Monitoring Tools for Microbial *in situ* Activity in Natural and Engineered Environmental Systems

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Summary

Extensive industrial and waste disposal activities since the last century have led to ubiquitous contamination of the subsurface with hazardous substances. Bioremediation is at the core of a suite of cost-effective strategies for the reduction of the contamination. Sound site-specific *in situ* assessment will promote those strategies and is even a prerequisite for approval of bioremediation by regulatory agencies. The main objective of this work was to specify microbial *in situ* activity in engineered bioremediation systems via improved labelling strategies for *in situ* identification of active degraders.

The first study aimed at characterisation of the benzene-degrading microbial community in the rhizosphere of a pilot-scale constructed wetland (CW), fed with benzene-contaminated groundwater. A combination of *in situ* microcosms (BACTRAPs) and RNA-SIP with in-depth phylogenetic analysis of 16S rRNA profiles revealed that members of the two genera *Dechloromonas* and *Zoogloea* played a key role in benzene metabolism on the BACTRAPs. However, limiting aspects became apparent concerning the approach: substantial amounts of unlabelled benzene and other organic compounds at the site restrained labelling and the identification of benzene degraders. Furthermore, BACTRAPs seemed to serve as *in situ* enrichment cultivation units with different properties than the surrounding environment so that it remained uncertain whether the detected benzene degraders are indeed of high relevance in the habitat or whether those are the best suited ones among all to colonize the BACTRAPs.

In the second study the SIP/BACTRAP approach was evaluated for its applicability for on-site characterisation of microbial bisphenol A (BPA) transformation. Although laboratory tests on useable concentrations, bioavailability and incorporation of BPA were promising, results of a test in a rhizodegradation pilot plant did not favour a recommendation for tracking BPA transformation via BACTRAPs at field sites.

In the third study a new approach for sensitive *in situ* identification of relevant and active key players at contaminated systems without prior phylogenetic information was developed and evaluated. 'MicroAutoRadiography of Encapsulated Cells (MAR-EC)' encompasses an incubation of a microbial sample with a ^{14}C-labelled substrate followed by encapsulation of cells together within an autoradiographic emulsion in microbeads with subsequent microautoradiography (MAR). Thus, compound-transforming microbes are spatially isolated and may be separated based on optical differences caused by MAR. To achieve their identification powerful multiple displacement amplification for single cells provides sufficient DNA. A proof-of-principle experiment demonstrated the potential of the approach. MAR-EC was realized with bacterial numbers at a level of a few cells.

Zusammenfassung

Zahlreiche industrielle Aktivitäten und aus heutiger Sicht fragwürdige Strategien der Abfallentsorgung des letzten Jahrhunderts haben zu omnipräsenten Kontaminationen des Untergrundes geführt, die teilweise einen geringen bis unsanierten Zustand aufweisen. Die biologische Sanierung einschließlich mikrobieller Abbauprozesse stellt eine kostengünstige, jedoch öffentlich meist nur unzureichend anerkannte Alternative zur Verringerung der Kontaminationen dar. Eine schlüssige *in situ* Bewertung der (mikro)biologischen Abbauleistung unterstützt die Anwendung dieser Strategien und ist Voraussetzung für eine behördliche Genehmigung. Das Hauptziel dieser Arbeit war, die *in situ*-Aktivität von schadstoffabbauenden Mikroorganismen in natur-nahe konstruierten Umweltsystemen zu bestimmen, hier mittels verbesserter Methoden zur *in situ*-Identifizierung von aktiven Schlüsselorganismen, die allesamt auf Isotopen-Markierungen beruhen.

Das Anliegen der ersten Studie dieser Arbeit war die Charakterisierung der benzolabbauenden mikrobiellen Gemeinschaft in der Rhizosphäre einer Pflanzenkläranlage. Die Pilotanlage wurde mit benzolkontaminiertem Grundwasser gespeist. Die Kombination von *in situ*-Mikrokosmen (BACTRAP-Technologie) und RNA-SIP mit tiefgehender phylogenetischer Analyse der 16S rRNA Profile ergab, dass die beiden besonders zwei Gattungen *Dechloromonas* und *Zoogloea* eine Schlüsselrolle im Benzolstoffwechsel auf den BACTRAPs einnahmen. Allerdings traten einige limitierende Faktoren des BACTRAP-Ansatzes auf: erhebliche Mengen an nicht-markiertem Benzol und anderer organische Verbindungen am Standort beschränkten die Markierung und damit die Identifizierung der Benzolabbauer. Darüber hinaus haben BACTRAPs andere spezifische Eigenschaften als die Umgebung und scheinen eher als *in situ*-Anreicherungskulturen zu dienen, bei denen unklar bleibt, ob die identifizierten Benzolabbauer tatsächlich von großer Bedeutung im Habitat waren oder ob diese unter allen *in situ*-Abbauern die für die BACTRAP-Kolonisation geeignetsten Mikroorganismen waren.

In der zweiten Studie wurde der SIP/BACTRAP-Ansatz hinsichtlich seiner Anwendbarkeit bei der Vor-Ort-Charakterisierung der mikrobiellen Bisphenol A (BPA) Transformation bewertet. Versuche zur Bestimmung nutzbarer Konzentrationen, der Bioverfügbarkeit und der Einbaurate des Substrates waren vielversprechend. Die Ergebnisse des Feldversuchs deuteten aber an, dass BACTRAP-Technologie zur Verfolgung der *in situ*-BPA-Transformation nicht geeignet ist.

Im Rahmen der dritten Studie wurde ein neuer Ansatz zur sensitiven *in situ*-Identifizierung relevanter und aktiver Schadstoffabbauer in kontaminierten Systemen ohne vorherige phylogenetische Information schrittweise entwickelt und beurteilt. ‘MicroAutoRadiography of Encapsulated Cells (MAR-EC)’ umfasst eine kurze Inkubation einer mikrobiellen Probe mit einem ^{14}C-markierten Substrat, gefolgt von einer Einkapselung von Zellen zusammen mit einer autoradiographischen Emulsion in Mikrokapseln mit anschließender Mikroautoradiographie (MAR). Damit sind die schadstoffumsetzenden Mikroben räumlich isoliert und können auf der Basis von optischen Unterschieden, die durch MAR verursacht werden, abgetrennt werden. Um eine gezielte Identifizierung zu ermöglichen, erlangt man durch die leistungsstarke ‚multiple-displacement amplification‘ an Einzelzellen ausreichend DNA. Experimente zur prinzipiellen Machbarkeit zeigten das Potential des Ansatzes. MAR-EC wurde mit geringen Zellzahlen bis auf wenige Zellen pro Mikrokompartiment gezeigt. Die enthaltene DNA blieb während des ganzen Prozesses stabil und konnte erfolgreich amplifiziert werden.

List of Abbreviations

ACPM:	alginate-cell-photoemulsion-mixture
BPA:	bisphenol A
cDNA:	complementary deoxyribonucleic acid
CSIA:	compound-specific isotope analysis
CsTFA:	cesium trifluoroacetate
CW:	constructed wetland
DAPI:	4',6-diamidino-2-phenylindole
DGGE:	denaturing gradient gel electrophoresis
DNA:	deoxyribonucleic acid
DOC:	dissolved organic carbon
EDC:	endocrine disrupting chemicals
EDTA:	ethylenediaminetetraacetic acid
FAME:	fatty acid methyl ester
FISH:	fluorescence *in situ* hybridization
GC-MS:	gas chromatography - mass spectrometry
HMN:	heptamethylnonane
HPLC:	high-performance liquid chromatography
LCA:	lowest common ancestor
MAR:	microautoradiography
MAR-EC:	microautoradiography of encapsulated cells
MBR:	membrane bioreactor
MDA:	multiple displacement amplification
MS:	mass spectrometry
MWCO:	molecular weight cut-off
NA:	natural attenuation
NanoSIMS:	nano-scale secondary ion mass spectrometry

OTU:	operational taxonomic unit
PBS:	phosphate-buffered saline
PC:	polycarbonate
PCR:	polymerase chain reaction
PDMS:	polydimethylsiloxane
PEEK:	polyetheretherketone
PFA:	paraformaldehyde
PLFA:	phospholipid fatty acid
PMMA:	polymethylmethacrylate
PP:	polypropylene
$PP_{i:}$	pyrophosphate
PTFE:	polytetrafluoroethylene
qPCR:	real-time quantitative polymerase chain reaction
RIP:	radioisotope probing
RNA:	ribonucleic acid
rRNA:	ribosomal ribonucleic acid
RT-qPCR:	reverse-transcription real-time quantitative polymerase chain reaction
SIMS:	secondary ion mass spectrometry
SIP:	stable isotope probing
SSCP:	single strand conformation polymorphism
TOC:	total organic carbon
T-RFLP:	terminal restriction fragment length polymorphism
WashSol:	washing solution
WGA:	whole genome amplification

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1 General Introduction

Extensive industrial and waste disposal activities in the last century have led to ubiquitous contamination of the subsurface with man-made, hazardous substances. While nowadays in Europe the creation of newly contaminated sites is constrained by strict regulations, there are still numerous sites with historical contamination that present unacceptable risks. The main contaminants in European groundwater and soils are heavy metals and substances derived from petroleum and its processing (EEA, 2014). According to the latest report from the European Commission's Joint Research Centre (EEA, 2014), 'Progress in the management of contaminated sites in Europe', approximately 2.5 million potentially contaminated sites in 39 member states of the European Environment Agency need to be investigated in detail. An estimated 342 000 of these possibly contaminated sites may pose a serious risk to human health, to water and groundwater ecosystems or to other targets. To date, only one third of these sites have been identified and only 15 % of these remediated. The broad need for action is obvious.

Traditionally, most common remediation techniques are based on the excavation of contaminated soil and its disposal in landfills (‘dig and dump’). Contaminated aquifers are often similarly *ex situ* treated physically and chemically (‘pump and treat’), which is time-consuming and expensive, and frequently not effective (EEA, 2014). Much more cost-effective is the reduction of the contamination without direct human intervention. This so-called Natural Attenuation (NA) includes volatilization, dilution, sorption and microbial degradation (National Research Council, 2000; Bombach et al., 2010a). Of these, the latter is typically the most desirable process because it indeed eliminates the contaminant from the system. Despite the general positive track-record of microbial-based NA, there is a need to validate its success at any given contaminated site and estimate its magnitude in comparison to transfer of the contaminant to another system or phase (volatilization, dilution, sorption) (Bombach et al., 2010a). Consequently, the use of microbial Natural Attenuation (NA) relies on sound site-specific *in situ* assessment and monitoring of the biological degradation.

