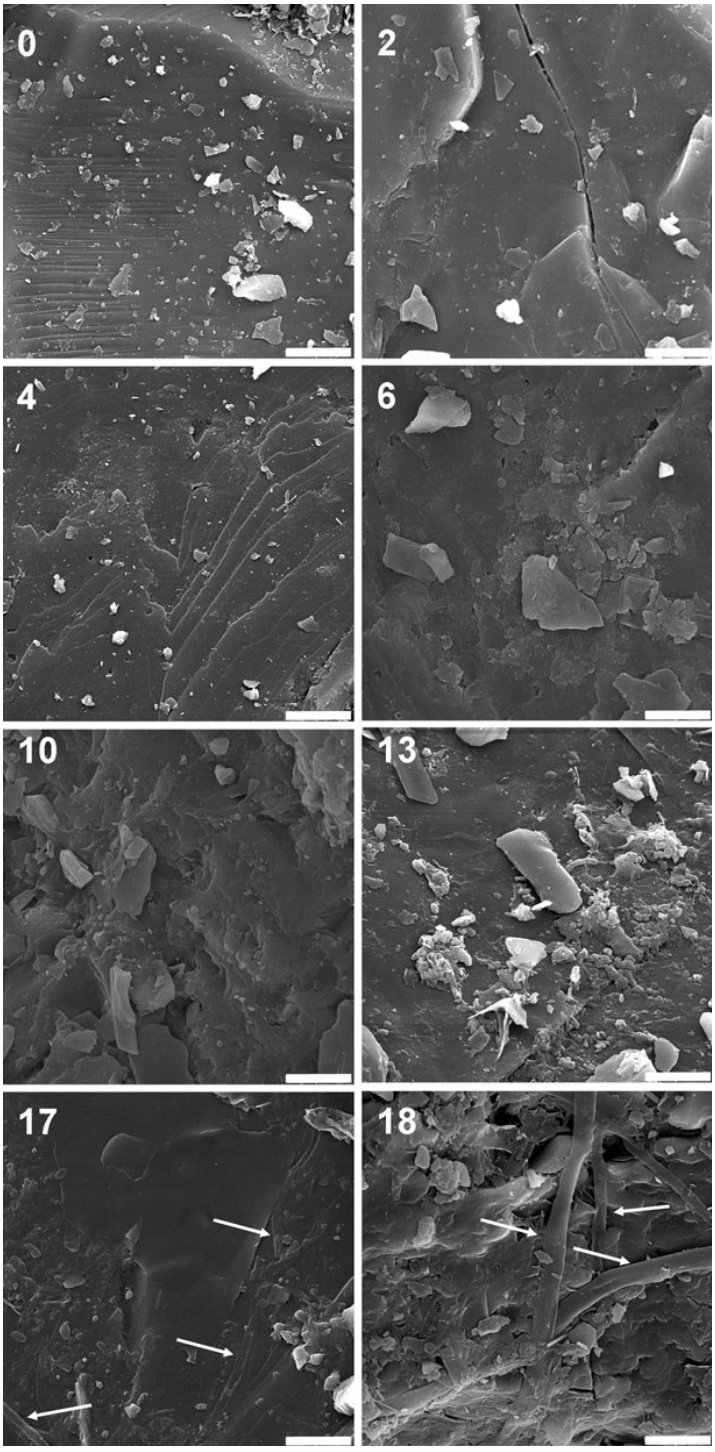

Fig. 3 SEM of soil samples from various sampling sites at the Damma glacier forefield (identical scale, *bar* = 6 μm). Samples from sites of increasing SOM age showed increasing coverage by cell-envelope fragments. Site numbers are shown in the *upper left* corners of the micrographs. Hypha are highlighted by *arrows* (for details, see methods and Table 1). Supplement A4 provides a higher resolution version of figure 3

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Stotzky (2002) estimate the surface of soil covered by bacteria to be about 1 %, so that the chance of finding intact bacterial cells is rather small. However, in samples 6, 17 and 18, fungal hyphae were present, indicating increasing colonization by fungi with increasing distance from the glacier. After cell death, these hyphae appear to integrate into the condensed organic fragment coating. This is evident because the hyphae are embedded into a condensed multilayer coverage of mineral surfaces.

The increase in number of the round-shaped bacterial cell-envelope fragments along this chronosequence was quantified by means of the evaluation scheme described in the methods (Fig. 4). Much of the variability observed can be attributed to the heterogeneity of the samples from a given site. This heterogeneity is particularly high at the sites of intermediate ages (sites 6, 10 and 13). In general, the coverage was very low in young soils and increased strongly with distance from the glacier from 0.014 $\mu m^2 \times \cdot \mu m^{-2}$ (site 0) to 0.68 $\mu m^2 \times \mu m^{-2}$ (site 17). This increase appears to end at the oldest sites 17 and 18 (110–150 a, $>$ 600 m) below the second moraine, as site 18 does not significantly differ from site 17. This may indicate saturation of surface coverage, since the majority of visible surfaces are covered with fragments, which is also reflected in the logistic regression curve of the coverage (Fig. 4). The amount of cell-envelope fragments, however, might have increased further, because we did not account for the visible multilayer coating. The importance of multilayer coatings can be assumed to increase towards the older soils with dense coatings by cell-envelope fragments, because it will be more probable that they are stacked on top of each other.

Carbon and nitrogen contents

Increasing C and N contents at the sampled sites are found to accompany the increasing coverage of mineral surfaces with microbial necromass and particulate cell-envelope debris (Fig. 5). The sample closest to the glacier contains 0.3 mg C and 0.05 mg N per g of soil. These values increased to 17.1 mg C g^{-1} soil and 0.9 mg N g^{-1} soil in the oldest sample. From logistic regressions (Fig. 5) it could be estimated that C and N contents are showing exponential increase between 50 and 80 years since deglaciation, while exhibiting a relatively stable level in the soils from the older sites. At the same time, the C/N ratio starts with a value of 6, indicating microbial biomass, and increases to 19 over the chronosequence,

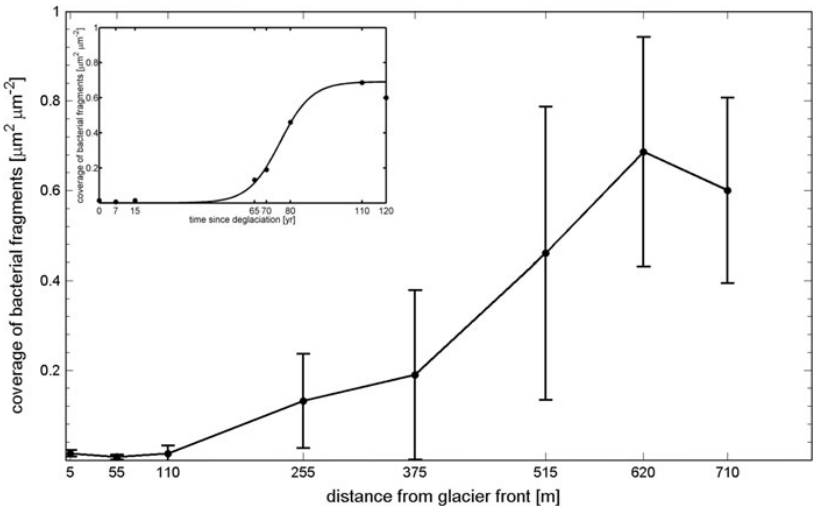

Fig. 4 Surface coverage of mineral particles by microbial cell-envelope fragments in the SEM of Damma glacier forefield samples related to the distance from the glacier front. *Error bars* represent the standard deviation of three independent analyses (for details see "Evaluation of bacterial cell-envelope fragments" section). A logistic regression of the surface coverage with cell envelope fragments and the time since deglaciation is shown along with the data points in the *small inserted figure* ($y = 0.691519/1\text{-}e^{-0.15112*(t-75.6477)}$, $r^2 = 0.99$)

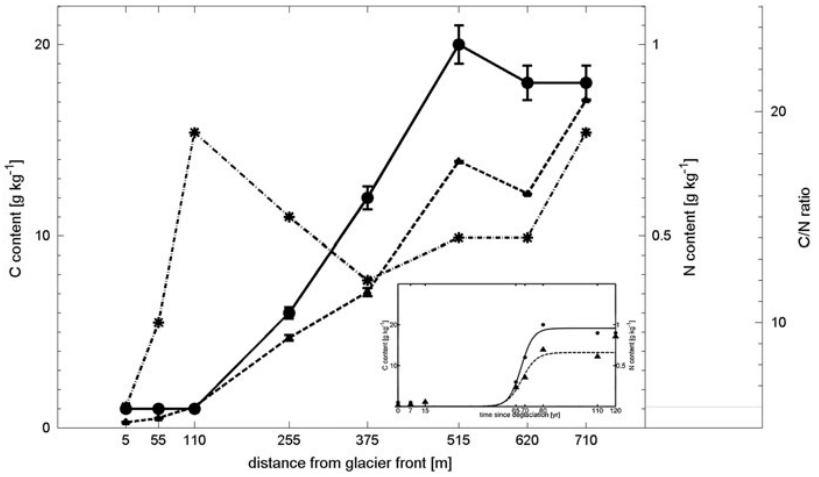

Fig. 5 Carbon (*filled triangle*) and nitrogen (*filled circle*) contents, and C/N ratio (*star*) in the soil samples from the Damma glacier forefield. *Error bars* represent the analytical error of the samples ($n = 3$). Error bars for C measurements are *hidden behind* the markers. The *inserted figure* depicts logistic regressions of C (–) and N (-) of the data ($C = 13.1976/1 + e^{-0.24725*(t-68.2965)}$ $r^2 = 0.97$; $N = 0.95663/1 + e^{-0.29843*(t-67.9038)}$ $r^2 = 0.99$)

demonstrating a transition from more microbial-derived ratios to higher influence of plant litter. The peak value of site 4 may possibly be due to higher uncertainty of the C/N ratios at low C and N concentrations (Fig. 5).

Lipid contents

In order to evaluate whether microbial residues are the major source of SOM, fatty acids, as direct and residue biomarkers for certain groups of organisms, were analyzed. Phospholipid fatty acid (PLFA) concentrations, known as indicators for living biomass (Frostegård & Bååth 1996; Pinkart et al. 2002; Frostegård et al. 2011), increased 30-fold from 2.7 nmol per g of soil at site 0, closest to the glacier front, to 94 nmol g_{soil}^{-1} at site 17 (Fig. 6). However, the concentration at the last sampling site 18 was lower (64 nmol g_{soil}^{-1}) than at site 17. The trend was similar for the total fatty acids (tFA) in the soil, with an increase from 5.5 nmol g_{soil}^{-1}

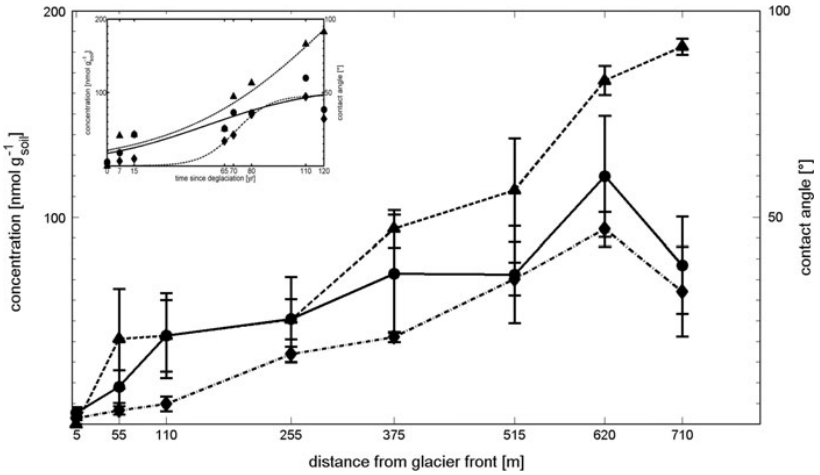

Fig. 6 Total amounts of detectable phospholipid fatty acids (PLFA) (*filled tetrahedron*), total lipid fatty acids (*filled circle*), and water contact angle (*filled triangle*) in the samples from the Damma glacier forefield. *Error* bars represent the standard deviation from triplicate extractions. The curves in the *inserted figure* represent logistic regressions of tFA (-), PLFA (-·-) and contact angle (–) with respect to time ($tFA = 110.0966/1 + e^{-0.030869*(t-54.8109)}$ $r^2 = 0.9$; $PLFA = 96.4615/1 + e^{-0.10398*(t-71.4133)}$ $r^2 = 0.96$; $contact\ angle = 213.4872/1 + e^{-0.022383*(t-131.6771)}$ $r^2 = 0.96$)

to 120 nmol g_{soil}^{-1} at site 17 and is consistent with the fact that tFA additionally represents fatty acids from non-living organic matter and microbial necromass. This is also reflected by the fact that the logistic regressions show a later entrance into the saturation phase on higher concentrations for tFA compared to PLFA. The PLFA/tFA ratios decreased at the three youngest sites (Fig. 6), but increased towards the oldest sample. This is most likely caused by the known higher turnover rates of tFAs in comparison to the bulk microbial C (Miltner et al. in press) in the older samples.

Soil wettability

The increasing organic coating on mineral surfaces is not only visible, but also reflected in the increasing water contact angle (Fig. 6). The hydrophobicity increased with increasing distance from the glacier front. While Sample 0 is completely wettable with a contact angle of 0°, samples 2, 4, 6, 10 and 13 show measurable contact angles in the subcritical range (0°− < 90° contact angle). The contact angle of sample 17 is around 90°; sample 18 is hydrophobic with a contact angle larger than 90°. A logistic regression of the contact angle data suggests that the contact angle is likely to increase further in older samples, ultimately reaching a stable level in older soils than the other presented measures.

Since organic compounds, such as lipids derived from bacterial cell-envelopes, are mostly hydrophobic, the rise in contact angle from 0° to 91° is in accordance with the results obtained by image analysis; C and PLFA content, showing direct positive correlations (see Supplementary Material Figure A1). In addition, other hydrophobic organic compounds, such as plant-derived lipids and leaf waxes, may contribute to the large contact angles measured on older samples with higher C contents.

The microscopic wetting properties of the material were characterized by condensation experiments in an ESEM. The dominantly mineral soil from site 0 showed complete wettability. Figure 7a shows the dry sample and Fig. 7b demonstrates that the condensing water forms a film covering the whole surface of this sample. In contrast, the soil from site 18 exhibits high hydrophobicity, as can be observed by the formation of a droplet during the condensation experiment (Fig. 7c, d).

PLFA biomarker analysis

The changes in hydrophobicity of the soil samples may also be related to the microbial lipid fatty acids, since not only the amount of the PLFA but also the relative pattern of the individual PLFAs changed over the chronosequence. This relates observed cell-envelope fragment formation to shifts in the composition of the microbial community. The proportion of branched PLFA relative to unsaturated PLFA, which are characteristic for Gram-positive and Gram-negative bacteria, respectively, increased from 0.2 at sampling point 0 to 0.5 at sampling point 18 (Fig. 8a). The proportion of 18:2ω9,12 PLFA, which is characteristic of fungi, relative to bacterial PLFA increased from 0 to 0.28 with time since deglaciation (Fig. 8b). However, sampling points 2 and 4 showed relatively higher contents of this PLFA than the other sampling points.

The proportion of methyl-branched PLFA, characteristic for actinomycetes, increased over the chronosequence (0 to 0.019, Fig. 8c), and show a positive correlation to PLFA ($r^2 = 0.77$, Supplement A2). The markers for actinomycetes at the youngest sites were below the detection limit. The older sampling sites showed no significant changes from site 10–18 (age of 70–120 years). The ratio of cyclopropyl fatty acids/unsaturated fatty acids, which is frequently used as an indicator for stress or starvation in Gram-negative bacteria, increased from 0 to 0.18 with distance from the glacier (Fig. 8d). At the three youngest sites, the cyclopropyl fatty acids were below the detection limit.

Discussion

In the present work, we show the increase of mineral surface coverage by microbial cell-envelope fragments and their abundance accompanying the development of SOM along the chronosequence in the forefield of the Damma glacier. This confirms the previously formulated hypothesis that SOM partially derives from microbial necromass, in particular cell-envelope fragments (Miltner et al. in press). The SOM development in this forefield was clearly documented by many soil-related parameters, such as cation exchange capacity, bacterial and fungal PLFAs, and decreasing pH values (Bernasconi et al. 2011; Dümig et al. 2011) and by the general increase in lipids (Smittenberg et al. 2012). It is also confirmed by the

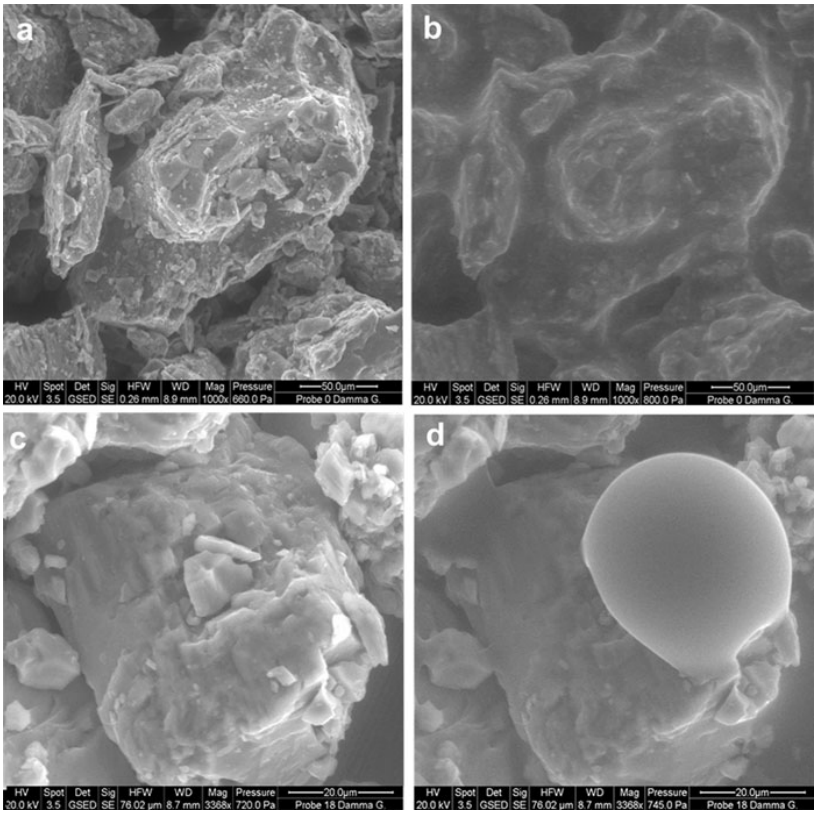

Fig. 7 Micrographs from condensation experiments in an environmental scanning electron microscope (ESEM), **a** sample from site 0 without condensed water, **b** sample from site 0 with water film, **c** sample from site 18 without condensed water, **d** sample from site 18 with condensed water droplet (water repellency)

PLFA, tFA, C, and N data of the samples presented here. In addition, SOM development is also accompanied by increasing macroscale contact angles of the soil samples, and thus by decreasing wettability on the microscale (Goebel et al. 2007). This increase in contact angles is also reflected by the results of the condensation experiment in the ESEM as presented in this study.

Change of C and N contents during SOM development

The increasing coverage of minerals by SOM with proceeding soil development correlated with the higher C and N contents in the present samples of this chronosequence. Increasing contents of total organic carbon with increasing soil age have been shown several times for the Damma glacier forefield and have also been found to be linked to the abundance and activity of microorganisms (Sigler and Zeyer 2002; Hämmerli et al. 2007; Miniaci et al. 2007). However, contrary to previous reports (Sigler and Zeyer 2002; Edwards et al. 2006), the C content in the present work is shown to increase continuously also in soil ages beyond 50 years. The N contents were found to increase over the sequence similarly to the descriptions in previous works (Sigler and Zeyer 2002; Edwards et al. 2006). However, detailed analyses of the soil age, as well as C and N contents, of all sampling sites of the BigLink project at the Damma glacier forefield showed that there is no stringent correlation between the age and the soil development (Göransson et al. 2011), although the overall analyses do show that there are consistent trends of increasing C

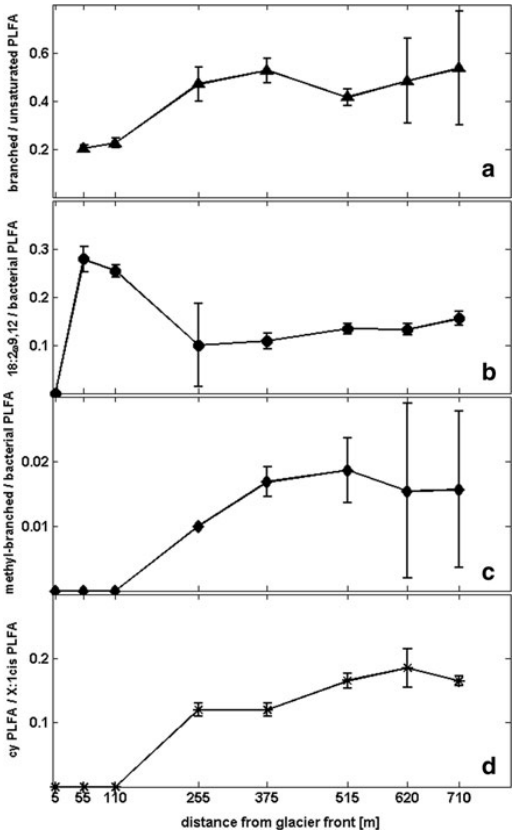

Fig. 8 Changes of the microbial community composition along the Damma glacier forefield, as indicated by PLFA analysis, **a** ratio of markers for Gram-positive bacteria to markers for Gram-negative bacteria, **b** ratio of fungal to bacterial markers, **c** ratio of markers for actinomycetes to total bacterial markers, **d** starvation markers (for details, see Table 2). *Error bars* represent the standard deviation from triplicate extractions

and N contents within the different age classes of the soil, caused by various advances and retreats of the glacier within the last 150 years (Bernasconi et al. 2011; Dümig et al. 2011). Bernasconi et al. (2011) reported exponential increase of total organic carbon (TOC) and total N within the two youngest age ranges, while the increase was found to be slower for the older soils. Therefore, the apparent increase of SOM along this forefield resulted in choosing the sampling sites with clearly distinguished increasing ages of soil development as documented by biogeochemical parameters (Bernasconi et al. 2011). Hence, the similar behavior of C and N observed in the present study is consistent.

C/N ratios increased from the typically bacterial value of 5–8 (Paul and Clark 1996) in the young samples up to a value of 19, representing a higher C input from the increasingly abundant plants, at the sites of higher SOM age. The lower C/N ratios in the younger soils of the chronosequence are associated with lower concentrations of both elements, which result in substantial loss of accuracy for the ratio. Nevertheless, Dümig et al. (2011) found a similar distribution of C/N ratios when studying soil samples from the Damma glacier forefield and concluded from this a bacterial contribution. Dümig et al. (2012) also found no major changes in the composition of SOM over the chronosequence, which is consistent with the similar structure of the observed patchy materials in the SEM of the present paper. Therefore, the presented data indicate the important microbial contribution to the SOM development in this chronosequence.

However, Göransson et al. (2011) did not find any pronounced increase in the C/N ratio for all samples of the glacier forefield; they only identified ratios of 11.4–15.8 with a diffuse distribution, whereas Bernasconi et al. (2011) found C/N ratios varying between 12 and 30 in their samples. In the present study, we also observed an increasing trend for the C/N ratio in the samples of consecutive soil ages, at least for the youngest sampling sites. In addition, the initial C/N ratios of 6–10 provide evidence for microbial carbon input, i.e. the dominance of microbial cell residues, within the soils of younger age. In later stages, the C/N ratio indicates a higher contribution of plant-derived material.

Accumulation of cell-envelope fragments during pedogenesis

The new aspect we provide here is the strong indication that SOM development is accompanied by the increasing abundance of flat, round-shaped, patchy organic material, which has been reported recently to consist of microbial cell-envelope fragments (Miltner et al. in press). An appropriate method for quantification of the cell-envelope fragments is an important prerequisite for providing quantitative data for comparison to the other soil related parameters. Our evaluation scheme for estimating the number of microbial cell-envelope residues in a sample is based on image analysis on SEM. Coupling these two techniques has recently been shown to be a powerful

tool, also for other soil chronosequences (Calero et al. 2009). Unlike Calero et al. (2009), the image analysis scheme based on optical analysis by several scientists, as applied in this study, is laborious and potentially biased by the subjective factor of fragment assessment. However, at present the patchy material cannot be identified with appropriate precision by automated optical or by EDX analysis. In addition, the bias introduced by the three-dimensional heterogeneity of soil cannot yet be compensated for by an automated image analysis. Even though the presented analytical procedure includes several uncertainties in addition to soil heterogeneity, the results show a clear trend of increasing bacterial cell-envelope fragments with time since deglaciation.

As the direct precursors of SOM imprint their molecular structures on SOM, an increasing contribution of cell envelope residues to SOM indicates an increasing contribution of their constituents, such as lipids, carbohydrates, proteins, and amino sugars, derived from the bacterial murein sacculus and fungal cell walls (Madigan et al. 2012). The increasing coverage of mineral particles by fragments of microbial cell-envelopes is linked to microbial growth and decay, which is also indicated by increasing microbial biomass and shifts in the microbial community. In the most developed soils of the chronosequence, the coverage of minerals by microbial necromass appears to form multiple fragment layers, finally causing microaggregates (Fig. 3). A coverage of mineral particles in marine sediments by a monolayer of organic matter has been suggested (Mayer 1994), but organic matter in soils appears to be distributed in a more patchy manner (Mayer and Xing 2001). Our SEM show that multiple layers of organic matter may form in the relatively dry soil systems. With increasing contents of organic matter, the soil must also contain increasing amounts of plant-derived organic matter as indicated by other analyses (Smittenberg et al. 2012). However, in the SEMs of our samples we did not find any indication for a significant contribution of fiber-like plant-derived particulate organic matter.

The macromolecular aggregation of the biocompounds in the microbial necromass profoundly affects the stability and degradability of the compounds (Miltner et al. in press). Without overall degradation of the murein sacculus of a microbial cell envelope, for example, a protein structure embedded within the macromolecular architecture of the sacculus may never be degraded by proteases. This is simply due to physical protection, because no proteolytic enzyme will have access to the molecule. A complementary mechanism is that lipids and fatty acids may protect the small-sized fragment particles by covering their surface after drying events. This potentially prevents the re-wetting of the material, as we show in this study by means of the contact angle and the condensation experiments in the ESEM. It is well-known that increasing SOM contents are accompanied by increasing contact angles of the materials (Goebel et al. 2011). This effect may be explained by the increased input of lipids from microbial cell-envelope fragments (see correlation to the macroscale contact angle Supplementary Material A1). It is further enhanced by the change in microbial community composition, since many Gram-positive soil bacteria show very high contact angles (van Loosdrecht et al. 1987). The macromolecular structure of the cell-envelope fragments also provides the matrix for incrustation with Fe or Si, as was indicated by electron disperse X-ray analyses of the SEM (data not shown). These organo–mineral associations are also evident from a study at a similar glacier forefield (Egli et al. 2010), where protein-enriched material was found mainly in the high-density fraction. Another study observed increasing amounts of sugars and amino acids in the SOM fractions co-precipitated and stabilized by Fe and Al (Dümig et al. 2012), which are considered to lead to the formation of amorphous non-crystalline minerals. However, our presented micrographs did not show any visible amorphous Fe structure and this suggests that the cell-envelope fragments may provide the incrustation matrix for Al and Fe.

Microbial community in the chronosequence and contribution of different microbial groups to SOM formation

Fatty acids, as biomarkers for living microbial biomass (PLFA) and total microbial biomass plus residues (necromass; tFA), provide a more detailed insight into the SOM development as well as its relation to microbial abundance and activity. Up to a soil age of ~120 a (site 17), the increasing PLFA concentration shows a positive correlation to the increase in the number of bacterial cell-envelope fragments ($r^2 = 0.87$, see Supplementary Material A1). However, both amounts decreased towards the

oldest site 18, which may be explained by the fact that the ratio of living microbial biomass to the total content of SOM is generally lower in mature soils. In addition, the turnover of fatty acids was shown to be more than twice as fast as that of the bulk carbon in soil (Kindler et al. 2009), which is also reflected when normalizing the tFA and PLFA values against total soil C. Consequently, carbon-normalized PLFA and tFA contents do not show a clear increase over the chronosequence (Göransson et al. 2011; Bernasconi et al., 2011). However, hardly degradable hopanes of bacterial origin consistently increased with SOM development over the entire glacier forefield (Smittenberg et al. 2012), clearly indicating the microbial contribution to SOM, although the carbon originally stems from plant primary production before being degraded by and transferred to the microorganisms.

Sigler et al. (2002) and Sigler and Zeyer (2002) also analyzed soil samples from the Damma glacier forefield for active bacteria by applying DAPI (4′,6-diamidino-2-phenylindole) staining methods and DNA contents. Although different biomarkers were applied in this study, a similar trend of increasing microbial community complexity was evident. Considering PLFA as a proxy for living microbes, the increase in microbial activity with increasing age can also be shown for the present samples, similar to the previous work applying dehydrogenase assays (Sigler et al. 2002; Sigler and Zeyer 2002). Increasing contents of PLFA have also been found in older successional stages of glacier forefields in Austria (Tscherko et al. 2004); the increase was shown to occur in soil samples from both the rhizosphere and the bulk soil, but PLFA concentrations were up to seven times higher in the rhizosphere than in bulk soil samples. For glacier forefields in North America, PLFA have also been shown to increase in colonized soils with time since deglaciation (Ohtonen et al. 1999; Tscherko et al. 2004; Margesin et al. 2009).

Interestingly, even the youngest samples of the forefield contain bacteria, supporting the hypothesis that there is already a microbial community at the glacier front (Carpenter et al. 2000; Christner et al. 2000; Skidmore et al. 2005; Xiang et al. 2009). This microbial community must be affected by ancient organic C from underneath the glacier, which is metabolized by heterotrophic bacteria after thawing (Bardgett et al. 2007), or by bacteria transferred to the newly exposed surfaces by deposition of eroded soil via wind or water flow.

The relative proportion of markers for Gram-positive bacteria (Table 2) in relation to markers for Gram-negative bacteria increased towards the older soils, as has also been found previously (Ohtonen et al. 1999; Margesin et al. 2009). This may be due to differing reproduction strategies of the two bacterial groups. While many Gram-negative bacteria in soil are r-strategists, most Gram-positive bacteria in soil frequently belong to the K-strategists (Madigan et al. 2012). This means that in the initial stages of soil formation, easily degradable organic C is consumed by Gram-negative bacteria, which thereby quickly form a relatively high amount of biomass (Elfstrand et al. 2008). The opposite can be observed for Gram-positive bacteria, which grow more slowly but are more competitive under the high-competition conditions typical for later stages of soil development. Often, the community in younger soils has been found to be dominated by Gram-negative bacteria, such as Cyanobacteria (Schmidt et al. 2008) and Acidobacteria (Xiang et al. 2009; Schütte et al. 2010), which are adapted to the unfavorable conditions at a glacier and its forefield. However, the increase of Gram-positive bacteria is not continuous; furthermore, it is influenced by the plant inputs and the related changes in nutrient supply (Tscherko et al. 2004; Miniaci et al. 2007) and microbial community structures.

The ratio of fungal to bacterial biomarkers did not consistently increase, but fungi tended to be more abundant in the older soils, which is consistent with the findings of Zumsteg et al. (2011). The low abundance in the C-poor soils can be explained by the need for organic C sources to feed heterotrophic metabolism. The relatively large proportion of fungal PLFA at sampling sites 2 and 4 may be due to fatty acids derived from lichens or from fungal networks associated with plant roots (Tscherko et al. 2004; Miniaci et al. 2007). Nevertheless, the low proportion of fungal markers in comparison to those of bacteria was also found by other authors (Ohtonen et al. 1999; Bardgett et al. 2007). This explains that fungal hyphae only appear in images from the last sampling points. The concentration of markers for Actinomycetes (Table 2) increased with increasing distance to the glacier front, and in parallel to the amount of bacterial cell-envelope fragments, which also correlates to the increase of Gram-positive bacteria.

The increase of cyclopropyl PLFA relative to monounsaturated PLFA suggests increasing stress or

starvation of Gram-negative bacteria with soil age. This is most probably caused by substrate limitation in microbial communities with higher diversity and microbial biomass, and is consistent with the rising abundance of Gram-positive bacteria. This shift in microbial community is related to the death of initially abundant bacteria, whose cell-envelope fragments can thus contribute to SOM formation. The results demonstrate that a primary succession is taking place from the initial sparsely microbially colonized soil to a fully developed ecosystem, whereby the primary source of organic matter input switches rapidly from bacteria to plants, as indicated by the decreasing C-normalized PLFA and tFA concentrations. However, also in developed ecosystems, bacteria continue to process the higher input of organic matter derived from plants, as indicated by the unchanged ratio between bacterial biomass and SOM at sites older than ca. 20 years. This may finally result in starvation due to consumption of the degradable plant input material, and again an increased formation rate of SOM derived from bacterial fragments.