Qualitative *in situ* assessment of biodegradation can be achieved by several geochemical methods [comprehensive review by Bombach et al. (2010a)]: A simple indirect indication of microbial transformation or biodegradation capacity can be gained by monitoring of geochemical changes e.g. via concentration changes of the contaminant as well as oxygen and other electron acceptors (Chapelle, 2000; Christensen et al., 2001).
A more elaborate approach involves the use of tracers (Ptak et al., 2004), which may prove and quantify biodegradation but requires extensive spatial and temporal monitoring

of cost-intensive compounds. A typically more cost-effective method is compound-specific isotope analysis (CSIA). Here, the change in the ratio of heavy-to-light isotope is quantified for the compound of interest. In several biotransformation reactions, the chemical bonds containing the light isotopes are preferentially cleaved, thereby the isotope ratio is altered (Meckenstock et al., 2004). In laboratory studies, even degradation pathways could be identified for the turnover of some compound (Kuder et al., 2005; Fischer et al., 2008; Abe et al., 2009). Quantification of biotransformation via CSIA is in principle possible, but strongly limited to known and appropriate isotope enrichment factors.

In situ biodegradation can be also proven by the evidence of metabolites (Beller et al., 1995). The detection of microbial transformation of parent compounds into these intermediate compounds requires a comprehensive knowledge of the specific degradation pathway, which is not yet available for several common contaminants (Chakraborty and Coates, 2004). Furthermore, difficulties arise when the metabolite can be associated with the biodegradation of several compounds present at the site (Beller, 2000) or when they occur at concentrations below the detection limit (Griebler et al., 2004).

All of these listed methods are time- or cost- intensive, or require high technical effort or prior information on biodegradation, but typically provide only qualitative evidence of overall transformation. Furthermore, active transformation pathways are typically not identified.

A more comprehensive understanding and characterization of the degradation processes allow for a better assessment of the potential for microbial transformation and, ultimately, site-restoration. Such knowledge is required by regulatory agencies for the approval of NA as remediation approach for a particular site. Furthermore, it may support enhanced NA via insights on how the *in situ* processes may be stimulated and accelerated. In addition, it may foster the development of innovative, more effective bio-process technologies e.g. bioaugmentation, enzyme technology, phyto- and rhizoremediation, or bioelectrochemical remediation, which again have to be monitored and characterized. Therefore, a suitable and sufficient description of the system and the active bio-processes is initially required. In particular, there is a strong need for directly linking the identification of microbes with the elucidation of their metabolism of compounds in the environment. These descriptive tasks belong to the core of microbial ecology, i.e. the understanding of structure, function, and dynamics of environmental systems. Among the fundamental challenges in microbial ecology are to identify which microorganisms are responsible for certain processes in the environment, the quantification of their functional contribution and the assignment of specific functions to one of the many uncultured microorganisms thriving in such

communities (Wagner, 2011). With emphasis on bioremediation, this suite of challenges translates into the identification *in situ* of key microbial players in compound turnover.

There are a number of microbial and molecular methods that provide information about microbial community structures and their catabolic potential. For a long time only laboratory cultivation from environmental samples was available. By isolation in pure cultures or enrichment in defined co-cultures, single organisms or consortia were phylogenetically, physiologically and biochemically characterised (Amann et al., 1995). Since less than 1 % of the microorganisms are considered to be cultivable (Amann et al., 1995), cultivation-based methods have given only limited insight into diversity and function. Consequently, Brock (1987) and Amann et al. (1995) recommended the use of culture-independent methods for *in situ* assessment of microbial communities. Since then 16S/18S rRNA as a taxonomic marker (Woese and Fox, 1977) and PCR-based methods for detecting the encoding genes in environmental samples (Olsen et al., 1986) have revolutionized the description and structure of microbial communities.

However, the detection of phylogenetic markers, e.g. via 16S rRNA sequencing approaches or catabolic genes do not necessarily provide information about *in situ* functionality (Okabe et al., 2004). The recently developed DNA-sequence-based metagenomics is a powerful approach for extensive identification of microbes in complex environmental samples and even metabolic potentials, but again does not provide information on *in situ* activities (Pinnell et al., 2011). The application of mass spectrometry (MS)-based metaproteomic investigations may help to resolve the metabolic activities of the community by linking their genetic potential with the actual function (Hettich et al., 2012). It accomplishes the analysis of the proteomes of specific, and in a best-case scenario, all species within the consortium of an environmental sample, which gives insight into their phenotypes (Pinnell et al., 2011; Seifert et al., 2012; Seifert et al., 2013; von Bergen et al., 2013). Currently, this modern high-throughput sequencing of whole communities and protein identification based on MS approaches is time-consuming, can become extremely costly and is very complex in terms of the production of huge datasets, where crucial information can get lost or is not specific enough for comprehensive identification of possibly active degraders out of environmental samples. More than a mere description of the microbial community and its metabolic potential is required. One particular state-of-the-art technology is based on isotope labelling of active microorganisms and their biomolecules (see also review of Neufeld et al. (2007a)). Compounds of interest, stable or radioactive labelled isotopes, are added to a microbial community of relevance and incubated under carefully selected conditions, *in situ* or mimicking the environment as closely as possible. Physiologically active microbes metabolise the substance and incorporate the isotopes into their macromolecules. Via

detection and analysis of the label in macromolecules or whole cells it is possible to link activity to identity.

Stable Isotope Probing (SIP) allows the tracking of a chemical's fate by following the flux of selected heavy isotopes into biomolecules (e.g. ^{13}C, ^{15}N or ^{18}N). It has been applied for a wide range of substrates and environments (Radajewski et al., 2000; Radajewski and Murrell, 2002; Evershed et al., 2006; Madsen, 2006; Whiteley et al., 2006; Dumont et al., 2011).

A bird's eye view can be achieved with PLFA-SIP (Boschker et al., 1998). The extraction of phospholipid fatty acids and the direct analysis of the isotope ratio using gas chromatography coupled with mass spectroscopy (GC-MS) or isotope ratio mass spectroscopy is a relatively quick and highly sensitive technology (for carbon detection of differences < 0.1 at. %, (Jehmlich et al., 2010)). It allows the reliable detection of the turnover of the microbial contaminant but gives only basic information on microbial clades (Maxfield and Evershed, 2011).

More sophisticated technologies such as DNA-SIP (Radajewski et al., 2000) and RNA-SIP (Manefield et al., 2002) provide in-depth phylogenetic information on specific microbes involved in contaminant transformation. Labelled DNA and RNA molecules are co-extracted with unlabelled ones, separated via isopycnic centrifugation and analysed using a molecular biological tool that is applicable for community analysis, such as DGGE, T-RFLP, metagenomics, or functional genomics. Both DNA- and RNA-targeting detection require several at. % isotopic substitution for successful separation from 'native' nucleic acids (Zemb et al., 2012). The incorporation into DNA depends on cell replication (Ostle et al., 2003; Manefield et al., 2011). RNA labels faster than DNA due to its higher cellular synthesis rates (Manefield et al., 2002; Neufeld et al., 2007b; Whiteley et al., 2007). Consequently, RNA-SIP requires less incubation time of the microbial sample with the labelled substrate, and has thus lower cross-feeding effects of label into non-degrading organisms (Neufeld et al., 2007a) and therefore lower misinterpretation; but as in DNA-SIP this phenomenon should not be underestimated. Based on the same sequence-based resolution for organism identification as DNA, RNA serves as a more responsive biomarker for use in SIP (Manefield et al., 2002), which in the literature is often stated as a better sensitivity of RNA-SIP compared with DNA-SIP.

Due to the comparably high content of up to 50 % cellular proteins (Gottschalk, 1986) and continuously improving measurement technology, the relatively new Protein-SIP (Jehmlich et al., 2008b; Jehmlich et al., 2008a) attains a higher sensitivity [~ 2 at. % for carbon (Jehmlich et al., 2010)]. By analysing proteins as executive biomolecules for physiological reactions it allows a more direct link to *in situ* function while also providing phylogenetic

information (Jehmlich et al., 2008a). With improved mass spectrometry for peptide identification and quantification, the metaproteomic analysis of complex environmental samples has become achievable (VerBerkmoes et al., 2009). However, the identification of peptides depends on sequence information about the associated metagenome or meta-transcriptome. This knowledge may be obtained either via large-scale sequencing efforts or a suitable selection of deposited nucleic acid or amino acid sequences from public databases. Simply feeding all available sequences from those databases into the peptide identification algorithms would currently require (too) much computational power during peptide analysis (Jehmlich et al., 2011; Seifert et al., 2013; von Bergen et al., 2013; Sachsenberg et al., 2015). Furthermore, there are still technical challenges in extracting proteins from environmental matrices (Jehmlich et al., 2011).

These SIP approaches are costly due to the labelled substrates and the expensive analytical equipment, and they require substantial amounts of time for analysis. Typically, the labelled substrate is not added directly to the natural habitat but rather laboratory microcosm investigations are carried out in order to limit the amount of labelled substrate needed. Although substrate concentrations and incubation times are adjusted as close as possible to the natural environment in microcosms (Neufeld et al., 2007a), these experiments are not *in situ* studies. In most cases, the simplification of the environmental parameters results in changes in the community and activity of relevant microbial members. Complexity and heterogeneity of a consortium are lost, and the availability of substrates, nutrients, and electron acceptors differs from the habitat (Mandelbaum et al., 1997; Roling and van Verseveld, 2002; Bombach et al., 2010a). The challenge in reproducing the complex environmental habitat may result in an underestimation of the *in situ* catabolic activity.

A possible solution to this problem is the application of *in situ* microcosms to study degradation processes directly in the field. Such microcosms have been designated as BACTRAP®s (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006). Combined with SIP, they may allow for the determination of degradation activity under *in situ* conditions without the necessity of applying large amounts of an expensive labelled substrate to the field and therefore may overcome stated difficulties of laboratory microcosms. The labelled organic substrate and, if required for evaluating biostimulation approaches, electron acceptor and nutrients, are loaded onto an inert carrier material and packed in perforated PTFE or stainless steel containers of a few centimetres in diameter and length. They are exposed directly at the environmental site over an incubation period of several weeks. Indigenous microbes colonize the carrier material and metabolize the offered labelled organic chemicals desorbing from the carrier, which enables the investigation of

the heterogeneous compound transforming microbial community by molecular biological tools. For phylogenetic identification of labelled microbes, preferable DNA or RNA-SIP (Bombach et al., 2010b), but also Protein-SIP (Herbst et al., 2013) is used.

The clear advantage of SIP in combination with nucleic acid sequencing, and to a certain extent proteomics, is that no previous information about the microbial community structure is needed. All methods described in the following description deliver information only about whether a specific microbe or clade already known is responsible for degrading the substrate of interest. They require at least some knowledge about respective community structure or catabolic activities (Manefield et al., 2011). These approaches use radioisotopes (e.g. ^{3}H, ^{14}C and ^{13}P) to follow the chemical's transformation and incorporation into biomolecules.