Conclusions

Using scanning electron microscopy and image analysis, we could provide a process-based description of the development of SOM. In the developing ecosystem at the glacier forefield, we observed a strong increase of microbial residues in SOM, in particular visible small-sized cell-envelope fragments. The increase in the abundance of the fragments is accompanied by increased C, N, PLFA and tFA contents as well as higher water repellency at both macro- and micro-scale. In the early stages of glacier forefield ecosystem development, most of the visible particulate SOM input appears to be derived from bacteria, in particular their fragmented cell envelopes. The fact that the shape of the small-size fragments did not change over the chronosequence, although their abundance increased with increasing SOM, is a strong indication of ongoing bacterial processing of SOM as part of the overall dynamics of SOM. It suggests that SOM input from plants in later stages of soil development is processed by bacteria, which after cell death form the typical bacterial cell-envelope fragments.

We observed a distinct increase of the water contact angle, indicating a proceeding coverage of mineral surfaces with hydrophobic materials probably derived from non-degraded microbial residues and plant lipids. In conclusion, the theory of SOM development from microbial cell-envelope residues, as documented in other studies (Miltner et al. in press), was validated in the field. The majority of the plant litter inputs in developed soils are rapidly consumed by microorganisms, which leads to starvation and consequently increasing amounts of decaying bacteria. This in turn leads to higher SOM formation rates, while the properties of the SOM are maintained from the initial stages of ecosystem formation to the most developed stages. Although the majority of SOM carbon in the ecosystem is ultimately derived from primary production by plants, we show here that part of the SOM actually consists of microbial necromass, which underlines the important processing role that bacteria play in every soil ecosystem.

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3 Discussion

The first paragraph of the discussion has the purpose to remind the reader of the most important results of this thesis. This is followed by a detailed discussion where the achievements of this thesis are highlighted by *italic* font.

The present thesis had the purpose of developing low invasive methods for the assessment of soil microbial activity under in situ conditions. In order to enable feasible sampling of these deeper layers of soil with respect to microbial activity, the already well established BACTRAP in situ microcosm system (e.g. Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006; Braeckevelt et al., 2007; Bombach et al., 2010a) was therefore adapted to the Direct Push technology (e.g. Loxham et al., 1988; van Ree and Olie, 1993; US-EPA, 2005; Zschornack and Leven, 2012) and formed the basis for a part of the further experiments in the framework of this thesis (Schurig and Kästner, 2012).

A field test revealed that the DP-BACTRAP probe is applicable in the aquifer and compares better to in situ soil conditions than the 'classical' BACTRAP system (Schurig et al., 2013a). Moreover, laboratory tests demonstrated that the DP-BACTRAP probe establishes hydraulic equilibration with the surrounding soil under vadose zone conditions within 2-3 days, thereby facilitating microbial colonisation of the supplied activated carbon (AC) (Schurig et al., 2013a). The demonstrated applicability in the vadose zone permits adapting the BACTRAP approach to previously inaccesible, hydrophobic, LNAPL forming contaminants, which are mainly accumulating in the vadose zone. Hexadecane was, therefore, used as a model compound for the crude oil contamination at a field site in order to enable a comparison experiment between the commonly used soil incubation microcosms and BACTRAPs (Schurig et al., 2013b). The results revealed similar performance and thereby demonstrate the general applicability of the approach with this type of contaminants under vadose zone conditions (Schurig et al., 2013b).

Microbial abundances are decreasing with increasing depth below ground, therefore, in the aquifer a higher proportion of microbes can be assumed to be involved in contaminant degradation, whereas in the topsoil their fraction might be very small (Schurig et al., 2013c). In order to check the feasibility of the DP-BACTRAP approach in systems with high microbial abundances, a visualisation approach of microbial activity was developed. This approach, indeed, showed that in a groundwater ecosystem most microbes were involved in contaminant degradation or cross-feeding on degrader microbes, whereas in the rhizosphere of a constructed wetland system only very few microbes were degrading contaminants. The remaining microbial community was either hypothesized to be dead or feeding on other organic compounds, such as root exudates or dissolved organic carbon (DOC) in the pore water (Schurig et al., 2013c).

The important role of microorganisms in soil can now be assed under in situ conditions by means of DP-BACTRAPs, however, as the NanoSIMS study showed, parts of this formerly active microbial community can already be dead upon biomarker extraction. A visualisation approach was therefore chosen to determine the ratio of living to dead or inactive microorganisms under in situ conditions. While this ratio is, on the one hand, of interest for contaminant degrading microbes, it is, on the other hand, a keyfactor in

natural ecosystems, because a high fraction of the newly built SOM at the chronosequence of soil development in the forefield of a receding glacier was of microbial origin, at least during initial soil formation (Schurig et al., 2013d). Although the input of plant material was strongly increasing in older soils, the general shape of microbial biomass residues, as markers for SOM, was not changing. These results demonstrate that during SOM formation no entirely new compound class is formed, but instead a high fraction of SOM most probably consists of dead microbial cell residues (Schurig et al., 2013d).

3.1 In situ microbial activity assessment in water saturated soils

Microcosm studies have a long tradition in environmental microbiology, because they help to elucidate and isolate relevant microorganisms in the laboratory. For example, Winogradsky's columns allow to grow certain groups of soil or sediment microorganisms along an artificial redox-gradient by amending a soil sample with a carbon source and exposing it to direct sunlight in a more or less controlled environment. While this method requires a rather long incubation period of several weeks, the contact slide array (Rossi et al., 1936) provides results within a week by exposing a microscopic slide to a glucose amended soil in the laboratory. Both of these methods lead to selective growth of certain groups of microorganisms at the expense of others (Pepper et al., 1995), but predicting which groups will be selectively enriched remains challenging for experiments with various soils and sediments. Selective detection of certain microorganisms, such as pathogens, in environmental samples can be achieved in the laboratory by plating, liquid, or multiwell culture approaches using special media (e.g. Atlas and Bartha, 1997), which exclude the majority of other microbes. While all these ex situ approaches are valuable for studies on certain groups of microbes, they are prone to bias resulting from sample extraction, sample handling and sample preparation in the laboratory.

Therefore, methods that avoid these sources of bias are clearly beneficial for the elucidation of in situ microbial activity. However, currently only few methods are available that enable measurements of microbial activity under in situ conditions in aquifers (Griebler et al., 2002; Bombach et al., 2010b). Some of these systems incubate a pure microbial strain of interest, separated from the aquifer microbial community by means of a membrane, in groundwater monitoring wells to investigate its physiological response to in situ conditions (Shati et al., 1996). This method, on the one hand, clearly excludes the indigenous microbial community (Griebler et al., 2002) and, thus, represents an artificial system prone to the cultivability bias (Amann et al., 1995). On the other hand, it is an improvement for the comparability of laboratory experiments to in situ conditions. Better correspondence to true in situ contaminant degradation can only be achieved by methods that adequately represent the aquifer's indigenous microbial community, such as the microcosm systems developed by Nielsen et al. (1996), Griebler et al. (2002) or BACTRAPs (Geyer et al., 2005). The Nielsen et al. (1996) in situ microcosm system consists of a reaction chamber that is pushed into aquifer sediment in order to fill an enclosed reaction chamber with sediment. This filled chamber is, subsequently, loaded

with contaminant-spiked groundwater. During the course of the experiment water samples are taken from the reaction chamber to analyse contaminant degradation and at the end of the experiment the incubated sediments can as well be extracted from the aquifer allowing for detailed analysis of the microbial community or further experiments in the laboratory (Nielsen et al., 1996). Similarly to BACTRAPs (Geyer et al., 2005), the Griebler et al. (2002) in situ microcosm system is exposed in groundwater monitoring wells at the level of the aquifer. In contrast to BACTRAPs and the Nielsen et al. (1996) probe, the Griebler et al. (2002) microcosms consist of un-spiked sand material from similar geological background as the aquifer material, which is exposed to a permanent stream of fresh aquifer porewater by means of pumping and is separated from the well-water by inflatable packers. The systems of Nielsen et al. (1996) and Griebler et al. (2002) avoid the bias of analysing an unrepresentative microbial community in the stagnant water of the groundwater monitoring wells via pumping or direct incubation in soil. The 'classical' BACTRAPs (Geyer et al., 2005), however, are incubated in stagnant water in groundwater monitoring wells, which can be assumed to be not really representative for the aquifer conditions (Chapelle, 1993; Griebler et al., 2002; Schurig et al., 2013a). *With the new DP-BACTRAP probe this bias could now be overcome (Schurig et al., 2013a) and, moreover, the advantages of the several systems can be combined. The new approach developed in this thesis allows for direct microcosm incubation in aquifer material, like with the Nielsen et al. (1996) probe. The opportunity to analyse labelled biomarkers, which enables resolving the active fraction of the indigenous microbial community (Geyer et al., 2005), and avoiding the bias of stagnant water in groundwater monitoring wells (Griebler et al., 2002). Another feature of the new DP-BACTRAP probe is that it allows sampling in aquifers of varying depth and thus overcomes the limitation of the Nielsen et al. (1996) probe, which is just applicable in shallow aquifers that are reachable with manual power. However, the application of the DP-BACTRAPs requires significant investments in DP-equipment. Moreover, in contrast to the Griebler et al. (2002) probe, no electricity is required for pump operation, which can be a significant advantage, especially, during sampling campaigns at remote field sites. Furthermore, these field sites are usually not equipped with a dense network of pre-installed groundwater monitoring wells, but, nevertheless, can now be analysed for their microbial contaminant degradation potential by means of DP-BACTRAPs, provided that the aquifer is accessible by the DP-technique. This clearly prohibits application of the approach in very deep aquifers or aquifers underneath rock surfaces.*

In summary, the BACTRAP method has been clearly improved with respect to several factors; however, some flaws inherent to the system remain to be solved. These flaws are related to the incubation of an activated carbon surface in the aquifer rather than using aquifer sediments, as the other methods do (Nielsen et al., 1996; Griebler et al., 2002). This procedure can be expected to render the colonising microbial community on the AC different compared to the indigenous microbial community of the aquifer (Bengtsson, 1989; Scholz and Boon, 1993; Bennett et al., 2000; Griebler et al., 2002). Differing microbial communities are resulting from the fact that the transfer of microorganisms from the aquifer to the AC surface of the DP-BACTRAPs is not solely a physical process, but rather is a function of the varying motility of certain microor-

ganisms. Moreover, the motility cannot be assumed to be correlating with the microbial activity, because van Loosdrecht et al. (1990) showed that sessile microbes in aquifers can exhibit higher activities than motile ones. *While this problem is of higher importance for meso- or eutrophic topsoil systems with per se higher microbial abundances, the results of Schurig et al. (2013c), furthermore, indicate that most of the previously visualised microbes from the aquifer (Bombach et al., 2010a; Miltner et al., 2012) are actively involved in contaminant degradation.* This finding seems reasonable, because natural aquifer systems are usually oligotrophic and, therefore, possess low microbial abundances, but contamination has been shown to cause an increase of motile microbes (Harvey et al., 1984; Godsy et al., 1992; Bekins et al., 1999). *Therefore the bias caused by the selective enrichment of motile microbes is reduced on DP-BACTRAPs compared to BACTRAPs (Schurig et al., 2013c).* Yet, only the activated carbon beads provide strong enough sorption capabilities for the contaminants of interest and thereby guarantees that the loaded ^{13}C contaminant stays in the BACTRAPs to be available for degrader microbes. *Results from scanning electron microscopy (SEM), furthermore, indicate that the microbial community settling on DP-BACTRAPs is more closely representing the one found in soil (Schurig et al., 2013c). Related to this, the 'classical' BACTRAP approach was not suitable for providing real in situ contaminant degradation rates. However, the new probe brings the AC surfaces into direct contact with the soil at the level of the aquifer and, thereby, the AC is directly embedded into the groundwater flow. Thus, the new results of Schurig et al. (2013a) indicate that at least semiquantative results can be obtained, because the bias with respect to in situ conditions is reduced.* Combining the results of DP-BACTRAPs with a groundwater sampling campaign and analysis for compound specific isotopic fractionation analysis (CSIA; Hunkeler et al., 2008; Braeckevelt et al., 2012), can lead to quantative results. A CSIA is relying on the fact that during biodegradation, contaminants with lighter isotopes show higher chemical activity and, therefore, are preferentially degraded by microorganisms. This process leads to an enrichment of contaminants with heavy isotopic signature in the residual contaminant mass. By means of this data, and laboratory derived enrichment factors, the already biodegraded fraction of the original contaminant amount can be calculated (Meckenstock et al., 2004; Hunkeler et al., 2008). Hence, a combined approach of DP-BACTRAPs and CSIA would provide data on both the past and present biodegradation potential at the field site (Schurig et al., 2013a).

Soil systems feature a high degree of heterogeneity and structural complexity on several scales (Odum, 1969). Therefore, van Ree and Carlon (2003) argued that the results of a site investigation should be interpreted with great care, especially, when data from methods with low spatial resolution is interpolated, such as measurements based on groundwater monitoring wells. While chemical and geophysical measurements independent from monitoring wells are already available by means of the DP-technique, microbiological methods, other than the Nielsen et al. (1996) probe, are still relying on pre-installed wells. Consequently, some of the variability inherited in the measured results can be attributed to spatial variability and synchronisation of results from the chemical and geophysical measurements to the microbiological data requires a kind of scaling approach, which itself is based on strong assumptions. Commonly these data interpolations are done by means of the Kriging approach (Krige, 1951), which

assumes a spatially homogeneous aquifer devoid of preferential flow paths and composed of spatially homogenous soil structure. *Clearly, these assumptions are not met in a real world field setting. The uncertainty related to interpolation can, therefore, only be decreased by increasing the density of the measurement grid for microbiological methods, which can now easily be done by means of the DP-BACTRAPs (Schurig et al., 2013a). Thus, for the first time, microbiological methods can be synchronised to a high resolution low invasive site investigation approach, as demanded by the US-EPA (2006) and later proposed by Kästner and Cassiani (2009).*
As described by the authors, this DP-based investigation approach can significantly decrease costs and time effort for a site investigation and, moreover, provides results at an early project stage. Another described disadvantage of the commonly used site investigation approach is that the methods used are usually not capable of monitoring remediation success during an early stage of an ongoing site remediation action (Kästner and Cassiani, 2009). *With the new DP-BACTRAP probe (Schurig et al., 2013a), however, decreasing microbial abundances along with decreasing microbial activity were detected during an on-going remediation action, which demonstrates the potential of the method for fast and accurate assessment of remediation success.* Additionally this provides site managers with the opportunity to revise their remediation approach during the ongoing treatment, if it shows unfavourable side effects, or to test the effect of remediation measures, like electron acceptor amendments, on the microbial activity of a small part of the field site before the start of a large scale application. *Further cost- and time savings during site investigation can be achieved by means of DP-BACTRAPs, because the method is capable of analysing more than one contaminant at the same time, if the contaminated layer has a sufficient vertical extent (Schurig et al., 2013a).* When using this approach, the method is, moreover, capable of accounting for the toxicity effect of a mixture of contaminants on microbes under in situ conditions, which might be very different compared to the toxicity of a single compound (Altenburger et al., 2004). Therefore, concentration decreases during a remediation action do not necessarily lead to lower toxicity, if just one component of the mixture is removed. *Since these effects are very complicated to mimic even under laboratory conditions, DP-BACTRAP tests at the field site prior to a remediation action could help to gain confidence on the applied remediation method prior to the start of the action.*
While DP-BACTRAPs are providing significant advantages for site managers and engineering companies, they feature also valuable characteristics for research questions. For example, DP-BACTRAPs can be incubated in an accessible ecosystem and later the colonised AC can be used for further laboratory cultivations. Compared to other sampling techniques, like drilling or taking water samples from wells, this approach would likely offer advantages with respect to comparability to in situ conditions and, additionally, no contaminated material reaches the soil surface. Bombach et al. (2010a) isolated an enrichment culture from the Zeitz aquifer and visualised it by means of confocal laser scanning microscopy. The authors reported that the microbial community on the AC after cultivation was mainly consisting of rod-shaped bacteria leading the authors to the conclusion that mostly rod-shaped bacteria are degrading the toluene in the Zeitz aquifer. Miltner et al. (2012) and Schurig et al. (2013c) visualised the microbial community settling on the AC directly after exposure in the same aquifer at the same

sampling spots and found a very diverse microbial community with a wide variety of cellular shapes. These results could mean that these non rod-shaped microorganisms are either not capable of toluene degradation or that they are not cultivable. *With the new NanoSIMS based visualisation method, as proposed by Schurig et al. (2013c), a direct visualisation of the microbial community after exposure at the field site is now available, which, furthermore, provides information on the activity of the visualised microbial cells.* Schurig et al. (2013c) reported that most of the visualised microbial cells were involved in toluene degradation or were cross feeding on degrader microbes. This allows the conclusion that the results of Bombach et al. (2010a) were either affected by the laboratory cultivation measures (Amann et al., 1995; Schurig et al., 2013c) or that the samples of Bombach et al. (2010a) were not representative for the aquifer, because of the previous exposure in groundwater monitoring wells (Chapelle, 1993; Griebler et al., 2002; Schurig et al., 2013a). Moreover, these new results seem more reasonable, because probably most of the microbes in the Zeitz aquifer are using the contaminants as their carbon source in an otherwise oligothrophic environment. A combination with the HISH-SIMS approach (Musat et al., 2008), applying more specific FISH-probes than Schurig et al. (2013c) used, should, thereby, help to elucidate the active degrader fraction of microbes together with information on the phylogenetic identity under in situ conditions, as Musat et al. (2008) did for a lake in Switzerland. This approach can be regarded clearly superior to approaches requiring cultivation, as the Bombach et al. (2010a) experiments, and the FISH-experiments of Tischer et al. (2012), which are providing information on the phylogenetic identity, but no information on contaminant degrading activity and the spatial arrangement of the microbes in their environment. Moreover, the Schurig et al. (2013c) approach is more likely to elucidate complex environmental relationships, like microbial consortia, because it is cultivating the indigenous microbial community under in situ conditions and analyses the labelling of microbial cells on the single-cell level, whereas the other approaches extract the biomarkers of the whole microbial community (Schurig et al., 2013c). Especially in meso- or even eutrophic ecosystems, such as the rhizosphere of constructed wetlands or topsoil, the proportion of microbes not related to the degradation of a specific compound is likely larger than in oligotrophic groundwater systems. Thereby, the extraction of labelled biomarkers with low phylogenetic resolution, such as the phospho-lipid fatty acids commonly used for the BACTRAP-approach (Schurig and Kästner, 2012), is rendered inadequate due to an attenuation of the labelled biomarkers in the unlabelled bulk-mass of biomarkers from inactive, unrelated or dead microorganisms (Schurig et al., 2013c). However, the analysis of labelled DNA or RNA requires an even higher isotopic enrichment than PLFA analysis in order to allow for the detection of the active microbial cells.

Extractable biomarkers from dead microbial residues can be a significant part of SOM (Miltner et al., 2012; Schurig et al., 2013d) and, thus, represent also a part of the extractable biomarkers in the BACTRAP approach, especially, in meso- or eutrophic, water saturated ecosystems like river sediments, swamps or the rhizosphere of constructed wetlands, where the data of Schurig et al. (2013c) demonstrate thorough surface coverage with dead microbial residues even after a short incubation time of 4 weeks. This means that, even under water saturated conditions, microbial biomass

fragments are accumulating and care should be taken during the interpretation of extracted biomarker patterns, especially, when biomarkers with low phylogenetic resolution, like fatty acids, are applied. In order to account for this uncertainty, and since analysis on the single cell level is not feasible for every experiment, future experiments in meso- or eutrophic ecosystems should, therefore, apply more species specific biomarkers, which are, moreover, highly unstable after cell death (Green and Scow, 2000; Pinkart et al., 2002). Since DNA, and also proteins, can be quite stable in soils (Miltner et al., 2012; Schurig et al., 2013d), RNA is the only suitable biomarker for this kind of analyses in meso- or eutrophic ecosystems. While the substitution of PLFA- with RNA analysis in these ecosystems seems practicable for research settings, it is associated with higher costs, which are of economical concern in commercial site management applications. However, these additional costs might be outbalanced by the cost savings associated to not necessarily needing a DP-machine for probe installation and retraction in shallow, water saturated ecosystems. Therefore, further experiments and calculations should focus on these problems in order to enable widespread application and to explore the full potential of the new DP-BACTRAP method.

3.2 Microbial activity in the vadose zone

During field site investigation in the framework of remediation actions, the main focus is usually on groundwater contaminations. These contaminations are threatening drinking water resources and contaminants are transported by the groundwater flow to previously unaffected environmental compartments, such as other parts of the aquifers or aboveground water bodies, like rivers and lakes. The groundwater flow is, moreover, smoothing the conditions in an aquifer, therefore, its absence in the vadose zone leads to an even higher spatial heterogeneity, due to slower transport processes and transport processes along preferential flow paths (Rivett et al., 2011; Schurig et al., 2013a). In such systems, an even finer sampling grid is required for adequate mapping and subsequent model construction for a field site. Although, the DP technology suite allows geophysical and chemical measurements in this zone of soil (Dietrich and Leven, 2005; Zschornack and Leven, 2012), at the beginning of this PhD work, no in situ methods existed for the measurement of microbial activity in deeper layers of the vadose zone (<1.5m). Even though, the natural attenuation site remediation approach (NA) is often just applied for groundwater contaminations, it can be regarded as an effective remediation measure for soil contaminations in the vadose zone. The relevance of microbial activity assessment in unsaturated, shallow layers of soil is even higher, because other natural attenuation processes, like advection and dispersion (Wiedemeier et al., 1999), are not efficient in these layers, due to the lack of effient transport process like groundwater flow. *The proposed DP-BACTRAP approach for microbial activity in the vadose zone (Schurig et al., 2013a), therefore, provides a valuable option for site managers and researchers and, moreover, serves the rising demand for such an in situ method in the context of increasing legislational focus on soil protection, with e.g. the new soil framework directive of the European Union (Kästner and Cassiani, 2009).*

In water saturated systems planktonic microorganisms may be transported to the AC by advection with the groundwater flow or microrganisms may actively move towards the AC surface. In the vadose zone, however, they require a continuous water film, or fungal hyphae, in order to actively migrate to the AC surfaces (Kohlmeier et al., 2005; Madigan et al., 2012). *The hydraulic equilibration of the AC in DP-BACTRAPs with the surrounding soil is, thus, of major importance in the vadose zone. The general applicability of the DP-BACTRAPs in the vadose zone with respect to hydraulic equilibration to the surrounding soil was successfully tested in the laboratory down to 20% of the soil water holding capacity (WHC) (Schurig et al., 2013a). Only after reaching its surface, microorganisms can colonise the AC and degrade the supplied contaminant. As 20% WHC represents very dry conditions for deeper layers of soil, this demonstrates the wide applicability range of DP-BACTRAPs under natural soil conditions (Schurig et al., 2013a).* Moreover, the data of Liang et al. (2009) shows that activated carbon surfaces are rapidly colonised by indigenous microbes in soil and, thereby, can enhance the soil's potential for microbial contaminant degradation. *Therefore, reason exists that the DP-BACTRAP probe should be applicable under vadose zone conditions, though the incubation time may have to be prolonged in order to enable sufficient bacterial colonisation of the AC (Schurig et al., 2013a). This may not be necessary under meso- or eutrophic topsoil conditions, where high microbial abundances enable fast colonisation of the AC (Schurig et al., 2013c). While the experimental data successfully showed the feasibility of the DP-BACTRAPs under vadose zone conditions in the laboratory, future studies should verify these results in the vadose zone of a contaminated field site (Schurig et al., 2013a).*

Results from the laboratory, nevertheless, indicate the general applicability of the approach for alkane contaminations under vadose zone conditions (Schurig et al., 2013b). Moreover, the BACTRAPs provided similar conclusions compared to the commonly used soil microcosm mineralisation assays (e.g. Towell et al., 2011; Adetutu et al., 2012; Aislabie et al., 2012). The degradation rates in soils from the Trecate (Italy) site were low compared to other studies (Towell et al., 2011; Adetutu et al., 2012), but this result was consistent between BACTRAPs incubated in soil microcosms and 'traditional' soil microcosm incubations. *This clearly indicates that the BACTRAPs reflect in situ processes in the vadose zone and thus gives reason for the potential of a vadose zone application of DP-BACTRAPs under in situ conditions.* As alkanes are mostly hydrophobic, they are frequently accumulating on top of the groundwater table as light non aqueous phase liquids (LNAPLs) and get dispersed in the soil and groundwater with variations of groundwater height (Molins et al., 2010). The affected soil volume can, therefore, be quite high even when water samples from the aquifer indicate low contamination due to the low solubility of these compounds (Eastcott et al., 1988). *Schurig et al. (2013b) obtained sediments from the Trecate (Italy) site, which shows high groundwater fluctuations due to rice cultivation in paddy fields, and, thereby, is an ideal test site for investigating this problem.* Adequate characterisation of field sites affected by alkane pollution, like the Trecate (Italy) site, cannot rely on groundwater monitoring wells alone, but instead requires sampling of the water unsaturated zone by means of expensive drilling operations or the more cost-efficient DP technique. Therefore, DP was the method of choice for the site managers of the Trecate site (ENI,

2008). Although alkanes are known to be readily degraded by microorganisms (e.g. Pirnik et al., 1974; Gallo et al., 2012), their potential in the framework of a NA site remediation approach was, prior to the start of this thesis, insufficiently explored by ex situ laboratory microcosm incubations, like the ones performed by e.g. Towell et al. (2011), Adetutu et al. (2012) and Aislabie et al. (2012). Kaufmann et al. (2006) and Höhener et al. (2006) presented in situ studies, in which they inserted contaminant spiked sediments into the soil in order to investigate the contaminant degradation. In spite of the advantage of being an in situ method, this procedure is prone to bias arising from the insertion into soil and is restricted to the upper layers of soil. *All these approaches clearly lack the resolution of the DP-technique, and, therefore, the adaptation of the DP-BACTRAP technique to these contaminants offers significant benefits for commercial site investigators (Schurig et al., 2013b). Additionally, the method provides conclusive proofs for a natural attenuation site remediation strategy, which can be presented to the government.*

Moreover, the DP-BACTRAP approach would allow to exclude several potential sources of bias inherent to the ex situ microcosms approach, such as cross contamination and mixing of soil layers during sampling, contact of formerly anoxic layers to oxygen, change of the microbial community due to storage and cultivation bias during laboratory scale incubations (Bombach et al., 2010b). A further interesting aspect, especially for research projects, is that the approach allows analysing biomarkers directly rather than measuring mineralisation as a proxy for microbial activity (Schurig et al., 2013b). These biomarker studies provide, depending on the biomarker analysed, data on the active fraction and the identity of uncultivable or cultivable microbial strains under field conditions. A combination of these data with the visualisation approach by means of NanoSIMS (Schurig et al., 2013c) would also be directly applicable with these kind of samples. As earlier described, this potentially fosters the understanding of trophic relationships in these environments under in situ conditions. Yet, this approach is visualising the microbial community settling on the AC rather then the one directly colonising the soil. However, the results of Schurig et al. (2013b) indicate good comparability between soil microcosm incubations and BACTRAPs in deeper soil layers and, as well as, between results from the aquifer zone and the vadose zone. Therefore, the BACTRAP approach is generally applicable.

Another part of the Schurig et al. (2013b) study was presenting the use of ^{2}H-labelled contaminants, rather than the traditional ^{13}C approach. The use of ^{2}H-labelled contaminants could be valuable for a future extension of the BACTRAP approach onto other contaminants, which might not be available with ^{13}C or ^{15}N label. However, only some microbial degradation processes lead to an integration of ^{2}H atoms along with carbon atoms into the microbial biomass and, as such, the ^{2}H labelling approach is only applicable for alkanes and styrene (Alexandrino et al., 2001), because parts of these compounds are directly transfered to the cellular membranes of the degrading microorganisms. For example, Schurig et al. (2013b) used pristane as a marker compound for recalcitrant branched chain alkanes (Pirnik et al., 1974) and this compound was commercially available only with ^{2}H-label at an affordable price. *Moreover, using deuterated compounds could increase the financial feasibility of the DP-BACTRAP approach, since ^{2}H-labelled compounds are usually cheaper than ^{13}C and ^{15}N labelled*

compounds. The downside of using ^{2}H-labelled contaminants, however, is that a higher relative label compared to ^{13}C-labelling approaches is required in order to avoid needing an expensive GC-C-IRMS device for measurements. However, Alexandrino et al. (2001) and Friedrich and Lipski (2010) successfully measured ^{2}H enriched fatty acids extracted from laboratory pure cultures by means of GC-MS. Also high ^{2}H enrichments can lead to a separation of peaks at natural isotopic abundance and labelled peaks at the GC column, because of the relatively high mass-difference between ^{1}H and ^{2}H compared to, for example, carbon. This could facilitate easier identification of the isotopic enrichment of certain biomarkers. However, the visualisation approach by NanoSIMS (Schurig et al., 2013c) is not applicable for ^{2}H-labelled contaminants due to instrumental restrictions.

3.3 Contribution of dead microbial biomass to non-living soil organic matter

Results from the NanoSIMS study indicated, that a part of the microbial biomass found on DP-BACTRAPs after 4 weeks of incubation in a constructed wetland was already dead (Schurig et al., 2013b). This finding highlights that, under in situ conditions, microbial proliferation and contaminant degradation is accompanied by the formation of dead microbial biomass residues. However, the DP-BACTRAP approach aims to elucidate the active fraction of microorganisms in soils and sediments and, therefore, requires data on the ratio of living to dead microbial biomass. Determining this ratio is of special concern, since DP-BACTRAPs have been shown to adequately represent the soil microbial community (Schurig et al., 2013a).
Recent results, however, indicate that the significance of microbial residues for soil organic matter (SOM) formation was previously underestimated (Miltner et al., 2012), and that SOM is consisting of microbial biomass fragments that accumulate at the soil mineral interfaces (Miltner et al., 2012; Schurig et al., 2013d). Moreover, these residues show similar physical and chemical properties compared to humic substances, which were previously thought to form the majority of SOM (Miltner et al., 2012; Schurig et al., 2013d).
While Miltner et al. (2012) provided evidence for their hypothesis from laboratory experiments and a field scale experiment using BACTRAPs, no results from a natural soil environment were presented. *Schurig et al. (2013d), however, could find similar residues in a chronosequence of soil development spanning soil ages of 0 to 130 years by means of a image analysis approach based on scanning electron micrographs. The authors thus supported the theory of Miltner et al. (2012) in field examinations.* Schurig et al. (2013d) still did not provide evidence on the chemical composition of these fragments due to instrumental restrictions. However, by means of atomic force microscopy - nano thermal analysis (AFM-nTA), Kunhi-Mouvenchery et al. (2013) found that the fragments in the samples of Schurig et al. (2013d) throughout the chronosequence had similar properties as pure microbial cell envelope material. As indicated earlier, these findings mean, that a successfull application of DP-BACTRAPs in topsoils require highly unstable biomarkers, and that fatty acid analysis, therefore, might be inad-

equate.
Similarly to degrading plant litter in natural ecosystems, organic xenobiotics are also degraded in soil by microbes. While it was previously believed, that non-extractable residues are formed during biodegradation (Barriuso et al., 2008), which are not easily amenable to chemical analysis, but commonly assumed to consist of the parent product or direct metabolites strongly sorbed to the soil matrix. Recently, Nowak et al. (2011) and Nowak et al. (2013) showed that a substantial portion of these NER formed from biodegradable xenobiotics, such as 2,4-D or ibuprofen, consist of microbial biomass and its residues, and are thus non-toxic components of SOM. Similar behaviour could be expected during the contaminant degradation in the DP-BACTRAPs, which, especially for easily degradable compounds, demands for the analysis of specific unstable biomass markers, because, otherwise, the active fraction of microbes cannot be accurately assessed (Schurig et al., 2013c). The Nowak et al. (2011) results, additionally, highlight that soil mineralisation studies should be accompanied by biomarker analysis, as presented here, in order to investigate what happens to the fraction of supplied substance that remains in soil. *As soil microcosm preparation and mineralisation assays, with the exception of the Kaufmann et al. (2006) and Höhener et al. (2006) systems, were the only means of studying microbial contaminant degradation in the vadose zone before the presentation of the DP-BACTRAPs (Schurig and Kästner, 2012; Schurig et al., 2013a), these DP-BACTRAPs represent a major improvement.*
Moreover, the fluorescence in situ hybridisation (FISH) data of Schurig et al. (2013c) suggests that most parts of the AC are covered with biomass, because they show high fluorescence. SEM images, however, reveal that the surface of the AC is covered with a biofilm like structure, which mostly consists of dead microbial biomass fragments, like the ones described by Miltner et al. (2012) and Schurig et al. (2013d), and that living cells are embedded in these structures. Subsequent NanoSIMS analysis revealed that these structures are labelled by F using the HISH-SIMS approach (Musat et al., 2008) and that they are depleted in phosphorous. *This indicates that these structures originate from microbial biomass, but that the cells are not alive anymore, because the phosphorous from the PLFA is already missing. These findings can, therefore, explain the frequent failure of the FISH method in soil studies, which is commonly attributed to the high autofluorescence of the soil material (Tischer et al., 2012); if the soil particles are covered with dead biomass fragments, everything containing suitable nucleic acids gets labelled and identification of intact cells, thus, gets complicated.* In addition, only bacteria on top of surfaces can be detected because the soil is opaque. These problems can only be overcome by the extraction of cells from the soil (Tischer et al., 2012), the use of very specific FISH probes or the use of a NanoSIMS based approach (Schurig et al., 2013c). *However, only the NanoSIMS approach without embedding presented here, allows for visualisation of the microbial community and its activity in three-dimensional sturctures with high topography.*
The results of Miltner et al. (2012) and Schurig et al. (2013c), furthermore, highlight that these microbial biomass fragments are accompanying microbial proliferation under water saturated conditions in both anoxic aquifers and aerated constructed wetlands and, therefore, their stability is of potential interest for studies of dissolved organic matter (DOM). Until recently, the molecular origins of SOM and DOM remained unclear, and,

thus, the microbial cell residue theory (Miltner et al., 2012; Schurig et al., 2013d) could foster further understanding of these important processes.