Microautoradiography (MAR) is a highly sensitive tool for direct visualisation of metabolising microbes at the single-cell level (Brock and Brock, 1966). Cells that may have metabolised a radioisotopically labelled organic compound are overlaid with a radiosensitive silver halide emulsion. Silver ions precipitate as black metallic silver grains next to cells that contain the label. In a combined application with rRNA-targeted oligonucleotide probes (Fluorescence *In situ* Hybridisation – FISH (DeLong et al., 1989), MAR-FISH directly links activity to image-based identification of specific microorganisms (Lee et al., 1999; Ouverney and Fuhrman, 1999). FISH analysis currently is the 'gold standard' for cultivation-independent identification and quantification of microorganisms in environmental and medical microbiology (Amann et al., 1996; Wagner et al., 2003), but requires previous information or ideas about the system and degraders for probe selection or design. Nevertheless, due to its high sensitivity MAR-FISH requires much shorter incubation times than SIP techniques. When applied with microcosms, it might be even considered as a 'quasi' *in situ* method. In addition, it is technically easier to perform than the SIP technologies (Wagner, 2011).

Another Radioisotope Probing (RIP) technology, the isotope array approach, allows the simultaneous identification of various labelled microbes in complex communities (Adamczyk et al., 2003). Again, hybridisation of probes to rRNA is used for identification. For determination of the radio-label in extracted RNA, the analysis using a fluorescence scanner is coupled to an analysis using a beta imager; both are comparable, cost-intensive pieces of equipment. By applying them in series in a microarray format, this approach allows high-throughput screening of communities with a range of probes, only restricted by limited spatial resolution of the beta imager (Wagner, 2011). The accessible phylogenetic resolution increases with increasing practical and technical effort, but again unknown or unexpected microbes cannot be identified of analyzed.

Several advanced approaches, combining stable or radioisotope-labelling with single-cell methodologies, are under development or have been used to some extent. The recent refinement of Secondary Ion Mass Spectrometry (SIMS), the NanoSIMS technology, attains resolutions far below 1 µm. and can be coupled with *in situ* hybridisation of oligonucleotide probes for identification of microbes (Orphan et al., 2001). The simultaneous detection and visualisation of different elements and isotopes is applied for activity measurement (^{13}C or ^{14}C) and simultaneously tracer-mediated probe detection. Consequently, it requires prior phylogenetic information (or good guesses). Cells are largely destroyed during the measurement, thus preventing any further microbiological analysis. To date, the technology has been mostly used to gain information on morphology and spatial arrangement of selected single cells (Musat et al., 2011; Musat et al., 2016).

A similar concept combines Raman microscopy and FISH to detect incorporation of stable isotopes into single cells [i.e. phenylalanine, proteins, nucleic acids (Huang et al., 2004)]. The application of hybridisation with specific probes verifies whether an expected organism is involved in the metabolism of a certain compound (Huang et al., 2007). Raman microscopy coupled to optical trapping and cell sorting of isotopically labelled cells may open the door to single cell identification without pre-information (Huang et al., 2009a), but this approach is still in the early stages of development (Read and Whiteley, 2011; Wang et al., 2016).

The identification of microorganisms that are active *in situ* by isotope-labelling, either stable- or radioisotopes, is a valuable approach to address questions in microbiology. Up to now, the compendium of single-cell technologies is at different stages of development. The various approaches are challenging due to environmental complexities, high costs, their substantial technical sophistication, or low sensitivity so that only highly abundant key players can be tracked. The identification of microbes depends mainly on prior nucleic acid sequence information. In this context it has been proposed to apply traditional SIP approaches (DNA/RNA, maybe proteins) to generate clues for oligonucleotide probe design (Huang et al., 2009b). In consequence, improved or even new strategies are needed.

1.1 Overview of Research Approach

The general aim of this work was to assess microbial *in situ* activity in engineered environmental systems treating water contaminated with organic pollutants. The driving factor steering this aim is the idea that a detailed description of microbial activities will, ultimately, support the rational advancement of treatment approaches. To this end, the spectrum of methodologies for *in situ* identification of key active microbial players that are not based on previous phylogenetic information was improved. Two strategies were pursued, whereby both were based on following the chemical's fate via an isotope labelling strategy.

PART A1 sought to analyze a benzene-degrading microbial community and especially to identify the key active microorganism in a constructed wetland pilot plant fed with benzene-contaminated groundwater. Based on the prevailing wetland conditions, two contrasting hypotheses were formulated: (i) anaerobic benzene-degraders may play a crucial role due to the strongly negative redox potential in the constructed wetland; (ii) aerobic benzene-degraders on the root surface, where they receive oxygen and maybe root exudates, dominate the degradation processes.
The methodical strategy attempted to expand the current ***in situ* incubation concept** for identification of relevant key degraders. The research approach used a combination of *in situ* microcosms (BACTRAPs) with RNA-SIP and metagenomic analysis in order to provide comprehensive in-depth phylogenetic information on microbial biodegradation of benzene in a constructed wetland system.

PART A2 was part of a project that tried to assess the extent to which bioremediation of endocrine disrupting chemicals (EDCs) like bisphenol A (BPA) can be applied directly within natural or engineered systems. The aim of this part was to assess the applicability of the BACTRAPs for on-site recording and characterisation of the microbial BPA transformation. Thereby, the limited sensitivity of the BACTRAP approach and the expected, comparable low incorporation rate of the low concentrated micropollutant BPA needed a previous and thorough estimation on feasibility. A successful evaluation of the BACTRAP approach for BPA in particular would open up new fields of application, which would ultimately target the *in situ* identification of key microbial degraders of micropollutants.

PART B dealt with an even more sophisticated idea of identification of contaminant-degrading and *in situ* active microbial microorganisms that would overcome current limitations on low sensitivity and environmental complexities of specifically SIP and in general. It therefore describes the development of a novel, easy, rapid and sensitive **identification approach for *in situ* active microorganisms** which is also independent of any form of cultivation and prior phylogenetic information.

The hypothesis of this part was as follows: active degraders can be specifically isotopically labelled. Secondly, based on the label, the active degrading cells can be exclusively selected and separated for identification.

2 PART A – Stable Isotope Probing
A1: RNA-SIP, using *in situ* microcosms combined with a metagenomic approach

2.1 Introduction

Monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene and the three isomers of xylene (BTEX) are important petroleum constituents. Improper and irresponsible storage, handling, transport and disposal by petrochemical industry have led to the prevalence of highly contaminated subsurface environments. BTEX are relatively highly soluble in water and therefore can directly affect drinking water reservoirs. Benzene, in particular, represents a high risk for humans due to its carcinogenicity.

Biodegradation of benzene has been studied extensively in the past, and several microbes from various taxonomic groups have been identified to degrade benzene. Metabolism under aerobic conditions is well understood and proceeds readily (Ridgway et al., 1990; Wilson and Bouwer, 1997; Jindrova et al., 2002; Fahy et al., 2006; Fischer et al., 2008). Anaerobic degradation was reported for the important electron acceptors nitrate (Burland and Edwards, 1999; Coates et al., 2001; Kasai et al., 2006), sulphate (Lovley et al., 1995; Phelps and Young, 1999; Oka et al., 2008), and ferric iron (Anderson et al., 1998; Jahn et al., 2005; Kunapuli et al., 2007), as well as via fermentation (Weiner and Lovley, 1998; Ulrich and Edwards, 2003). The anaerobic turnover is much slower than the aerobic degradation, but more relevant for the subsurface environment where benzene is more persistent.

Up to now little is known about the *in situ* catalytic activity role of those contaminant degraders that have been isolated, enriched and identified via enrichment cultures and microcosm studies in the laboratory, which has been exemplified by Xie et al. (2011) in a SIP study with microcosms from mixed community samples. This study tried to close this gap and targeted the *in situ* identification of key active microbial benzene degraders at the environmental site via an improved *in situ* incubation approach.

Here, microbial benzene degradation was investigated in pilot-scale constructed wetland systems. Constructed wetlands (CWs) are artificial wetlands especially engineered and applied for the purpose of decentralised and ecological treatment of various kinds of contaminated waters (Williams, 2002; Haberl et al., 2003), including domestic, agricultural and industrial wastewater (Konnerup et al., 2009; Vymazal, 2009; Vymazal and Kröpfelová, 2009), landfill leachate (Bulc, 2006; Yalcuk and Ugurlu, 2009) and

groundwater contaminated with organic chemicals (Braeckevelt et al., 2008; Seeger et al., 2011). CWs, especially for groundwater remediation, are less invasive, less cost-intensive and economically more efficient than conventional treatment approaches such as 'pump-and-treat', and thus allow for an efficient remediation strategy of contaminated waters. They are near-natural phytoremediation systems, where plants enhance natural microbial attenuation by supplying oxygen and root exudates into the rhizosphere where contaminant transformation takes place (Williams, 2002; Stottmeister et al., 2003). This so-called 'rhizospheric effect' can result in effective treatment of benzene-contaminated groundwater in CWs (Seeger et al., 2011).

The combined application of SIP and *in situ* microcosms ultimately aims at providing conclusive evidence for contaminant degradation as well as generating deeper insights into the involved microbial community even at complex sites. Using these approaches, the success rate of cost-effective clean-up strategies may be enhanced.

SIP coupled to BACTRAPs have been successfully applied *inter alia* for benzene-contaminated field-site characterisation for several years now (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006). More recently they have also been applied in water-saturated and water-unsaturated zones of soil (Direct-Push-BACTRAPs) (Schurig et al., 2014). However, in those experiments the specific identification of key active degraders was not in focus. Fatty acid methyl esters were isolated, which provide only limited taxonomic information. Rough investigation of the community's diversity was done by molecular biological analysis of DNA from applied BACTRAPS and Single Strand Conformation Polymorphism (SSCP) fingerprinting analysis (Kästner et al., 2006; Stelzer et al., 2006).

The specific aim of this project was to characterise the benzene-degrading microbial community in a constructed wetland pilot plant fed with benzene-contaminated groundwater (↗ Chapter 2.2.2), and with special emphasis to identify the active microorganism involved in the degradation process. Special emphasis was on the identification of active microorganism involved in the degradation process.

Both will give insight into the effective microhabitats of the near-natural phytoremediation system that has a limited level of oxygen. In order to gain detailed taxonomic information on the microorganisms that are active *in situ*, a metagenomic approach (high-throughput sequencing) was combined with SIP and BACTRAPs. BACTRAPs were assumed as suitable tools for site assessment. Due to the higher sensitivity of RNA-SIP, we primarily analysed the 16S rRNA profile of the microbes that colonised the microcosm.

2.2 Material and Methods

2.2.1 Chemicals

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany) and were of analytical grade quality. [$^{13}C_6$]-benzene (≥ 99 atomic percent) was obtained from Campro Scientific (Berlin, Germany).

2.2.2 Field site

This study was carried in part out at the pilot-scale treatment plant 'Compartment Transfer' ('CoTra') in Leuna (51°18'30"N 12°01'19"E; Saxony-Anhalt, Germany). In 2007 horizontal-flow constructed wetland (CW) systems were built next to the former refinery and industrial site, which was strongly contaminated with the fuel constituent benzene, to investigate near-natural remediation strategies. Detailed description of the CW systems was given in previous studies (Seeger et al., 2011; Seeger et al., 2013).