4 Summary

Soils are an important compartment in the global carbon cycle and soil protection is of major importance to preserve the manifold soil functions. Despite their small abundance in terms of carbon, microorganisms are keyplayers in this ecosystem, due to their transformation capabilities towards many organic compounds that are entering the soil. While the importance of these microbes for contaminant degradation, on the one hand, is already well accepted and commercially used in the framework of the natural attenuation site remediation strategy in contaminated aquifers. In natural systems, on the other hand, their influence on SOM formation, and thereby their degradative capabilities towards plant litter, is often assumed to be low. This underestimation might arise from the lack of few suitable methods, which allow for direct in situ measurements and visualisation of microbial activity and microbial biomass.
In order to address these issues the purposes of this thesis were:

- to improve the BACTRAP method, in order to allow for in situ measurements of microbial activity at field sites without pre-installed wells and at varying depths of soil and sediment
- to use this new device to measure microbial contaminant degradation under in situ conditions in aquifers
- to enable fast in situ measurements of microbial activity in the vadose zone
- to compare this new probe with the commonly used ex situ laboratory incubations with vadose zone sediments
- to adapt the BACTRAP approach to LNAPLs like alkanes, since hydrophobic contaminants are usually accumulating in the vadose zone due to their high hydrophobicity
- to visualise soil microbial activity, by means of a combination of BACTRAPs, SEM and NanoSIMS, in order to permit comparison between aquifer and wetland conditions regarding the active fraction of microorganisms
- to use this visualisation approach to investigate the fate of microbial biomass after cell death

In order to achieve these goals, the BACTRAP method was coupled with the Direct Push (DP) technology and a new probe called DP-BACTRAP was developed. This new probe was compared to the 'classical' BACTRAP approach in a field test and showed superior performance and better comparability to in situ soil conditions at the level of the aquifer. In order to adapt this new method to contaminations mainly occuring in the vadose zone, which were very difficult to access for in situ measurements of microbial activity prior to the start of this thesis, additional experiments had the task to adapt the BACTRAP approach to alkanes, with hexadecane as a model compound for crude oil pollutions. The results of these experiments provided reason for the conclusion that BACTRAPs are now applicable with these compounds under vadose zone conditions

and, moreover, provide similar results compared to the previously used soil mineralisation assays. The application of DP-BACTRAPs under in situ conditions avoids the bias inherent in ex situ laboratory microcosms resulting from sampling, the handling of the material in the laboratory and the limited cultivability of microorganisms under laboratory conditions. DP-BACTRAPs were also incubated in a BTEX contaminated aquifer and in the rhizosphere of a constructed wetland treating benzene contaminated groundwater. These samples were later visualised by means of NanoSIMS and showed that in the aquifer most microbes were actively degrading contaminants, while in the constructed wetland only a small fraction of microbes was actively degrading the benzene and the rest of the microbial biomass was either dead or inactive. Visualisation experiments were performed in order to investigate the fate of the microbial biomass after the carbon incorporation from organic molecules. SEM analysis on soil samples from a chronosequence of soil development provided evidence that the quantity of microbial biomass residues is increasing in accord with increasing C, N, fatty acid contents and water contact angle readings.

In conclusion, this thesis provided approaches to detect and visualise soil and sediment microbial activity under in situ conditions. The earlier developed BACTRAP appproach (Geyer et al., 2005) formed the basis for the developed DP-BACTRAPs (Schurig and Kästner, 2012). This new approach avoids the bias of measuring in stagnant water in groundwater monitoring wells and, therefore, provides representative results compared to the aquifer sediments. Moreover, DP-BACTRAPs are applicable in the vadose zone above the aquifer (Schurig et al., 2013a). Measurements in the water-unsaturated zone of soil provide the opportunity to investigate microbial contaminant degradation activity towards hydrophobic contaminants, like alkanes arising from an oil spill, under in situ conditions in the framework of a DP-based site investigation approach (Schurig et al., 2013b). The DP-BACTRAPs, additionally, formed the basis for visualising soil microbial contaminant degradation activity in groundwater and constructed wetland ecosystems, by means of NanoSIMS, on samples incubated under in situ conditions (Schurig et al., 2013c). The study showed that the number of actively contaminant degrading microbes is similar between an oligotrophic aquifer and the rhizosphere of a constructed wetland and that samples with a high degree of topography not neccessarily need to be resin-embedded prior to analysis by NanoSIMS (Schurig et al., 2013c). Furthermore, it was shown, by means of scanning electron microscopy together with an image analysis approach, that dead microbial biomass residues are an important part of SOM also under in situ conditions in soils, sediments and aquifers (Schurig et al., 2013d).

5 Zusammenfassung

Böden und Sedimente sind nicht nur wichtige Teile des globalen Kohlenstoffhaushalts, sondern sie erfüllen zu dem eine Vielzahl anderer, wichtiger Funktionen, weshalb die Aufrechterhaltung und Verbesserung dieser Funktionen das Ziel eines jeden Bodenschutzkonzepts sein sollte. Obwohl Mikroorganismen nur einen sehr kleinen Teil der organischen Substanz im Boden ausmachen, hängt die Funktionalität der Böden als Ökosystem zu großen Teilen von ihnen ab, da Bodenmikroorganismen fast alle, in den Boden eindringende, organische Substanzen umwandeln können. Das Potenzial des mikrobiellen Schadstoffabbaus im Rahmen des natürlichen Schadstoffabbaus (natural attenuation) ist deswegen schon weitestgehend als kommerzielle Sanierungsstrategie für kontaminierte Böden und Sedimente akzeptiert. In natürlichen Systemen geht man aber nur von einem geringen mikrobiellen Abbaupotenzial pflanzlicher Streu, und somit einem geringen mikrobiellen Beitrag zur organischen Bodensubstanz, aus. Dieses Unterschätzen eines potentiell wichtigen Prozesses könnte auf einen Mangel an geeigneten Methoden zur Visualisierung mikrobieller Aktivität und Biomasse unter *in-situ* Bedingungen zurückgehen. Die, sich aus diesen offenen Themengebieten ergebenden, Ziele dieser Arbeit waren daher:

- die BACTRAP-Methode weiter zu entwickeln, um *in-situ* Messungen der mikrobiellen Aktivität an jedem Feldstandort und in verschiedenen Bodentiefen zu ermöglichen
- diese neuentwickelte Methode zu verwenden, um mikrobiellen Schadstoffabbau unter den gegebenen Bedingungen in einem Grundwasserleiter nachzuweisen
- das Ermöglichen von *in-situ* Messungen der mikrobiellen Aktivität in der ungesättigten Bodenzone über Grundwasserleitern (vadose zone)
- diese neue Methode mit den, üblicherweise verwendeten, sedimentgefüllten *ex-situ* Labormikrokosmensystemen zu vergleichen
- die BACTRAP-Methode an LNAPLs wie Alkane anzupassen, weil sich diese hydrophoben Schadstoffe häufig in der ungesättigten Bodenzone über dem Grundwasserleiter anreichern
- die Visualisierung der mikrobiellen Aktivität, mittels einer Kombination von BACTRAPs, Rasterelektronenmikroskopie (SEM) und NanoSIMS, um Vergleiche zwischen Grundwasserleitern und Pflanzenkläranlagen hinsichtlich der aktiven Mikroorganismen ziehen zu können
- das Verwenden dieses Ansatzes um das Schicksal toter mikrobieller Biomasse im Boden zu untersuchen

Um diese Ziele zu erreichen wurde die BACTRAP-Methode mit der Direct-Push (DP) Technologie verbunden und eine neue, DP-BACTRAP genannte, Sonde entwickelt. In einem Feldtest zeigte diese Neuentwicklung, im Unterschied mit der klassischen BACTRAP-Methode, vergleichbare Ergebnisse hinsichtlich der, sich darauf ansiedelnden, mikrobiellen Gemeinschaft verglichen mit der im Grundwassersediment. Die

Möglichkeit mit der neuentwickelten Sonde unkompliziert in der ungesättigten Zone unter *in-situ* Bedingungen zu messen, ermöglichte die Adaption des BACTRAP-Ansatzes an hydrophobe Schadstoffe (Alkane), welche sich nicht im Wasser lösen. Für diese Experimente wurde Hexadekan als Modellalkan für Rohölkontaminationen in der ungesättigten Bodenzone verwendet, wobei die BACTRAP-Ansätze unter Laborbedingungen den üblichen Mikrokosmen-Ansätzen ähnliche Ergebnisse lieferten, was die Schlussfolgerung erlaubt das, mit Hexadekan beladene, BACTRAPs unter ungesättigten Bedingungen im Boden funktionieren. Die Ausbringung von DP-BACTRAPs unter Feldbedingungen würde zu noch besser vergleichbaren Ergebnissen führen, da Fehler durch die Probennahme, die Probenverarbeitung und die mangelnde Kultivierbarkeit von Mikroorganismen unter Laborbedingungen ausgeschlossen werden können. In einem Feldexperiment wurden DP-BACTRAPs in einem BTEX-kontaminierten Grundwasserleiter und in einer Pflanzenkläranlage inkubiert, wobei durch Visualisierung der mikrobiellen Gemeinschaft mittels NanoSIMS gefunden wurde, dass im Grundwasserleiter ein Großteil der visualisierten Mikroorganismen am Schadstoffabbau beteiligt war, während in der Probe aus der Pflanzenkläranlage nur ein kleiner Teil der Mikroorganismen Schadstoffe abbaut und der Rest der mikrobiellen Gemeinschaft entweder inaktiv oder tot ist. Der Visualisierungsansatz wurde außerdem gewählt um das Schicksal kohlenwasserstoffabbauender, mikrobieller Biomasse nach dem Zelltod zu untersuchen. Anhand der rasterelektronenmikroskopischen Untersuchung von Bodenproben einer Bodenentwicklungsreihe (Chronosequenz) konnte dabei gezeigt werden, dass die Menge an mikrobiellen Biomassefragmenten mit steigendem Bodenalter zunimmt und außerdem, dass dies im Einklang mit zunehmenden C-, N- und Fettsäuregehalten, sowie Wasserkontaktwinkeln in den Proben geschieht.

Zusammenfassend wurden in dieser Dissertation Methoden zur Detektion und Visualisierung mikrobieller Aktivität in Böden und Sedimenten direkt am Standort entwickelt. Basierend auf den gebräuchlichen BACTRAPs (Geyer et al., 2005) wurde eine neue DP-BACTRAP Sonde (Schurig & Kästner, 2012) entwickelt, welche repräsentativere Ergebnisse liefert, in dem sie nicht im stehenden Wasser im Grundwasser inkubiert wird, sondern direkt im Sediment, und welche zudem erstmals Messungen in der ungesättigten Bodenzone über Grundwasserleitern ermöglicht (Schurig et al., 2013a). Damit können nun auch erstmal hydrophobe, organische Kontaminanten, wie aus einem Ölunfall stammende Alkane, mit der Methode unter *in-situ* Bedingungen im Rahmen eines innovativen DP-basierten Standorterkundungsverfahrens untersucht werden (Schurig et al., 2013b). DP-BACTRAPs waren zudem die Basis für weitergehende Experimente zur Visualiserung mikrobieller Schadstoffabbauaktivität im Grundwasser und in Pflanzenkläranlagen mittels NanoSIMS (Schurig et al., 2013c). Mit diesen Experimenten konnte gezeigt werden, dass die Anzahl aktiv schadstoffabbauender Mikroben in beiden Systemen ähnlich ist und das auch Proben mit einer hohen Topographie vor der NanoSIMS-Analyse nicht unbedingt in Harz eingebettet werden müssen. Außerdem konnte mit einem Visualisierungsexperiment mittels SEM gezeigt werden, dass tote mikrobielle Biomassefragmente, auch unter *in-situ* Bedingungen, ein wichtiger Teil der organischen Bodensubstanz in Böden, Sedimenten und Grundwasserleitern sind (Schurig et al., 2013d).

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Eigenständigkeitserklärung

Hiermit erkläre ich, Christian Schurig, geboren am 29.02.1984 in Dresden, das ich mit der Promotionsordnung der Fakultät für Biologie und Pharmazie der Friedrich Schiller Universität Jena vertraut bin. Außerdem erkläre ich, dass ich die hier vorgelegte Dissertation mit dem Titel "Assessment of soil microbial activity by non- or low invasive methods" eigenständig, im Einklang mit den Vorgaben der Universität, ohne unerlaubte Hilfe angefertigt habe und verwendete Quellen gekennzeichnet habe. Die Dissertation wurde in der vorgelegten, oder ähnlicher, Form noch bei keiner anderen Institution eingereicht. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen und Dritte haben keine geldwerten Leistungen für Arbeiten zu dieser Dissertation erhalten. Ich habe bisher keine erfolgreichen oder erfolglosen Promotionsversuche unternommen, um den akademischen Grad doctor rerum naturalium zu erlangen. Keine Teile dieser Dissertation wurden an der Friedrich Schiller Universität Jena, oder einer anderen Hochschule, zum Zweck der Graduierung oder der Erlangung von Prüfungsleistungen eingereicht.

Declaration of Independent Work

Herewith I, Christian Schurig, born on the 29.02.1984 in Dresden, confirm that I am familiar with the promotion regulations of the faculty of Biology and Pharmacy of the Friedrich-Schiller-University Jena. I further approve, that this present doctoral thesis represents my own work in accordance with the university regulations and no other support than listed were used in this work. The help of a promotion consultant was also not demanded and I did not generate any monetary perquisites which are in conjunction with the content of my thesis. This doctoral thesis was only submitted at the faculty board of the faculty of Biology and Pharmacy of the Friedrich-Schiller-University Jena and nowhere else for scientific evaluation or dissertation. This work is neither identical nor partly identical with work, which was already presented at the Friedrich-Schiller-University Jena or at any other university for obtaining an academic degree or academic credits.

Leipzig, 24. Juni 2013

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08/2007 04/2009	-	**University of Helsinki**, Finland Master of Science in Meteorology, within framework of international study program 'Atmosphere-Biosphere-Studies'. Thesis supervised by Prof. Timo Vesala and adj. Prof. Janne Rinne: Methane dynamics at a boreal fen measured by eddy covariance technique. Attended biogeochemical cycles line of study, studies mainly in Finnish Centre of Excellence for Physics, Chemistry, Biology and Meteorology of Atmospheric Composition and Climate Change.
04-06/2008		**University of Lund**, Sweden Study exchange period for course in ecosystem hydrology organised by Prof. Torben Christensen and Dr. Harry Lankreijer.
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10/2003 02/2007	-	**Dresden University of Technology**, Germany Bachelor of Science in Forestry. Thesis supervised by Prof. E. Gert Dudel : Uranfestlegung an *Cladophora* spec. unterschiedlicher Vitalität unter besonderer Berücksichtigung von Sulfat. (Incooperation of Uranium at *Cladophora* spec. of different vitality with special emphasis on sulfate.)

Work experience

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04/2009 09/2012	-	**Helmholtz Centre for Environmental Research - UFZ**, Germany Research assistant in the field of environmental biotechnology.
10/2007 02/2009	-	**University of Helsinki**, Finland Worked as research assistant at Department of Physics, Division of Atmospheric Sciences and Geophysics, in the fields of aerosol research and micrometeorology.
09-10/2006		**WWF Caucasus**, Tbilisi, Georgia Obtained knowledge about the structure and the tasks of an international nature conservation organisation.
08-09/2006		**Georgian State Agricultural University**, Tbilisi, Georgia Worked in a research project of Dresden University of Technology, collected data about Georgian forest enterprises and the general situation of forestry in Georgia.
08-09/2005		**Staatliche Umweltbetriebsgesellschaft**, Radebeul, Germany Sampled on former uranium mines and tailings in Saxony, obtained knowledge on the monitoring of radionuclides in the environment.
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Publications

Articles

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Utility patent

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Oral presentations

Dienemann, C., Dienemann, H., Schurig, C., Dudel, E.G. (2007). Uranium fixation with Cladophora spec. 2nd Annual Meeting of the Society of Wetland Scientists Europe.

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Appendix

The following section provides the reader of the thesis with the factsheets from the ModelPROBE book (chapter 2.1) and the supporting information for the rest of the manuscripts.

Contents

Factsheets (chapter 2.1)

2.15

BACTRAPS

(IN SITU MICROCOSMS)

MAIN OBJECTIVES

- Identification of contaminants degradation or retention
- Assessment of microbial activity
- Quantification of microbial activity

BRIEF DESCRIPTION

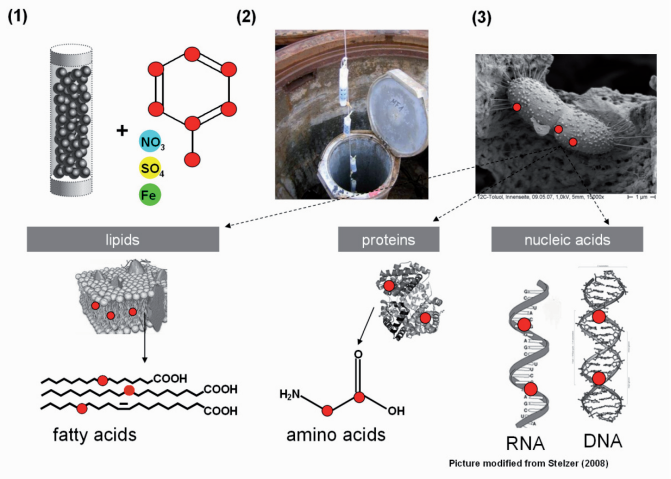
Main principle

Assessing the potential for microbial contaminant degradation at contaminated field sites is crucial for evaluating remediation options. For this purpose, an in situ microcosm system (BACTRAP®) has been developed. These in situ microcosms consist of a perforated Teflon tube filled with an activated carbon matrix and closed by glass wool stoppers. ^{13}C-labeled contaminants are loaded on the activated carbon and the microcosms are then exposed in groundwater wells. After the (usually 6 week long) exposure, the microcosms are extracted and the composition of the bacteria settled on the activated carbon matrix is analyzed by means of phospho-lipid fatty acid (PLFA) analysis or DNA/RNA analyis. For the commonly used ^{13}C-PLFA analysis, the incorporation of the ^{13}C label into the microbial biomass is evaluated by means of GC-C-IRMS or GC-MS techniques.

Main results

Microbial contaminant degradation activity can be measured by the method. Furthermore, by supplying BACTRAPs with terminal electron acceptors (Fe^{3+}, NO^{3-}, SO^{2-}), enhanced degradation potentials can be evaluated. The microcosms can also be used for repeated monitoring of microbial activity during a monitored or enhanced natural attenuation site remediation approach.

Sketch of measuring principle / concept



1. BACTRAPs are loaded with ^{13}C enriched substrate (and terminal electron acceptors)
2. Exposure takes place inside the contaminated aquifer
3. ^{13}C label is integrated into degrading microbes and can be identified by means of ^{13}C-PLFA, ^{13}C-amino acids or DNA/RNA analysis. PLFA analysis is of major interest for field application

C. Schurig & M. Kästner

REQUIREMENTS AND APPLICATION RANGE

Site requirements

- **Operation media**: Groundwater
- **Infrastructure requirements**: Groundwater monitoring wells in which the BACTRAPs will fit

Sample requirements

- None (samples are the microcosms themselves)

Data/information necessary for reliable interpretation of results

- **Geology**: None
- **Groundwater**: Level and flowing direction
- **Contamination**: Main contaminants which are likely, plume extension
- **Basic biogeochemical data**: Data on electron acceptors

APPLICATION RANGE ("OPERATING WINDOWS")

(Ideal) operative range for applicability

- Applicable in all aquifers accessible by groundwater monitoring wells
- Applicable for BTEX, MTBE, MCB and all compounds available with stable isotope labels (^{13}C, ^{15}N), and that are used as carbon or nitrogen source by bacteria

STANDING RELATED TO OTHER METHODS

Substitution and complementation of conventional methods

- Geochemical approaches
- Tracer tests
- Metabolite analysis
- Laboratory-scale microcosms

Advantages

- Less bias compared to off-site laboratory microcosms
- No bias introduced from taking soil or water samples
- No need to take soil samples for laboratory microcosms

Limitations compared to conventional methods

- Need for access to already installed wells
- Long exposure times needed
- Isotope ratio MS needed (specialized and expensive equipment)
- Expensive stable-isotope-labeled compounds needed
- Small release of 13C contaminants in the environment, implying that the use of $\Delta^{13}C$-fractionation to demonstrate degradation is not possible for a certain time period

STATE OF DEVELOPMENT

Field applicability

- Fully applicable and accepted by German and international authorities

Provider (selected)

- Department Environmental Biotechnology, UFZ

Isodetect GmbH
Ingolstädter Landstr. 1
D-85764 Neuherberg
Tel. +49 (0)89 3187-3086
Fax +49 (0)89 3187-3590
E-Mail: eisenmann@isodetect.de

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Geyer R, Peacock AD, Miltner A, Richnow HH, White DC, Sublette KL, Kästner M (2005) *Environ. Sci. and Technol*. 39:4983-4989.
Kästner M, Fischer A, Nijenhuis I, Geyer R, Stelzer N, Bombach P, Tebbe CC, Richnow HH (2006) *Eng. Life Sci.* 6:234–251.
Nijenhuis I, Stelzer N, Kästner M, Richnow H-H (2007) *Environ. Sci. Technol*. 41:3836–3842.

Expenditures

- 480-580€ per sample

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C. SCHURIG & M. KÄSTNER

2.16

DIRECT-PUSH BACTRAPS (IN SITU MICROCOSMS)

MAIN OBJECTIVES

- Identification of contaminants degradation or retention
- Assessment of microbial activity
- Quantification of microbial activity

BRIEF DESCRIPTION

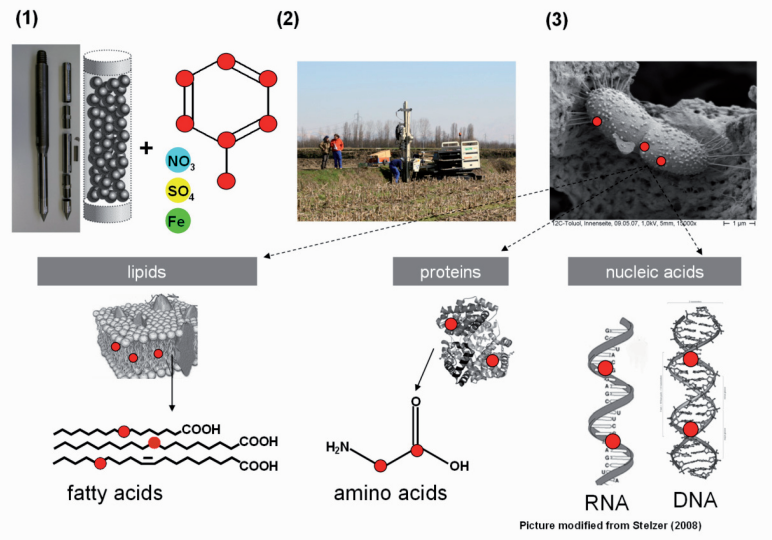
Main principle

Assessing the potential for microbial contaminant degradation at contaminated field sites is crucial for the evaluation of remediation options. Therefore, the in situ microcosm system (BACTRAP®) has been developed. In order to overcome some limitations of the "classical" BACTRAP approach (need for established wells, no access to the vadose zone) (see Fact Sheet 15), the system has been adapted to the Direct-Push technology (for details about Direct-Push see Fact Sheet 8). This allows for applicability at field sites without monitoring wells and also inside the vadose zone.

Main results

Evidence for microbial contaminant degradation activity inside aquifers as well as in the vadose zone can be provided by the method. Furthermore, by supplying DP-BACTRAPs with terminal electron acceptors (Fe^{3+}, NO_3^-, SO_4^{2-}), degradation potentials by various redox processes can be evaluated. Also, the microcosms can be used for repeated monitoring of microbial activity during a monitored or enhanced natural attenuation site remediation approach.

Sketch of measuring principle / concept



1. Direct-Push BACTRAPs prototypes are loaded with ^{13}C enriched substrate (and terminal electron acceptors)
2. exposure inside the contaminated aquifer/soil by means of Direct-Push rod probe (prototype)
3. During microbial degradation the ^{13}C label is integrated into degrading microbes, which can be identified by phospholipid fatty acid (PLFA), amino acids or DNA/RNA analysis for specific groups of microorganisms. Usually, ^{13}C-PLFA analysis is used for field applications.

REQUIREMENTS

Site requirements

- **Operation media**:Unconsolidated material, aquifer and vadose zone
- **Infrastructure requirements**: Energy supply (electricity or fuel), Direct-Push machine and tubing

Sample requirements

- None (samples are the microcosms themselves)

Data/information necessary for reliable interpretation of results

- **Geology**: None
- **Groundwater**: Level and flowing direction
- **Contamination**: Main contaminants which are likely, plume extension
- **Basic biogeochemical data**: Data on electron acceptors (inside aquifer)

APPLICATION RANGE ("OPERATING WINDOWS")

(Ideal) operative range for applicability

- Applicable on all sites in all depths accessible by a Direct-Push machine
- Applicable for BTEX, MTBE, MCB and all compounds available with stable isotope labels (^{13}C, ^{15}N), and that are used as carbon or nitrogen source by bacteria
- See also "classical" BACTRAP approach (Fact Sheet 15)

STANDING RELATED TO OTHER METHODS

Substitution and complementation of conventional methods

- Geochemical approaches
- Tracer tests
- Metabolite analysis
- Laboratory-scale microcosms
- Classical BACTRAPs

Advantages

- Less bias compared to off-site lab microcosms, avoiding bias resulting from obtaining soil or water samples
- No need for obtaining soil samples by drilling and consequently no need for lab microcosms anymore
- No wells or infrastructure necessary at the site
- As many BACTRAPs as desired are easily installed
- Installation of BACTRAPs can be done simultaneously with water level determination

Limitations

- Direct push machine is needed
- Pushing rod has to stay at the site for the exposure time
- Long exposure times needed
- Isotope ratio MS needed
- Expensive stable isotope labeled compounds needed
- Minor release of ^{13}C contaminants into the environment, implying that the use of $\delta^{13}C$-fractionation to demonstrate degradation is not possible for a certain time period

C. Schurig & M. Kästner

STATE OF DEVELOPMENT

Field applicability

- Prototype ready for field application, currently tested

Provider

- Department Environmental Biotechnology, UFZ

References (from classical BACTRAPs)

Geyer R., Peacock A.D., Miltner A., Richnow H.H., White D.C., Sublette K.L. & Kästner M. (2005) - Enviro Sci.Technol., **39**: 4983-4989.
Kästner M., Fischer A., Nijenhuis I., Geyer R., Stelzer N., Bombach P., Tebbe C.C. & Richnow H.H. (2006) - Eng Life Sci., **6**: 234–251.
Kästner M. & Richnow H.H. (2010) - In: Timmis K.N., McGenity T., van den Meer J.R., de Lorenzo V. (Hrsg.) - *Handbook of hydrocarbon and lipid microbiology*, Vol. *5 Experimental proto cols and appendices*, Part 1 Study Systems, Springer,
Berlin, S. 3504-3511.

Expenditures

- To be determined

CONTACT PERSON

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- Christian Schurig (christian.schurig@ufz.de)
- Department Environmental Biotechnology, UFZ

REMARKS

Also see Fact Sheet 15 for classical BACTRAPs

Supporting information (chapter 2.2)

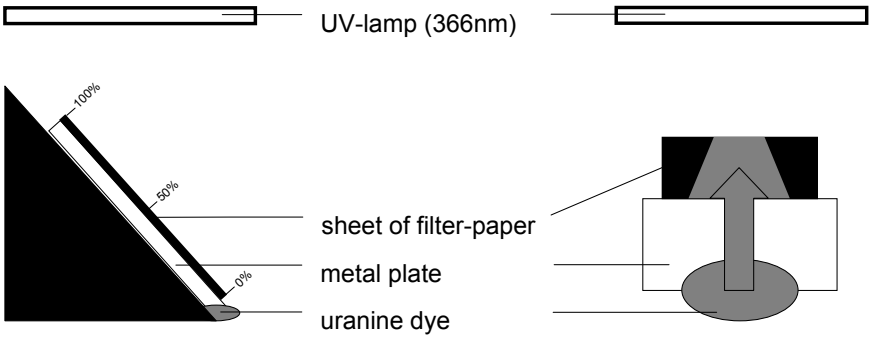


Figure S 1: Sketch of the measurement principle for wettability measurements

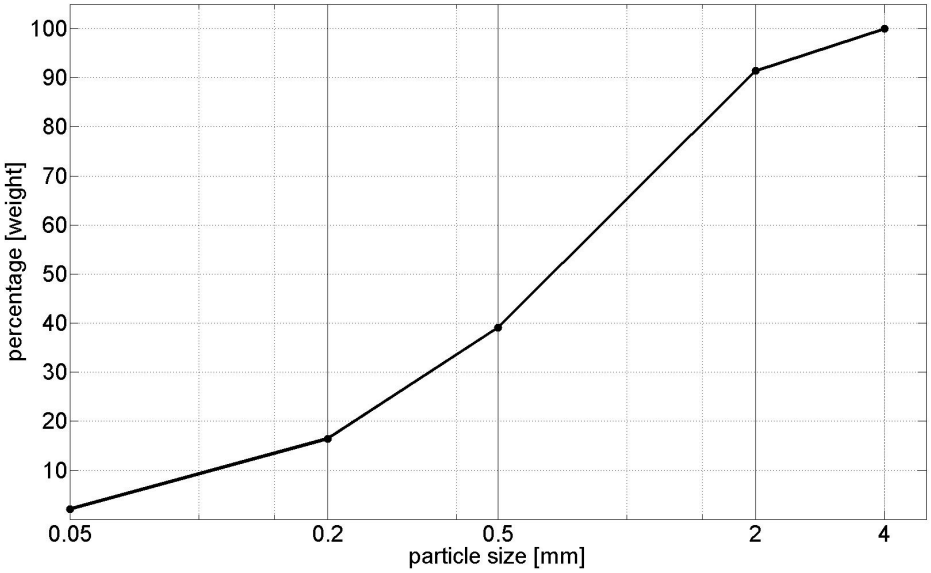


Figure S 2: Particle size distribution of the soil used for the laboratory experiments

Supporting information (chapter 2.3)

Table S1: Concentration of individual PLFA upon incubation with unlabelled or labelled hexadecane within traditional microcosms and BACTRAPs after 61 days of incubation. Blank represents incubation for 61 days without hexadecane addition.

	^{12}C		^{13}C		Blank		^{12}C		^{13}C		Blank		^{12}C		^{13}C		Blank	
PLFA	**vadose**	**STD**	**vadose**	**STD**	**vadose**	**STD**	**aquifer**	**STD**	**aquifer**	**STD**	**aquifer**	**STD**	**BACTRAP**	**STD**	**BACTRAP**	**STD**	**BACTRAP**	**STD**
										[µmol g_{soil}^{-1}]								
12:0	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.03	0.01	0.02	0.01	0.06	0.02
9:0 Diacid	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.03	0.01	n.d.		n.d.	
13:0	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.02	0.00	n.d.		n.d.	
i14:0	n.d.		n.d.		n.d.		0.07	0.01	0.12	0.02	n.d.		n.d.		n.d.		n.d.	
14:0	0.15	0.04	1.13	0.21	0.06	0.01	0.16	0.04	0.74	0.04	0.07	0.03	0.15	0.05	0.24	0.10	0.33	0.13
i15:0	0.01		0.18	0.02	0.01	0.01	0.12	0.02	0.16	0.02	0.05	0.01	0.03	0.02	n.d.		n.d.	
a15:0	0.02		n.d.		0.01	0.01	0.16	0.02	0.32	0.07	0.06	0.01	n.d.		n.d.		n.d.	
15:0	0.04	0.01	n.d.		0.01	0.01	0.09	0.02	0.30	0.07	0.07	0.01	n.d.		0.04	0.01	0.04	0.00
i16:0	n.d.		n.d.		n.d.		0.35	0.05	0.15	0.05	n.d.		n.d.		n.d.		n.d.	
16:1w 7	0.05	0.01	n.d.		0.03	0.02	0.32	0.12	n.d.		n.d.		n.d.		n.d.		n.d.	
16:1w 5	n.d.		0.15	0.04	0.02	0.00	n.d.		0.46	0.12	0.16	0.03	n.d.		n.d.		n.d.	
16:0	0.69	0.04	5.01	0.16	0.49	0.02	1.62	0.12	5.24	0.20	0.98	0.03	0.93	0.04	0.84	0.11	1.40	0.16
10Me16:0	0.04	0.01	n.d.		0.01	0.02	0.11	0.02	0.26	0.06	0.07	0.03	n.d.		n.d.		n.d.	
i17:0	0.04	0.02	0.14	0.02	0.02	0.00	0.09	0.02	0.19	0.04	0.06	0.02	n.d.		0.03	0.01	n.d.	
a17:0	0.07	0.02	0.39	0.05	0.03	0.01	0.32	0.09	0.30	0.10	0.03	0.01	n.d.		n.d.		n.d.	
cy17:0	0.04	0.01	n.d.		0.01	0.02	0.36	0.07	0.60	0.13	0.17	0.06	n.d.		n.d.		n.d.	
17:0	0.08	0.01	0.41	0.07	0.03	0.00	0.14	0.02	0.28	0.06	n.d.		0.03	0.01	0.05	0.02	0.05	0.00
18:2w 6,9	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		0.04	0.01	n.d.		n.d.	
18:1w 12	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
18:1w 9	0.48	0.07	2.58	0.09	0.27	0.05	0.42	0.12	2.44	0.04	0.26	0.09	0.18	0.10	0.25	0.15	0.76	0.31
18:1w 7	0.38	0.06	2.32	0.26	0.24	0.03	0.97	0.06	2.43	0.10	0.50	0.15	0.18	0.08	0.13	0.09	0.51	0.20
18:0	0.81	0.18	7.32	0.28	0.62	0.15	0.81	0.10	5.38	0.81	0.60	0.15	1.17	0.21	0.96	0.26	1.76	0.82
cy19:0	n.d.		n.d.		0.01	0.01	0.72	0.10	0.74	0.18	0.33	0.10	n.d.				n.d.	