The CW (5 m x 1.1 m x 0.6 m) was filled with fine gravel (1-3.2 mm) and planted with *Phragmites australis*. Inflow water was supplied from the contaminated groundwater well, which is located nearby and which contains benzene at a concentration of up to 8 mg L^{-1}. Table 2-1 summarises relevant physical and chemical parameters of the inflow and outflow water as well as the porewater characteristics in a depth of 0.2 m during the investigation period.

Table 2-1: ***Physical and chemical characteristics of inflow water from a benzene-contaminated groundwater well in Leuna. Summarised data from July to September 2012. Total organic carbon (TOC) was calculated by summing purgeable and non-purgeable organic carbon.***

Parameter [unit]	Inflow	Distance from inflow			Outflow
		1 m	2.5 m	4 m	
rH (Ag/Pt) [mV]	-191±47	-291±35	-338±16	-343±20	-320±37
T [°C]	17.8±1.9	20.9±3.7	21.1±3.9	20.0±3.4	19.5±1.7
pH	7.1±0.1	7.0±0.1	6.9±0.1	6.9±0.01	6.9±0.1
benzene [mg L^{-1}]	5.70±1.77	2.99±1.99	2.24±0.92	1.68±1.41	1.53±1.48
TOC [mg L^{-1}]	31.84±2.59	n.d.	n.d.	n.d.	20.80
Fe(II) [mg L^{-1}]	2.95±0.24	n.d.	n.d.	n.d.	0.22±0.03
Fe(III) [mg L^{-1}]	2.64±0.18	n.d.	n.d.	n.d.	0.08±0.06
nitrate [mg L^{-1}]	< 0.07	n.d.	n.d.	n.d.	< 0.07
sulphate [mg L^{-1}]	99.7±2.1	n.d.	n.d.	n.d.	13.5±4.2

n.d. = not determined

2.2.3 Experimental design

For specific identification of key microbial players in benzene turnover, *in situ* microcosms (BACTRAP®-systems) were applied. For comparative microbiological analysis and characterisation of the rhizosphere of the CW, representative samples of the root-sediment-gravel-mat were taken.

BACTRAPs were prepared following a previously described experimental design (Geyer et al., 2005). Briefly, granulated activated carbon ('AC') (AUF 540, Adako GmbH, Düsseldorf, Germany; granules of approximately 0.3 x 0.3 x 0.6 mm) was used as carrier material and prepared by heating to 300 °C for at least 4 h to remove biogenic organic carbon residues and afterwards autoclaved at 121 °C for 20 min in order to sterilize and re-hydrate it (Schurig et al., 2014). The air-dried granulate was put into a small desiccator and was loaded with the substrate via vapour exposure under partial vacuum. Air was exchanged against nitrogen gas (N_2) by alternate evacuation and flooding with N_2. Afterwards, under a continuous stream of N_2, an open vessel with 88 mg per g_{AC} of either $^{13}C_6$-labelled or unlabelled benzene was placed inside by briefly opening the container. The desiccator was evacuated to 50 mbar and held at this pressure until the substrate was completely evaporated from the reservoir vessel. AC for 'blank'-microcosms was treated similarly, but without any substrate. For incubations of the *in situ* microcosms in the rhizosphere of the CW, perforated and threefold segmented cages (stainless steel, 120 x 50 x 10 mm), were designed and framed in spades. The blank- (microbial background control without relevant substrate), ^{12}C- (comparative control with unlabelled substrate) and ^{13}C-labelled AC granules were separately transferred into the segments of two cages, which were finally exposed perpendicularly to the flow direction, one for 6 and the other for 28 days at a depth of 150 mm, 1.5 m downstream of the systems inflow.

At the beginning and at the end of the exposition time, representative wetland rhizosphere samples were taken from the area surrounding (up to 100 mm distance) the installed BACTRAP-systems. A special cylindrical with closable sampler was pushed to the intended sampling point and the aperture was opened. By moving the instrument mixed samples of gravel, sediments and roots were taken and after closing the aperture removed from the system.

All *in situ* microcosms and wetland samples were frozen immediately and stored at -80 °C until analysis.

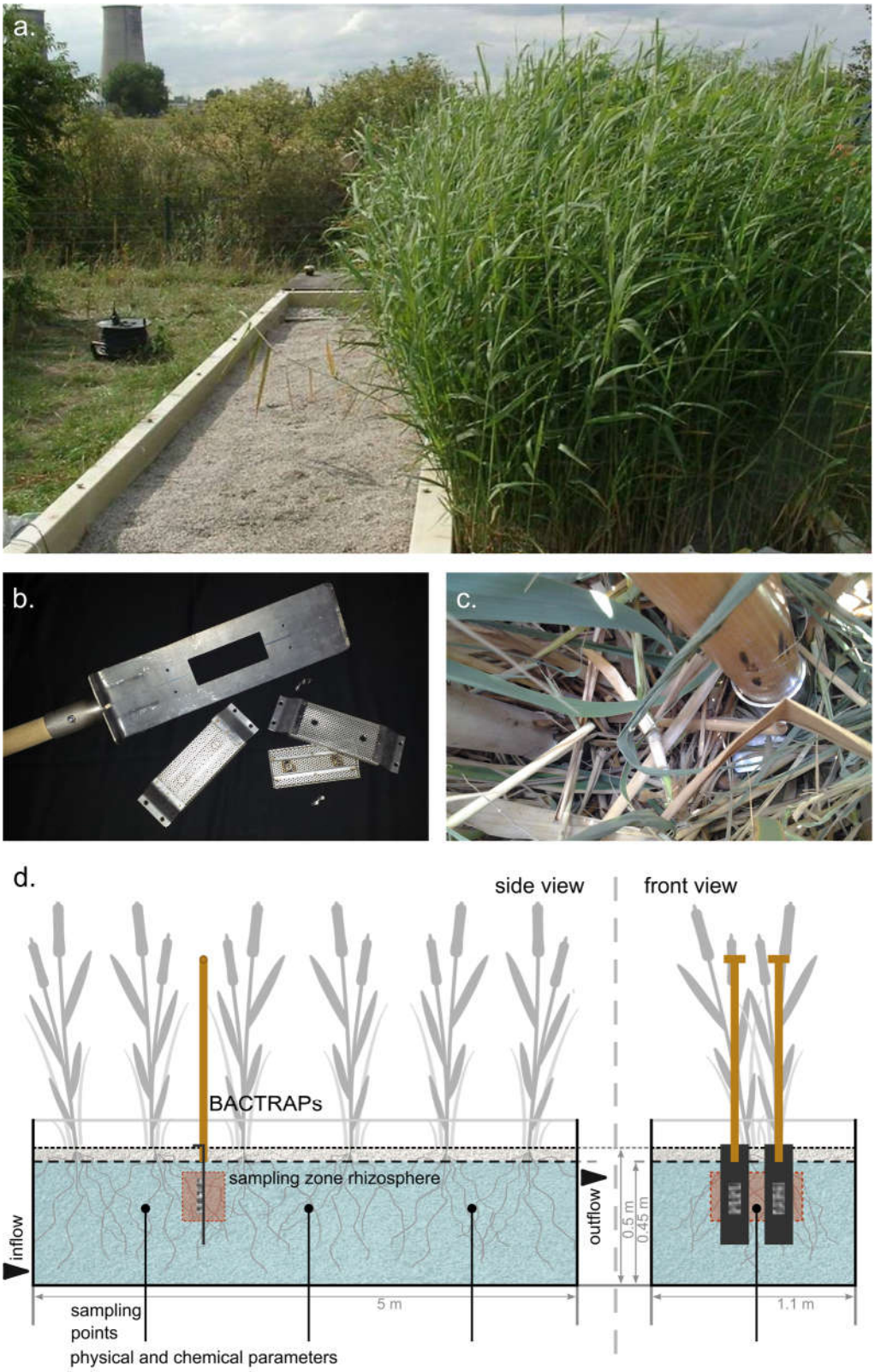

Figure 2-1: ***Experimental setup of the SIP-BACTRAP experiment in a pilot-scale constructed wetland in Leuna. a. Exemplary photography of the system (right: planted, left: unplanted reference). b. Special threefold segmented BACTRAPs for parallel exposition of blank, ^{12}C and ^{13}C framed in a spade for insertion into overgrown rhizosphere and c. their position in the system. d. Schematic drawing of the wetland rhizosphere sampling zones and the incubation of the two BACTRAPs.***

2.2.4 RNA extraction, isopycnic centrifugation and cDNA synthesis of BACTRAP-samples

RNA from BACTRAPs was extracted from the activated carbon according to a modified protocol from Bombach et al. (2010b). 3 g sample of activated carbon was distributed to twelve 2-mL bead-beating vials each filled with 0.2 mL of a 1:1 mixture of 0.1 and 1.0 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) and mixed with 300 µL sodium acetate buffer (50 mM Na-acetate pH 5.3, 10 mM Na-EDTA), 200 µL of 200 mM tri-sodium phosphate solution, 50 µL 20 % (w/v) sodium dodecyl sulphate solution and 450 µL phenol preheated to 65 °C. After incubation at 65 °C for 10 min cells were disrupted by bead-beating twice at 6.5 m s^{-1} for 60 s in a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA), frozen at -80 °C for 5 min and after briefly thawing, centrifuged at 16 100 rcf and 4 °C for 5 min. From each tube 400 µL of the upper aqueous phase was transferred to Phase Lock Gel Heavy tubes (Eppendorf, Hamburg, Germany). Acetate buffer (200 µL) was added to each remaining phase, the mixtures were carefully vortexed and centrifuged again at 16 100 rcf and 4 °C for 5 min. 400 µL of each upper aqueous phase, combined with the 400 µL previously transferred phase was extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 [v/v/v]) and centrifuged at 16 100 rcf and 4 °C for 5 min. All extracted RNA was pooled and re-extracted with the clean-up protocol of the RNeasy Mini Kit including DNA on-column digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany). The RNA was eluted in water and its concentration was determined by spectrophotometry (Nanodrop, Thermo Scientific, Wilmington, NC, USA) or Lab-on-a-Chip electrophoresis (Agilent Bioanalyzer, Agilent RNA 6000 Nano Kit, Agilent Technologies, Santa Clara, CA, USA).

The extracted RNA was fractionated by equilibrium density gradient centrifugation and gradient fractionation following a modified protocol of Lueders et al. (2004). To prepare the Cesium Trifluoroacetate (CsTFA) gradient 750 ng of RNA per sample was mixed with up to 970 µL gradient buffer (0.1 M Tris-HCl pH 8, 0.1 M KCl, 1 mM EDTA), 5.3 mL CsTFA (IllustraCsTFA, GE Healthcare, Chalfont St Giles, Great Britain), and 200 µL formamide. The mixture's density was adjusted to 1.79 g mL^{-1} (AR200 Automatic Digital Refractometer, Reichert Analytical Instruments, Depew, NY, USA) and it was transferred to Quick-Seal® Polyallomer tubes (Beckman Coulter, Brea, CA, USA). The tubes were centrifuged at 39 000 rpm (≈ 130 000 rcf) and 20 °C for 69 h in an ultracentrifuge (OPTIMA™L-90 K, Beckman Coulter) using a Near-Vertical Rotor (NVT 65.2, Beckman Coulter). Subsequently, they were fractionated in 12 fractions by displacement with water from below as described previously (Manefield et al., 2002; Lueders et al., 2004). For each collected fraction the density was determined using a refractometer. The RNA was

precipitated from the fractions by using the RNeasy Mini Kit (Qiagen, RNA clean-up protocol) and eluted in water.

The fractionated RNA was reverse-transcribed using the Omniscript® or the Sensiscript® Reverse Transcription Kit (Qiagen) with random hexamer primer (10 µM) for sequencing, with the primer Uni909R (1 µM), 5'-CCG YGA ATT CMT TTR AGT-3' (modified from Wang and Qian (2009)) for 16S rRNA gene quantifications via qPCR and with ASP1 (1 µM), 5'-C MAT GCC GAT YTC CTG RC-3' (Kuntze et al., 2008) for *bamA* gene quantification via qPCR, respectively.

2.2.5 RNA extraction and cDNA synthesis of wetland rhizosphere samples

RNA from wetland samples was extracted with the PowerSoil® Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells in the sediment samples were lysed using silica carbide beads, lysis buffers, and phenol:chloroform:isoamyl alcohol. Total RNA was bound on a column matrix, washed and eluted in water. Co-extracted DNA was digested using the Ambion® DNA-free™ Kit (Lifetechnologies, Carlsbad, CA, USA) and the RNA-extract purified with the RNeasy Mini Kit (Qiagen).
For sequencing, the extracted RNA was transcribed into cDNA using the Invitrogen™ SuperScript® III Reverse Transcriptase Kit (lifetechnologies) with random hexamer primer (10 µM).

2.2.6 Amplicon library preparation and Illumina sequencing

Amplicon library construction was performed following the general procedure of Camarinha-Silva et al. (2014). Briefly, the V1-2 region of the 16S rRNA-derived cDNA was amplified using the universal primers 27F, 5'-AGA GTT TGA TCM TGG CTC AG-3' (Lane, 1991) and 340R, 5'-TGC TGC CTC CCG TAG GAG T-3' (Etchebehere and Tiedje, 2005) in a first amplification (V_{PCR} = 20 µL, 20 cycles). The amplification mix contained 5x PrimeSTAR™ buffer (Clontech Laboratories, Mountain View, CA, USA) each deoxynucleoside triphosphate at a concentration of 0.25 mM, the primers at a concentration of 0.5 µM each and 1 unit of PrimeSTAR™ HS DNA polymerase (Clontech Laboratories). The PCR was performed as follows: initial denaturation step of 95 °C for 3 min and the indicated number of cycles comprised of denaturation at 98 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 45 s. In a second (V_{PCR} = 20 µL, 15

cycles) and a third reaction (V_{PCR} = 50 µL, 10 cycles) the primers were extended with specific sequences to insert sample-tracking barcodes and indices and mandatory process-specific Illumina sequences [see further details in Camarinha-Silva et al. (2014)]. Non-template controls were free of any amplification products after all 3 PCR. PCR amplicons were purified, quantified, and prepared for Illumina sequencing exactly as described (Camarinha-Silva et al., 2014), including positive control samples containing known 16S rRNA gene sequences: control #1 comprised 16S rRNA gene amplicons derived from *Staphylococcus aureus* (DSM3463) genomic DNA and from a pGEM-T easy vector containing the 16S rRNA gene from *Corynebacterium accolens* (GU074966), and control #2 comprised 16S rRNA gene amplicons derived from pGEM-T easy vectors containing the 16S rRNA genes from *Moraxella nonliquefaciens* (JF927886) and *Staphylococcus epidermidis* (JF927883). The amplicon library was 250 nt paired-end sequenced on a MiSeq System (Illumina, San Diego, CA, USA). Image analysis and base calling were accomplished using the Illumina Pipeline (version 1.7).

2.2.7 Bioinformatic analysis

As described in detail in Camarinha-Silva et al. (2014), obtained sequences were first filtered according to specific quality requirements. Subsequently they were trimmed to 120 nt and the read pairs were combined to 240 nt-long sequences. In downstream analyses they were collapsed, clustered, and finally filtered to consider exclusively phylotypes (maximum 1000 most abundant) that: (i) were present in at least one sample at a relative abundance >0.1 % of the total sequences of that sample; or (ii) were present in at least five samples at a relative abundance >0.01 % for a given sample.

2.2.8 Sequencing data analysis

Obtained operational taxonomic units (OTUs) and their abundances were used in rarefaction analysis with R software (R Core Team, 2013) using the VEGAN package (Oksanen et al., 2013). Chao1 richness estimators and Shannon diversity indices were calculated with the EstimateS software package (Colwell, 2013). OTUs were compared to taxonomy databases using the Naïve Bayesian Classifier [RDP-Classifier, (Wang et al., 2007)] of the Ribosomal Database Project (RDP) with an assignment cut-off of 70 % confidence threshold. Further analysis of classified sequences was done with MEta Genome ANalyzer (MEGAN 5, (Huson et al., 2007; Huson et al., 2011)). The dataset was processed by lowest common ancestor (LCA) algorithm using the following LCA

parameters: min support: 1; min score: 70.0; max expected: 100.0; top percent: 30.0; LCA percentage: 100 and Min complexity: 0.44.

Dissimilarities in the various community structures were analysed with the non-parametric test of the general multivariate hypothesis of differences in the relative abundances of organisms of different species in samples from different groups (Anderson, 2001), available in the R software, VEGAN package as ADONIS function.

2.2.9 Quantitative real-time PCR

Synthesized cDNA was analysed via SYBR Green-based quantitative real-time PCR (qPCR), performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The total number of bacterial 16S rRNA genes was quantified with the universal primers 519F, 5'-CAG CMG CCG CGG TAA NWC-3', (Lane, 1991) and Uni909R, 5'-CCG YGA ATT CMT TTR AGT-3' (modified from Wang and Qian (2009)) (= qPCR I). The number of *Geobacteraceae* sp. 16S rRNA genes was determined with the specific primers Geo564F, 5'-AAG CGT TGT TCG GAW TTA T-3', (Cummings et al., 2003) and Geo840R, 5'-GGC ACT GCA GGG GTC AAT A-3' (Cummings et al., 2003)) (= qPCR II). Furthermore the transcription of *bamA*, encoding for 6-Oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolases, one key enzyme of the anaerobic benzoyl-coenzyme A (CoA) degradation pathway, was investigated with the degenerate oligonucleotide gene primers SP9 (5'-CAG TAC AAY TCC TAC ACV ACB G-3') and ASP1 (5'-C MAT GCC GAT YTC CTG RC-3'), that were developed by Kuntze et al. (2008) and used for detection of benzene-degrading anaerobes, including especially *Geobacter* species (Kuntze et al., 2011; Staats et al., 2011) (= qPCR III).

In addition to template cDNA, PCR reaction mixtures contained 0.2 µM of each primer in qPCR I (519F and Uni909R), and 0.3 µM in qPCR II (Geo562F and Geo840R) and qPCR III (SP9 and ASP1), and 1x of the KAPA™ SYBR® FAST qPCR MasterMix (PEQLAB Biotechnology GmbH, Erlangen, Germany) (V_{PCR} = 11.5 µL).

PCR cycle parameters were as follows: initial denaturation step of 95 °C for 2 min, followed by 40 cycles comprised of denaturation at 95 °C for 3 s, annealing at 52 °C (qPCR I) respectively 57 °C (qPCR II) and 59 °C (qPCR III) for 20 s and extension at 72 °C for 20 s. At the end of the qPCR analysis, a DNA melting curve was recorded by short denaturation at 95 °C for 3 s, heating at 60 °C for 1 min and then increasing the temperature to 95°C by 0.3 °C/min.

Standard curves were prepared using a cloned 16S rRNA fragment (27F-1378R) of *Pseudomonas putida* MT2 for qPCR I, a purified 16S rRNA fragment (27F-1492R) of

Geobacter metallireducens amplified during PCR for qPCR II and a *bamA* gene, isolated from a toluene-degrading consortium of a planted fixed-bed reactor, cloned and re-amplified for PCR III. Standard curves were linear (R^2 > 0,999) over at least six orders of magnitude and amplification efficiency was > 85 % for qPCR I and II and 70 - 80 % for qPCR III. No signals were detected in controls without template.

2.2.10 Sequencing of *bamA* genes

For further qualitative characterisation of the amplified *bamA* gene, qPCR III products were selected according to obtained melting curves, pooled and purified using the MinElute PCR Purification Kit (Qiagen) and quantified via spectrophotometry (Nanodrop, Thermo Scientific). Fragments were ligated into pGEM®-T Easy Vector (Promega, Madison, WI, USA) and transformed into competent cells of *E. coli* following the manufacturer's instructions. Positive clones were selected by colour screening on indicator plates (blue/white screening of recombinants), and the inserts were amplified via PCR using the pUC/M13 forward and reverse sequencing primer sites of pGEM®-T Easy Vector. The amplification mix contained 10x QIAGEN® PCR buffer™ (Qiagen) each deoxynucleoside triphosphate at a concentration of 0.2 mM, the primers M13F, 5′-GT AAA ACG ACG GCC AG-3' and M13R, 5'- G GAA ACA GCT ATG ACC AT-3' at a concentration of 0.25 µM each and 2.5 units of *Taq* DNA Polymerase (Qiagen). PCR was performed as follows: initial denaturation step of 94 °C for 3 min and 32 cycles comprised of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s and extension at 72 °C for 60 s. PCR products were checked by agarose gel electrophoresis, and sent for standard plate sequencing to Macrogen Europe (Amsterdam, the Netherlands).

2.3 Results and Discussion

Within the framework of this project, the bacterial community in the rhizosphere of a pilot-scale constructed wetland, fed with benzene-contaminated groundwater, was characterised. Special emphasis was placed on the identification of key microorganisms involved in benzene turnover. A combination of *in situ* microcosms (^{13}C-benzene-loaded BACTRAPs), RNA-SIP and high-throughput Illumina® Sequencing was applied. The BACTRAPs were incubated in the wetland for 6 and 28 days. In order to characterize the rhizosphere microflora, representative wetland samples were also taken at days zero and 28 and 16S rRNA profiles were analysed via sequencing.

2.3.1 Fractionation of RNA

With the rationale of selecting ^{13}C-labelled RNA for molecular biological analysis, the RNA was fractionated via isopycnic centrifugation.