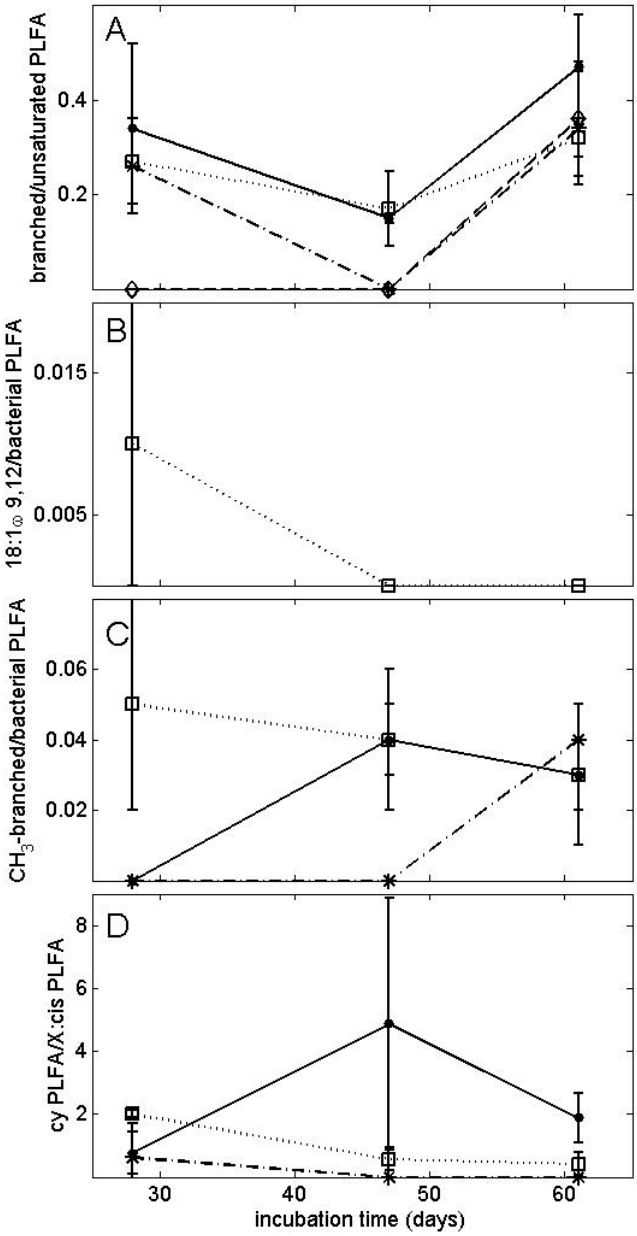


Fig. S1: *Changes of the microbial community composition with respect to time since incubation, as indicated by PLFA analysis: vadose zone (^{13}C: •; blank: □) and the aquifer zone (^{13}C: ⋆; blank: ▽): A) ratio of markers for Gram-positive bacteria to markers for Gram-negative bacteria; B) ratio of fungal to bacterial markers; C) ratio of markers for actinomycetes to total bacterial markers; D) starvation markers (for more details see: Schurig et al. 2013b). Error-bars represent the standard deviations for triplicate experiments.*

Supporting information (chapter 2.4)

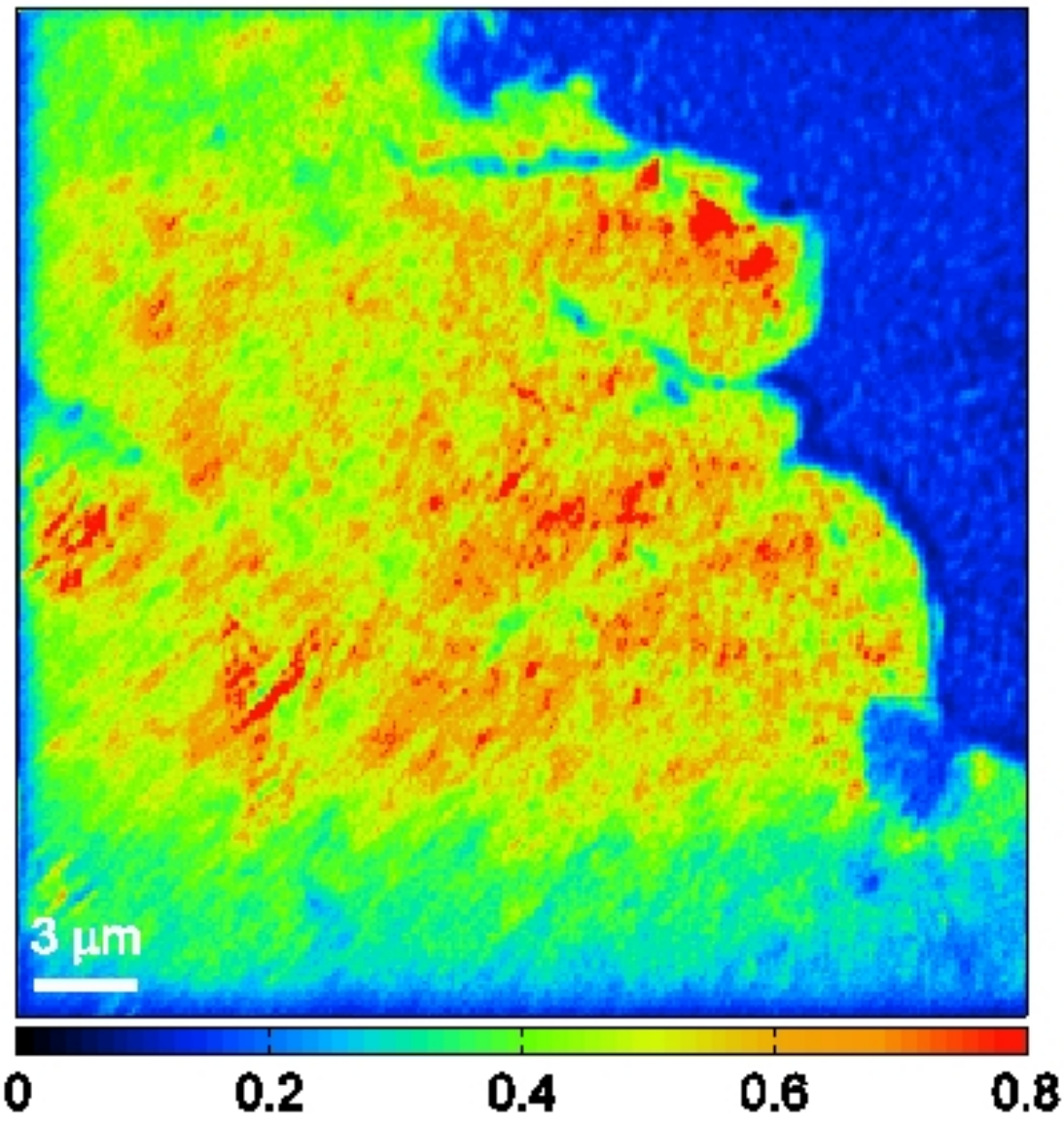


Figure S 1: $^{12}C/^{14}N$ ratio of the analysed area in order to check discrimination between embedding resin and AC.

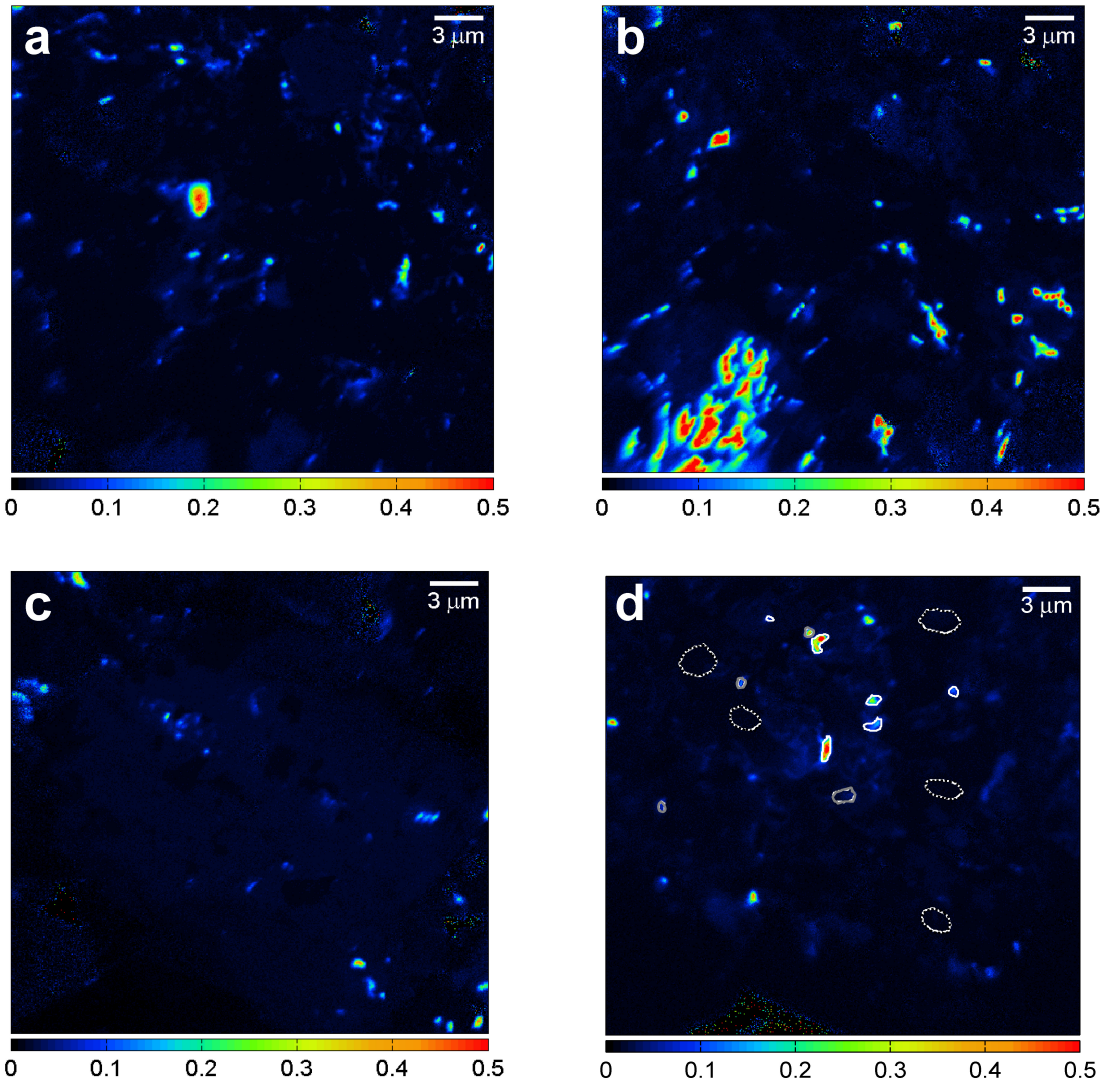


Figure S 2: panel a&c: $^{13}C/^{12}C$ ratio of mantle area indicate only few highly ^{13}C- labelled spots of microbial phenotype; b&d: $^{13}C/^{12}C$ ratio images of inner surfaces of the Biosep-beads reveal similar quantity of highly enriched spots of microbial phenotype compared to mantle areas (ROIs: class a: dotted line, class b: solid white line, class c: solid gray line)

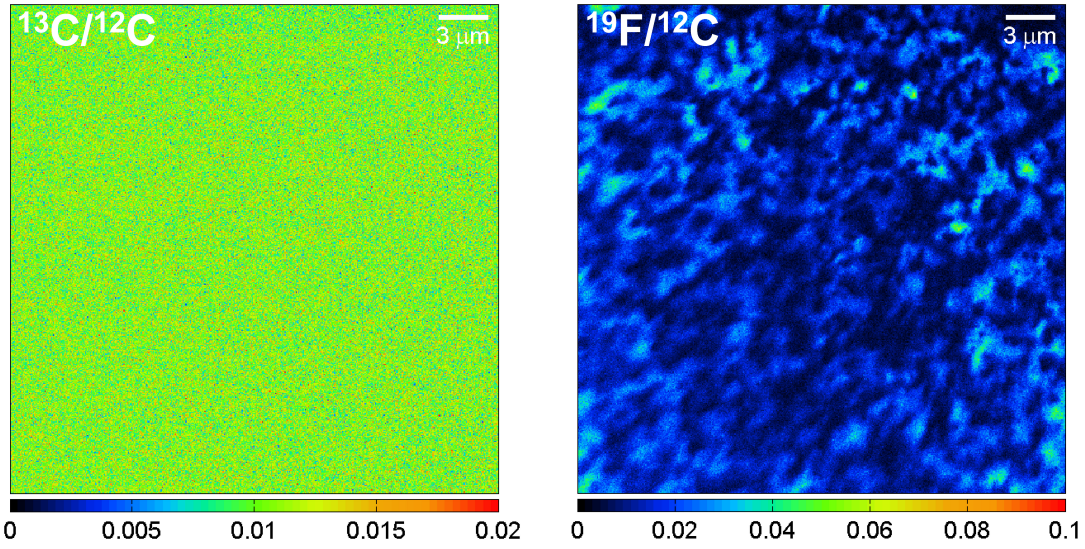


Figure S 3: Background check of the adhesive carbon foil used to mount the sample to the sample holder: panel a: $^{13}C/^{12}C$ ratio reveals equal distribution of carbon of natural isotopic abundance; panel b: $^{19}F/^{12}C$ ratio reveals a patchy but thorough ^{19}F background, which adversely affects the HISH-SIMS method.

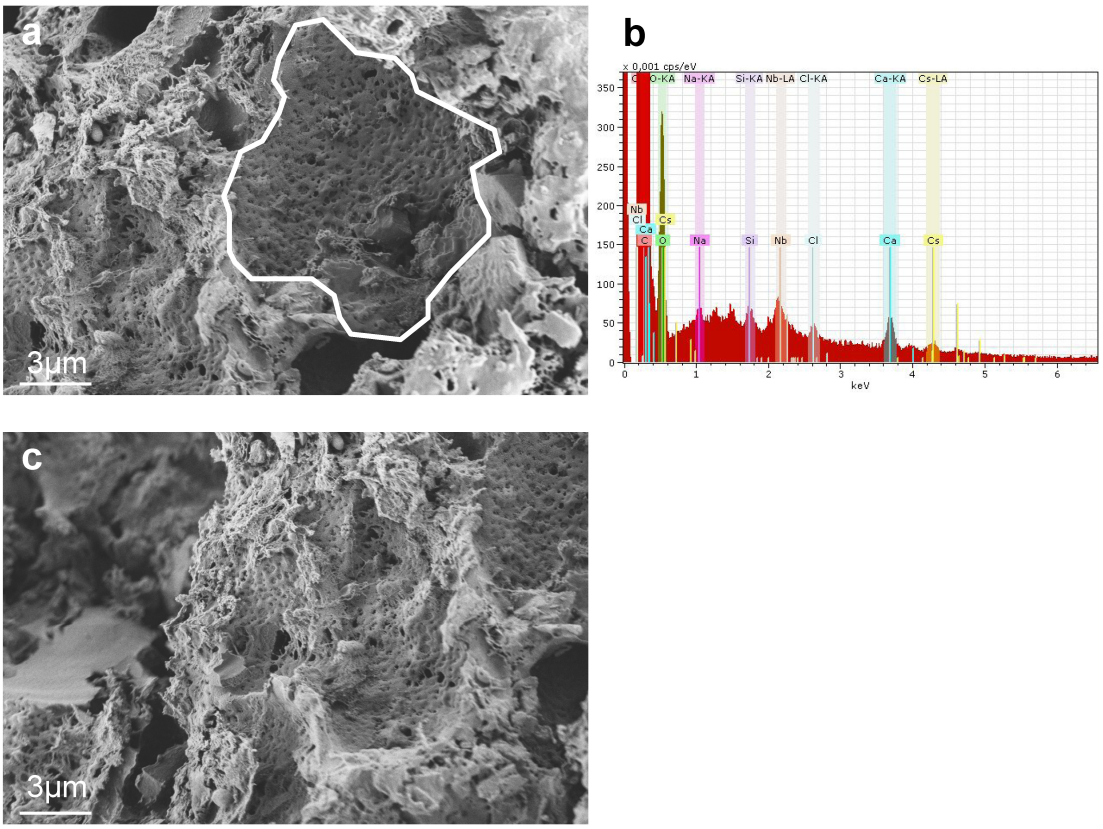


Figure S 4: SEM images from spots analysed by means of NanoSIMS after the measurements: panel a: no phenological identification of microbes is possible after the NanoSIMS measurements; panel b: EDX data indicates residual caesium, and thus shows that the spot was altered by the NanoSIMS; panel c: represents another analysed spot, thus indicating reproducible patterns of beam damage.