In RNA isolated from BACTRAPs incubated for 6 days, no incorporation of the provided stable isotope-labelled carbon into RNA was observed.

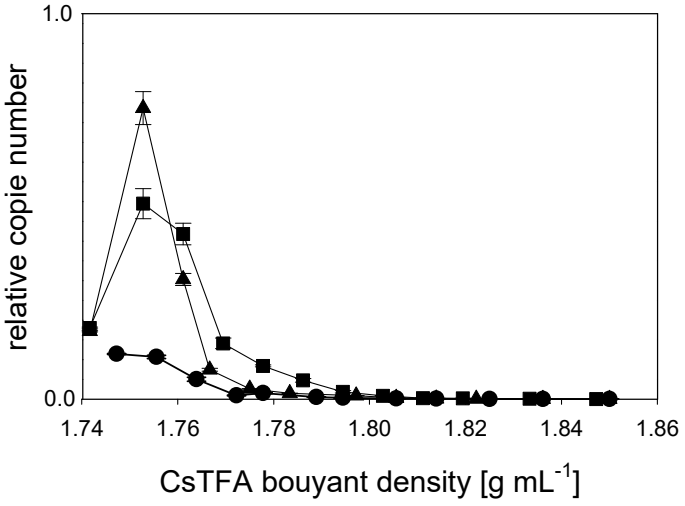

Figure 2-2: ***16S rRNA extracts, fractionated over buoyant density via isopycnic centrifugation in CsTFA. Shown are the 16S rRNA density profiles of the BACTRAP compartments loaded with ^{13}C- (■) respectively ^{12}C-benzene (▲) and unloaded blank (●), incubated for 28 days in the pilot-scale constructed wetland.***

After 28 days of incubation the buoyant density of the RNA-extract of the ^{13}C-BACTRAP was shifted to higher values (1.77-1.80 g mL^{-1}) compared to the ^{12}C-control and the blank (↗ Figure 2-2). No highly labelled RNA fractions at around 1.82 g mL^{-1} (Lueders et al., 2006; Whiteley et al., 2007) were detected. Relative RNA copy-numbers of the blank fractions were comparatively low, which perhaps was due to a limited colonisation of unloaded carrier.

For specific key player identification all fractions with detected and relevant amounts of 16S rRNA were analysed by high-throughput sequencing. This included the fractions 12 - 7 of the ^{13}C-benzene-BACTRAP (≡ 1.74 – 1.79 g mL^{-1}), fractions 12 - 9 of the ^{12}C-benzene-control (≡ 1.74 – 1.77 g mL^{-1}) and as unlabelled comparison the fractions 12 - 10 of the blank-BACTRAP (≡ 1.74 – 1.77 g mL^{-1}).

In addition, the non-fractionated 16S rRNA of the BACTRAP incubated for 6 days and 16S rRNA of the wetland rhizosphere samples were analysed. By this means differences of wetland and BACTRAP community and temporary changes of the latter were identified.

2.3.2 Analysis via Illumina Sequencing

Description and evaluation of the dataset

A total of 321 655 sequence-reads of a length of 240 bp were obtained after quality filtering protocols. The number of reads in all 18 samples ranged from 6 724 to 28 060 per sample. Those high-quality reads translated into a total of 1003 operational taxonomic units (OTUs). The number of OTUs per sample, and the calculated species richness (nonparametric Chao1 estimator) and species diversity (Shannon index) are reported in Table 2-2. Rarefaction plots can be found in Appendix 1.

The number of reads and OTUs obtained from the wetland rhizosphere samples was considerably smaller compared to those from the BACTRAP samples. Nevertheless, as predicted by Chao1 estimator and rarefaction curves (↗ Appendix 2), the number of reads was high enough for all samples to reach satisfactory diversity coverage of the microbial community.

Table 2-2: ***Summarised sequencing results. Number of sequences analysed, observed richness and diversity for 16S rRNA sequencing per sample or fraction.***

Sample*	Density [g mL^{-1}]	Reads	No. of OTUs**	Chao1 richness	Shannon diversity
CW_0d	-	6724	302	332	4.99
CW_28d	-	7604	565	730.05	5.05
BT_6d_BL	-	23296	754	851.62	4.57
BT_6d_12C	-	23287	712	816.86	4.14
BT_6d_13C	-	21528	677	806.46	4.18
BT_28d_BL_10	1.764	28060	730	817.08	3.62
BT_28d_BL_11	1.756	22125	843	905.53	4.71
BT_28d_BL_12	1.747	21881	863	912.63	4.80
BT_28d_12C_09	1.769	23863	852	927.98	4.26
BT_28d_12C_10	1.761	10358	801	897.83	4.83
BT_28d_12C_11	1.753	18634	843	921.28	4.48
BT_28d_12C_12	1.742	18342	841	948.62	4.48
BT_28d_13C_07	1.786	18240	762	870.74	3.76
BT_28d_13C_08	1.778	11214	754	841.78	4.40
BT_28d_13C_09	1.769	10327	767	866.26	4.80
BT_28d_13C_10	1.761	16480	856	906.5	4.98
BT_28d_13C_11	1.753	17764	881	943.13	4.91
BT_28d_13C_12	1.742	21928	890	955.64	4.87

** Sample designations are defined from the following sample parameters: SampleType (CW or BT)_SamplingTimePoint_BACTRAPType (BL, 12C or 13C)_FractionNumber; CW = constructed wetland, BT = BACTRAP, BL = blank; ** OTUs = operational taxonomic units*

General microbial structure of wetland and BACTRAP communities

The 1003 ribosomal sequences were taxonomically assigned using the RDP classifier with a 70 % confidence cut-off. 1001 sequences were of bacterial origin and after further LCA processing with MEGAN affiliated to 12 phyla (88 % of all sequences), 22 classes (76 %), 42 orders (71 %), 77 families (59 %) and 133 different genera (47 %). A full list of taxa that have been identified as the closest relatives of the obtained sequences (with available 16S rRNA sequence in the RDP database) is provided in Appendix 3.

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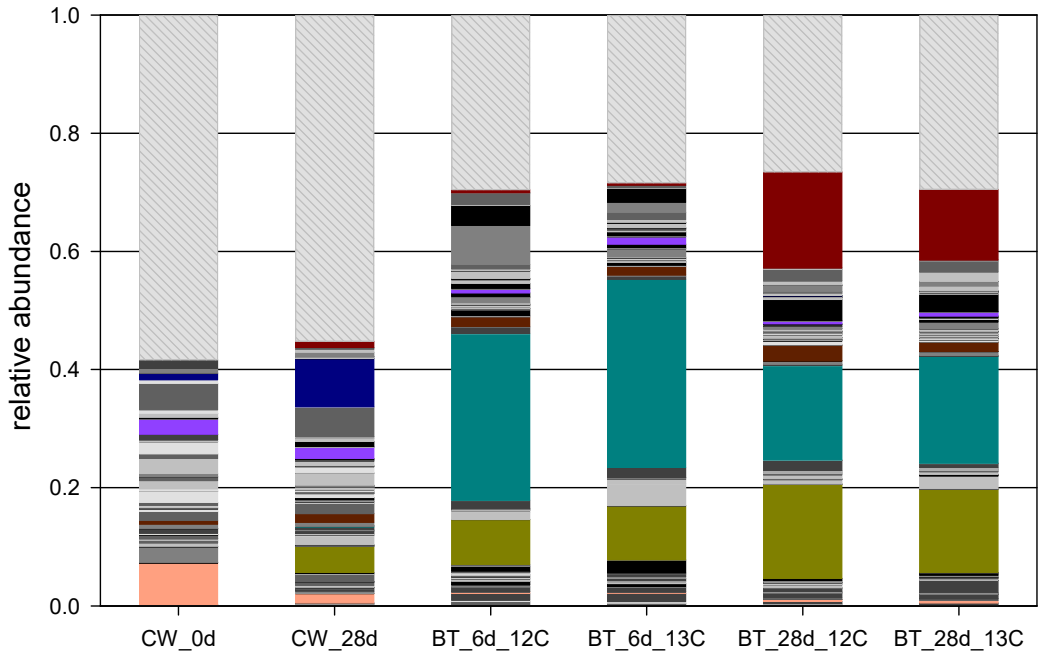


Figure 2-3: ***Distribution of relative abundances of microbial taxa at genus level in the constructed wetland and BACTRAP samples after 0, 6 or 28 days of incubation. Highlighted in colour are* Amaricoccus *(), Dechloromonas (), Geobacter (), Hydrogenophaga (), Pseudomonas (), Steroidobacter (), Zoogloea () and the sum of all sequences unassigned at genus level ().***

The wetland and BACTRAP communities showed a high level of diversity. As expected, the wetland microflora was composed of a number of taxa typically found in (semi)aquatic habitats and soils.

Already at the level of phyla significant differences between wetland and BACTRAP samples were detected. Whereas in all samples most of the community comprised *Proteobacteria* (wetland sample, day 0: 76.1 %; wetland sample, day 28: 80.8 %; BACTRAP, day 6: 70.1 %; BACTRAP, day 28: 74.8 %), *Actinobacteria* were decreased on the BACTRAPs (wetland sample, day 0: 10.3 %; wetland sample, day 28: 3.4 %; BACTRAP, day 6: < 1.3 %; BACTRAP, day 28: <1.6 %). *Bacteroidetes* and *Firmicutes* increased (wetland sample, day 0: 5.2 % / 0.1 %; wetland sample, day 28: 5.6 % / 0.5 %; BACTRAP, day 6: 6.1 % / 3.6 %; BACTRAP, day 28: 10.6 % / 3.8 %). *Fusobacteria* were strongly enriched on the BACTRAP incubated for 6 days (wetland sample, day 0: 0.0 %; wetland sample, day 28: 0.1 %; BACTRAP, day 6: 16.8 %; BACTRAP, day 28: 2.6 %). Additionally, *Acidobacteria*, *Chloroflexi*, *Fibrobacteres*, *Nitrospirae*, *Planctomycetes*, *Spirochaetes* and *Tenericutes* were represented with low abundance.

For a more detailed analysis, mainly the genus level was considered. Among all 61 assigned genera in the day zero rhizosphere sample, only one was detected with an

abundance of more than 5 % (*Amaricoccus* spp., 7.0 %). 28 days later again only one of the 104 identified genera had an elevated abundance (*Steroidobacter* spp. 8.1 %). Neither genus has previously been described as containing members associated with benzene degradation.

Bacterial phyla and their relative abundance significantly differed at the genus level in the representative wetland rhizosphere samples (sample group 1 = day 0 and 28) and the BACTRAPs incubated for 6 (= sample group 2), and 28 days (= sample group 3), respectively, as statistically shown with the non-parametric test for dissimilarities in the community structure of various groups of samples [Anderson (2001), R software, VEGAN package, ADONIS function].

The sequences of the communities on the BACTRAPs were mostly assigned to members from three genera, namely *Dechloromonas* (mean BACTRAP ^{13}C and ^{12}C, day 6: 8.3 % / day 28: 15.0 %), *Geobacter* (30.0 % / 17.0 %) and *Zoogloea* (0.5 % / 14.1 %), all of which contain species that have been associated with benzene metabolism: Members of the genus *Dechloromonas*, e.g. *D. aromatica* strain RCB (Coates et al., 2001), have been isolated and described as facultative anaerobes that grow and oxidise benzene coupled to the reduction of either oxygen, chlorate, or nitrate (Coates et al., 2001; Chakraborty and Coates, 2005; Chakraborty et al., 2005). Selective enrichment of microorganisms of the genus *Geobacter* in benzene-degrading sediments/aquifers suggested that they may play an important role in anaerobic benzene degradation (Anderson et al., 1998; Rooney-Varga et al., 1999; Yun et al., 2011). The transformation was reported for an axenic culture (Zhang et al., 2012) and some evidence was also obtained in microcosm studies (Botton et al., 2007; Holmes et al., 2007). A link between *Zoogloea* spp. and benzene degradation has been rarely reported (Takahata et al., 2006; Weelink et al., 2007), but a recent aerobic microcosm study coupled to SIP indicated that it could be an important benzene degrader in some microbial communities (Jechalke et al., 2013).

Many of the other (more than 100) taxa of the BACTRAP communities were present with similar, low abundance in the wetland samples. To gain a more comprehensive overview about the remaining, potentially benzene-degrading community, the list of affiliated taxa was screened in the literature with respect to benzene degradation. In comparable microcosm studies partly coupled to the SIP approach, or enrichment and cultivation experiments, some other taxa repeatedly emerged in association with possible benzene degradation: *Acinetobacter* spp., cultivated on phenol from an industrial aerobic wastewater bioreactor, also used benzene as substrate (*Acinetobacter johnsonii*) (Bramucci and Nagarajan, 2000); *Arthrobacter* spp. was enriched, in a microcosm study with groundwater samples (Aburto and Peimbert, 2011) and several species of this genus showed benzene degradation under aerobic conditions (Alvarez and Vogel, 1991; Greene

et al., 2000; Ma et al., 2013); three genera of the *Comamonadaceae* were found on the BACTRAPs and have been described previously as being capable of, or at least being associated with, benzene degradation; *Acidovorax* spp. occurred in aerobic benzene-degrading microcosms with benzene-contaminated groundwater samples (Fahy et al., 2006; Aburto et al., 2009; Aburto and Peimbert, 2011); *Rhodoferax* spp. are frequent members of communities of water-treatment plants and have been reported to be highly abundant in aerobic benzene-degrading consortia (Fahy et al., 2006; Aburto and Peimbert, 2011) and were detected in the heavy fraction of an aerobic SIP experiment (Jechalke et al., 2013). Some sequences affiliated with the genus *Hydrogenophaga* as closest related taxon, increased proportionally in the heavy RNA fractions of the BACTRAP. This genus was found previously in aerobic microcosms and cultivations from benzene-contaminated groundwater samples (Fahy et al., 2006; Fahy et al., 2008; Aburto et al., 2009; Aburto and Peimbert, 2011) via DNA-SIP, RNA-SIP and Protein-SIP (Aburto and Ball, 2009; Jechalke et al., 2013). In contrast, the abundance of the genus *Pseudomonas*, which dominated in aerobic microcosm studies and isolation experiments (Fahy et al., 2006; Aburto and Ball, 2009; Aburto and Peimbert, 2011) and was found via DNA-SIP with sediment samples from a coal-tar-waste contaminated site (Liou et al., 2008), slightly decreased on the BACTRAPs from about 2 % to less than 1 %. *Azoarcus* spp. were detected via RNA-SIP in anaerobic microcosms with benzene and other contaminated groundwater under denitrifying conditions (Kasai et al., 2006; Liou et al., 2008). As indicated in further SIP studies, members of the family of *Desulfobacteraceae* seem to play a key role in anaerobic benzene degradation under sulphate-reducing conditions (Oka et al., 2008) and the family of *Desulfobulbaceae* was found during anaerobic transformation under iron-reducing conditions (Kunapuli et al., 2007). All of them occurred with an abundance of less than 1 % on the *in situ* microcosms and were not significantly enriched for ^{13}C.

Among sequences that were unassigned at the genus level (in the following marked with*), similar distributions as for the whole community were detected. However relative abundances of *Proteobacteria** strongly decreased when *Proteobacteria* with deeper phylogenetic assignment, in particular *Dechloromonas* spp., *Geobacter* spp. and *Zoogloea* spp. increased in their abundance. At deeper phylogenetic levels of the phylum *Proteobacteria**, the class of *Betaproteobacteria** accounted for up to half of all *Proteobacteria** and were mainly comprised of the orders *Burkholderiales** and *Rhodocyclales**. The former includes the family of *Comamonadaceae* and the latter the *Dechloromonas* spp. and *Zoogloea* spp. The class of *Deltaproteobacteria**, which include the order *Desulfuromonadales* with *Dechloromonas* spp. were poorly represented.

In summary, in the wetland community there were only a few, low-abundance sequences from organisms connected to benzene degradation. In contrast, the communities on the BACTRAPs showed a strong, enrichment of three microbial genera, namely *Dechloromonas, Geobacter* and *Zoogloea*, which have been described in the literature as closely related to benzene degradation.

Identification of benzene degraders based on RNA-SIP

For assessing whether the detected taxa play a key role in benzene turnover in the constructed wetland system, a detailed analysis of the fractionated RNA of the BACTRAP communities of day 28 was carried out.

In this experiment with a relatively short exposure time of the BACTRAPs in the constructed wetland, the observed amount of ^{13}C incorporation into biomass was low and there was no exclusive enrichment of RNA from degraders in heavy RNA fractions. A slight shift (1.77-1.80 g mL^{-1}) between RNA from ^{12}C-benzene-loaded and ^{13}C-benzene-loaded BACTRAP was detected, but no highly labelled RNA (expected at 1.82 g mL^{-1}) was found. In general, a longer exposure time can result in incorporation of more label but it can also mask the insight into the contaminant-degrading community due to cross-feeding (Neufeld et al., 2007a), i.e. microbes that are not degrading benzene may nevertheless become labelled with ^{13}C due to their transformation of metabolites and decaying biomass from benzene degraders. Here, the comparably short exposure time of 28 days was chosen based on previous data (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006; Kästner and Richnow, 2010). In those investigations a substantial microbial incorporation of label had occurred after an exposure time of 6 weeks in the inflow aquifer system. Furthermore, benzene was effectively eliminated in the constructed wetland (Seeger et al. 2011, 2013), suggesting the presence of a highly active microbial community. In the present experiment the incubation time was sufficient for incorporation of ^{13}C into biomass (although low) and not too long to over-represent microbial food webs. The low incorporation may have been due to low benzene turnover, or to the use of unlabelled benzene or some other DOC available in the wetland (Table 2-1), hence resulting in a relatively weak ^{13}C-incorporation into RNA. From an experiment using *in situ* microcosms combined to PLFA-SIP for assessment of the biodegradation potential for benzene in a contaminated anaerobic aquifer, Geyer et al. (2005) calculated that for a specific fatty acid marker for gram-negative bacteria approximately only 17 % of the carbon was derived from labelled benzene. The rest was derived from other sources, namely unlabelled benzene and other organic substrates from the aquifer or CO_2. The

notion that unlabelled organic compounds including benzene from the surrounding pore water may have contributed to microbial growth on the BACTRAP was supported by the similarity of blank- to ^{12}C- and ^{13}C- BACTRAP communities (↗ Figure 2-4). Although no substrate was added experimentally to the blank, the relative profiles of the active communities on the three BACTRAP compartments did not differ significantly [as deciphered by ADONIS, sample group 1 = RNA-extract BL-BACTRAP (6d), sample group 2 = RNA-extract ^{12}C-BACTRAP (6d), sample group 3 = RNA-extract ^{13}C-BACTRAP (6d); equivalent for 28d incubation - sample group 1 = RNA-fractions BL-BACTRAP (28d), sample group 2 = RNA-fractions ^{12}C-BACTRAP (28d), sample group 3 = RNA-fractions ^{13}C-BACTRAP (6d)] or follow any meaningful regularities. Unfortunately, due to generally problematic low yields in RNA-extraction from BACTRAPs all carrier material was used in the RNA extraction and no verification of this proposition by determination of residual ^{13}C-benzene could be carried out.

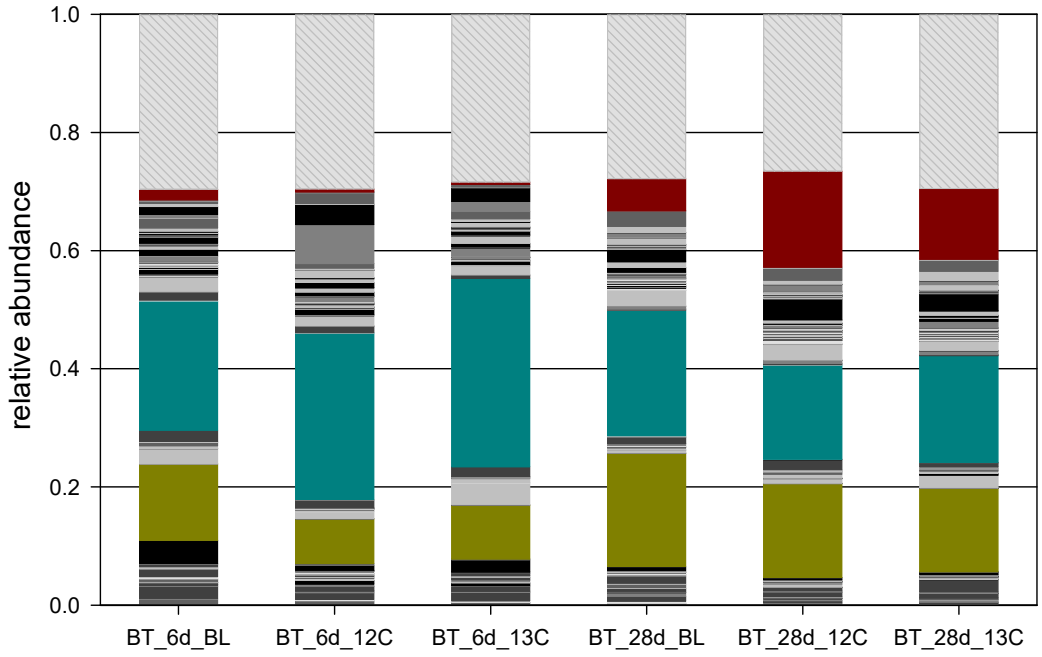


Figure 2-4: ***Visual comparison of relative abundances of microbial taxa at the genus level on the three BACTRAP compartments after 6 and 28 days of incubation. Highlighted are* Dechloromonas *(■), Geobacter (■), Zoogloea (■) and the sum of all sequences unassigned at the genus level (■).***

Ultimately, due to the lack of highly labelled fractions, only a tentative identification of benzene-degrading microbes that were active on the BACTRAPs, and which may be active in the constructed wetland, was possible for the majority of the assigned

community. Due to very low microbial abundance and their scope of variance, an enrichment on benzene-loaded compartments and therefore active participation in benzene transformation by all the low-abundance taxonomic groups could not be statistically reliably assumed. As stated before, the genera that were clearly enriched on the BACTRAPs were the potential benzene-degrading *Dechloromonas*, *Geobacter* and *Zoogloea*. To confirm the assumption of their active benzene metabolism, further analysis was done of the heavier RNA fractions of the ^{13}C-exposed BACTRAP incubated for 28 days.