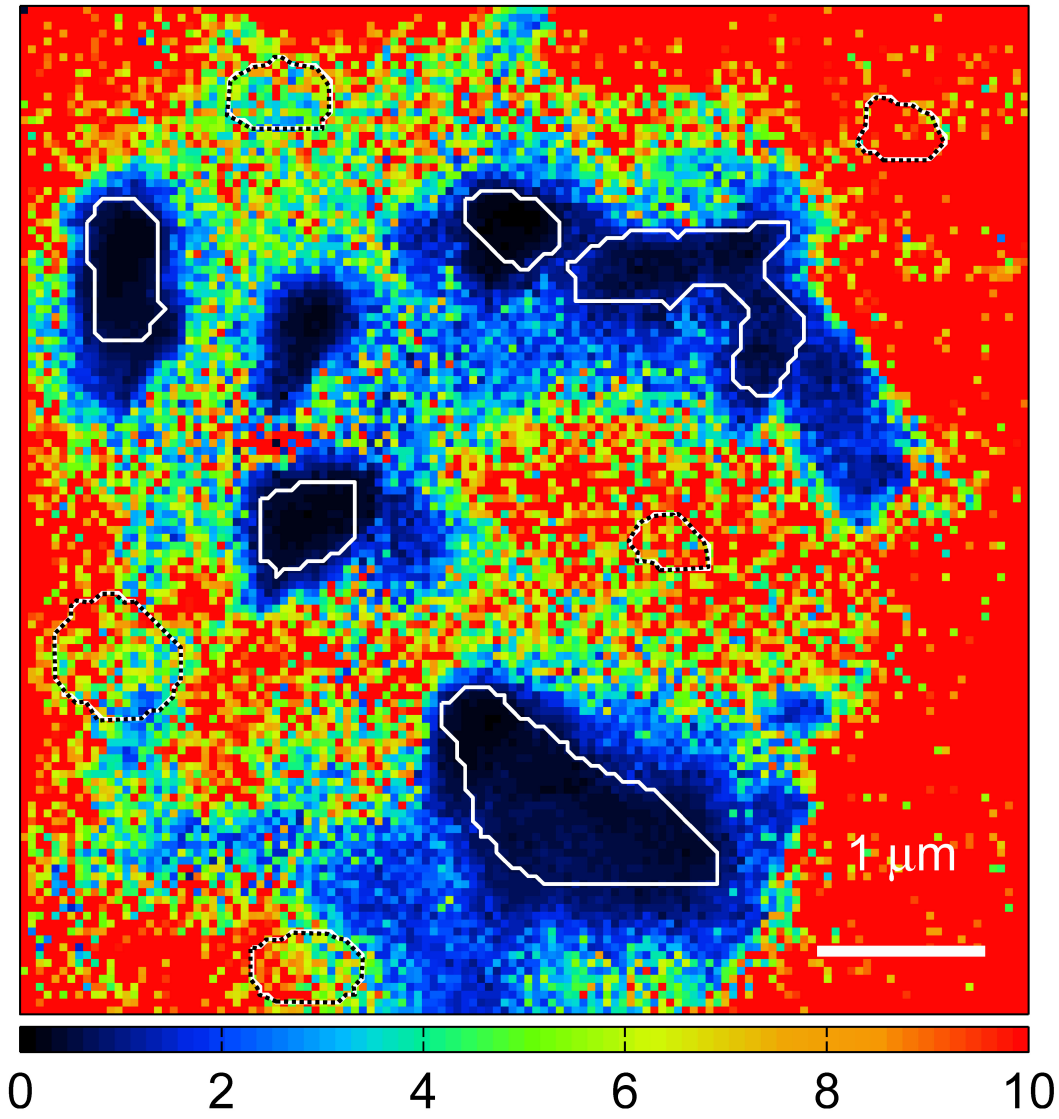
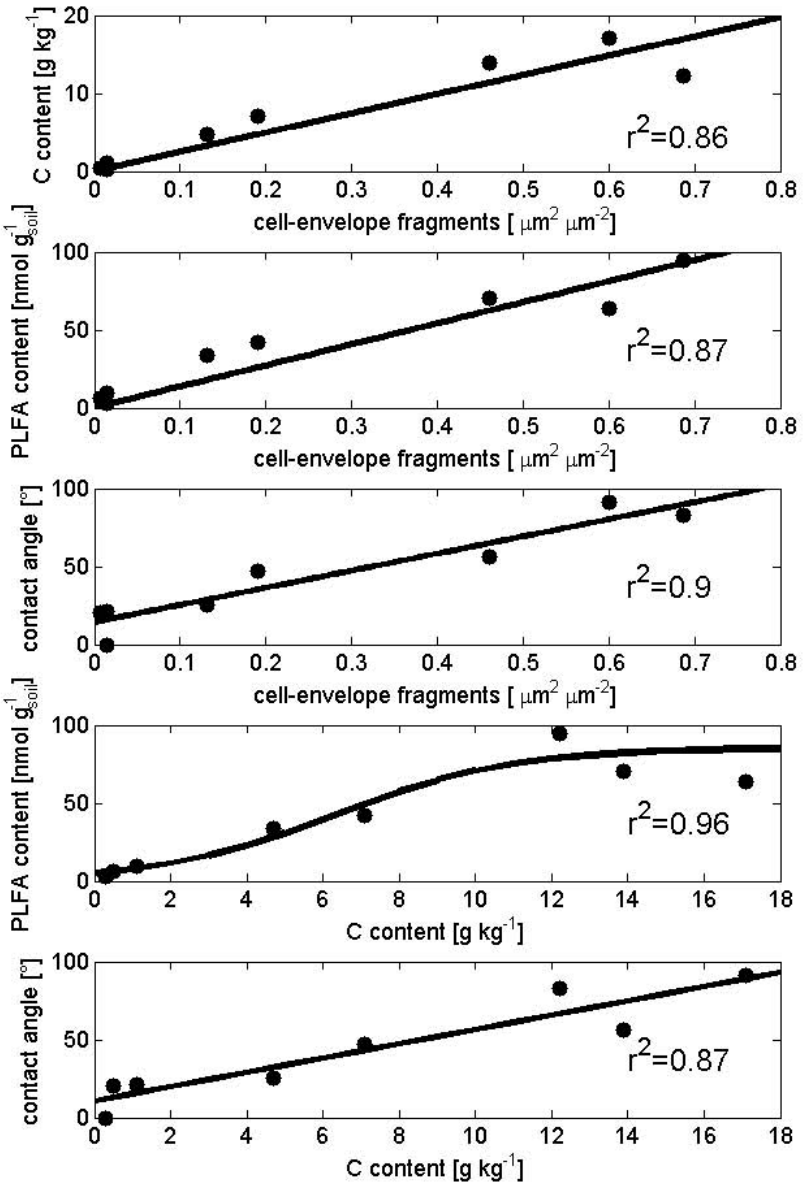
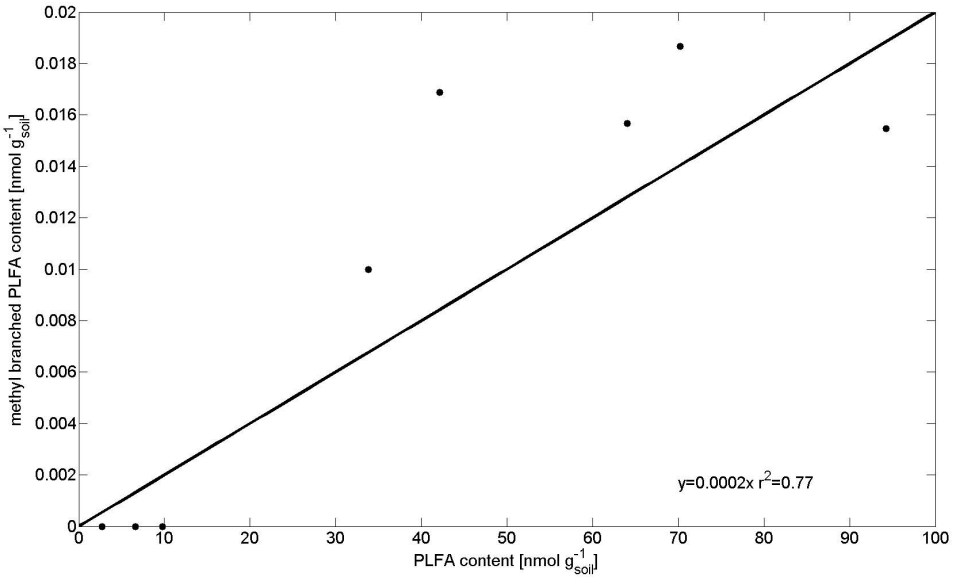


Figure S 5: Detailed $^{19}F/^{31}P$ ratio NanoSIMS image (6*6μm) of highly ^{13}C enriched microbial cells, ROIs: white line labelled biomass, dotted line background.

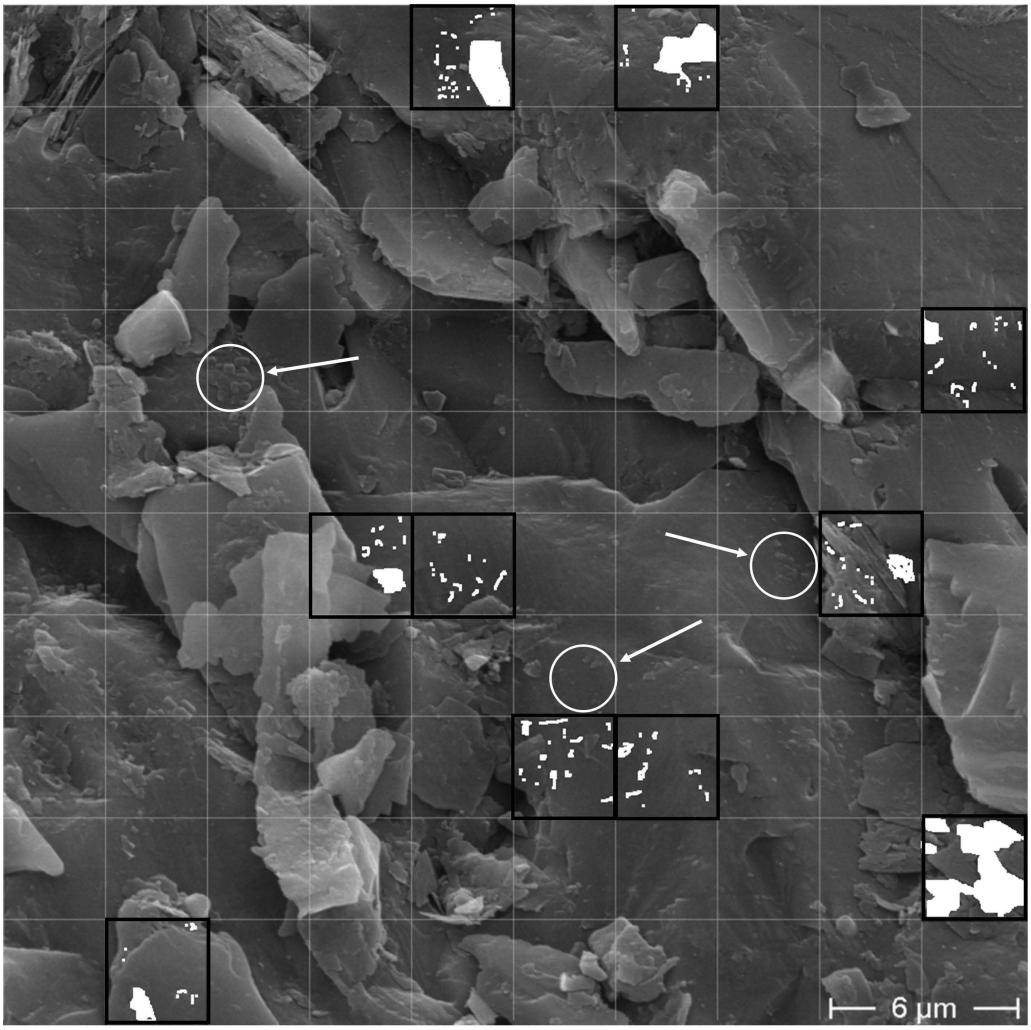
Supporting information (chapter 2.5)



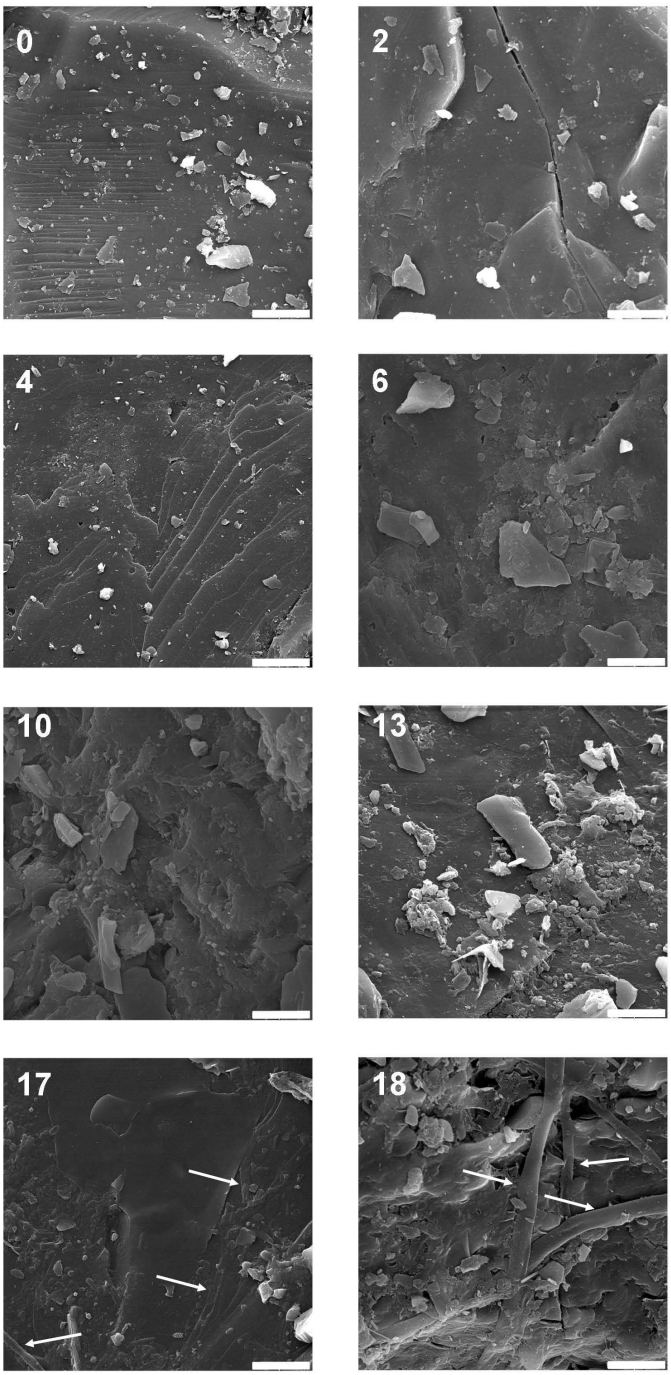
Supplement A 1: Correlation analysis along with regressions of important parameters from the Damma glacier forefield.



Supplement A 2: Methyl branched PLFA content as a function of total PLFA content.



Supplement A 3: Higher resolution version of Fig. 2 (upon reviewers request): Example of scanning electron micrograph (SEM) analysis of the soil samples from the Damma glacier forefield (age: 64 years; site 6). The encircled areas indicated by the arrows contain target fragments. The surface coverage of soil particles by fragments of bacterial cellenvelopes is estimated by laying a 10 x 10 grid over equally scaled SEM. Out of this grid 10 fields are selected randomly for analysis (for details see text); these fields are analyzed for bacterial cellenvelope fragments (marked in white).



Supplement A 4: Higher resolution version of Fig. 3 (upon reviewers request): SEM of soil samples from various sampling sites at the Damma glacier forefield (identical scale, bar = 6 μm). Samples from sites of increasing SOM age showed increasing coverage by cell-envelope fragments. Site numbers are shown in the upper left corners of the micrographs. Hypha are highlighted by arrows (for details, see methods and Table 1).