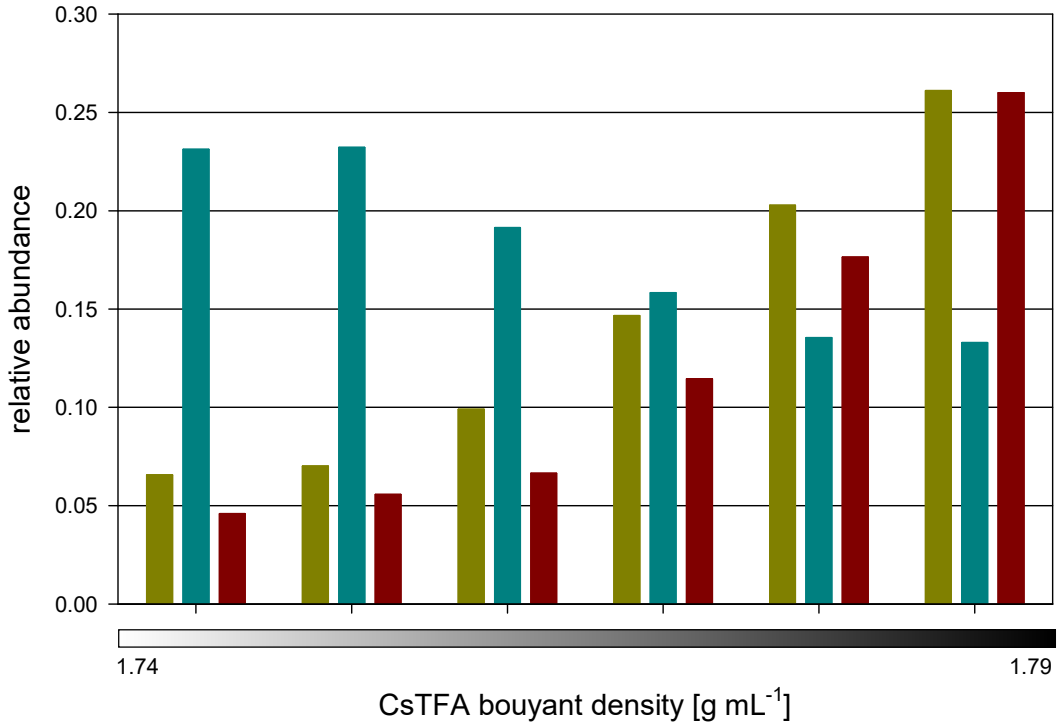


Figure 2-5: ***Relative abundance of the potential benzene-degrading genera in the different density fractions of the ^{13}C-BACTRAP compartment after 28 days of incubation. Presented are* Dechloromonas *(■),* Geobacter *(■),* Zoogloea *(■).***

Figure 2-5 shows an increasing abundance for *Dechloromonas* spp. and *Zoogleoa* spp. when fraction density was increasing. In contrast, the relative abundance of *Geobacter* decreased in the heavier fractions. Additionally, as shown in Figure 2-3 and Figure 2-4, the overall abundance of *Geobacter* sequences declined on the BACTRAP compartments from 6 to 28 days incubation. These correlations imply that for the first two taxa 16S rRNA were partly labelled with the ^{13}C isotope, and that the respective microbes play a key role

in the degradation of benzene on the BACTRAPs. In contrast, *Geobacter* might not be involved in benzene degradation.

2.3.3 Analysis via quantitative real-time PCR

Quantification of *Geobacteraceae* in the fractionated RNA

Since Illumina sequencing might not be 100 % reliable for the quantification of taxa due to biases associated with sample preparation (including one PCR and two following nested PCR reactions), the abundance of *Geobacteraceae* was quantified in fractionated RNA from exposure day 28 by following the published 16S rRNA qPCR assay of Cummings et al. (2003).

Compared to total bacterial 16S rRNA, the copy number of *Geobacteraceae* 16S rRNA was smaller by one order of magnitude (↗ Figure 2-6). When overlaying both abundance profiles, they were congruent for the ^{12}C compartment. In the ^{13}C-fractionionated 16S rRNA samples, the amount of *Geobacteraceae* decreased more steeply in the heavy fractions compared to the amount of 16S rRNA of all bacteria.

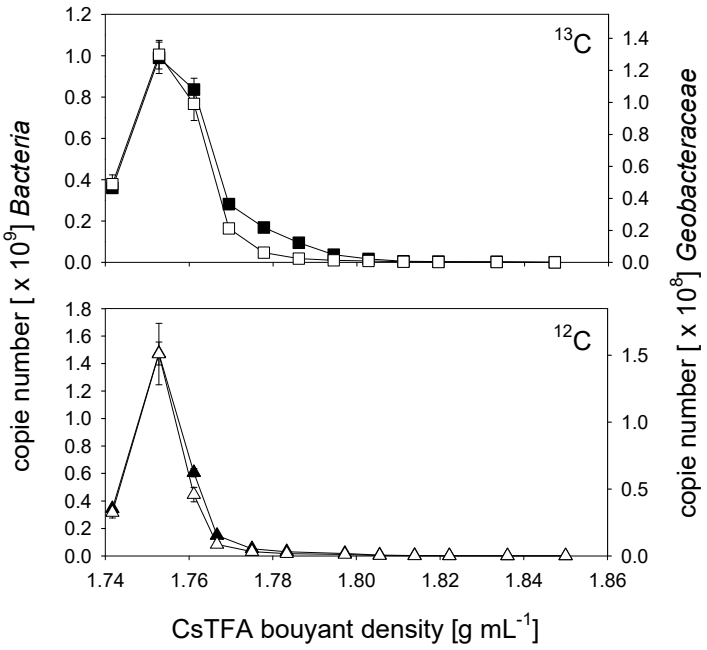


Figure 2-6: ***Comparison of 16S rRNA copy numbers of all* Bacteria *(^{13}C: ■, ^{12}C: ▲) and* Geobacteraceae *(^{13}C: □, ^{12}C: △) as extracted, fractionated and qPCR-quantified from the BACTRAP incubated for 28 days. Error bars for the values from denser fractions are hidden by the symbols.***

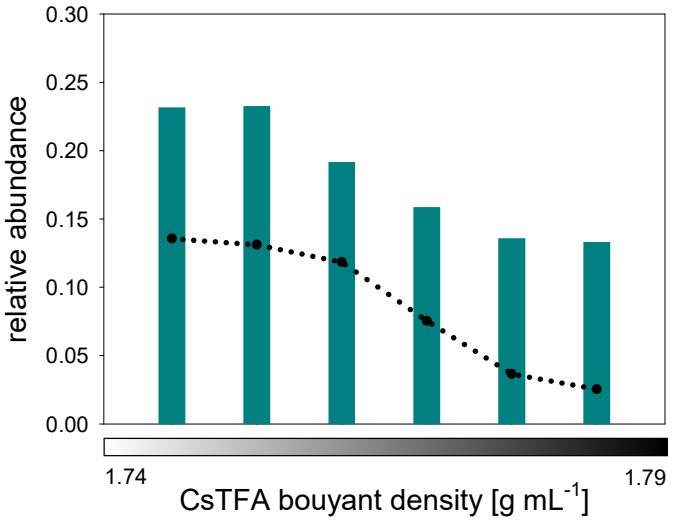


Figure 2-7: ***Comparison of sequencing and qPCR results. The courses of the relative abundance of* Geobacter *spp. in sequencing results (▬) and of* Geobacteraceae*, determined via qPCR analysis (···●···) in the 16S rRNA density profile of the ^{13}C-BACTRAP are demonstrated for the relevant fractions.***

Compared to the sequencing results, the qPCR assay recorded less *Geobacteraceae* than sequences that were assigned to *Geobacter* (↗ Figure 2-7). This might have been caused by the high specificity of the primers used, so that some genera and species were not taken into account. Nevertheless, a good correlation of qPCR quantification and abundance determination via Illumina sequencing was observed.

Consequently, in contrast to members of the genera of *Dechloromonas* and *Zoogloea*, *Geobacter* apparently did not incorporate the label. In spite of the fact that they were strongly enriched on the BACTRAPs, these microbes did not seem to colonize the *in situ* microcosm based on the degradation of benzene. *Geobacter* spp. are known to be able to grow on graphite in fuel cells (Williams et al., 2010; Lovley and Nevin, 2011). The granulated activated carbon used in this experiment seemed to be an excellent carrier for the colonisation and enrichment of these species.

As there are no 16S rRNA-based qPCR assays available for *Dechloromonas* and *Zoogloea*, the detected correlations of increasing density and increasing abundance could not be validated.

Analysis of the *bamA* gene in the fractionated RNA

Presumably, anaerobic degradation of benzene occurs via the benzyl-CoA pathway (Abu Laban et al., 2010; Luo et al., 2014) Oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolase (6-OCH-CoA hydrolase) is an enzyme of the benzoyl-CoA pathway (Fuchs et al., 2011). Its encoding *bamA* gene has been used as a molecular marker to query contaminated sites for the presence of anaerobic bacteria capable of degrading toluene (Kuntze et al., 2008). To support further the interpretation concerning *Geobacter* on the BACTRAP, the *bamA* gene was assayed by reverse-transcription qPCR (RT-qPCR) in RNA fractions and by sequencing RT-qPCR products. The used primers were valid for *Geobacter* spp. (Kuntze et al., 2011; Staats et al., 2011).

No evidence for the presence of *Geobacter*-type *bamA* was obtained. All sequenced RT-qPCR products were derived from 16S rRNA genes. Via sequence analysis some complementarity of at least one primer (ASP1) to 16S and 23S rRNA of *Geobacter metallireducens* was found (↗ Figure 2-8). When taking the formation of wobble base pairs into account, sequences for 16S rRNA were even more complementary. There was probably unspecific binding of the *bamA* gene primer to the rRNA during cDNA synthesis. To date, this assay has not been published in conjunction with RNA. Nevertheless, the unspecific binding including wobble base pairing does suggest that the transcript number of the intended target gene, *bamA*, was very low, if at all present. Apparently, *Geobacter* spp. were degrading benzene only in very low amounts or even not at all. The electron donor used for growth is uncertain.

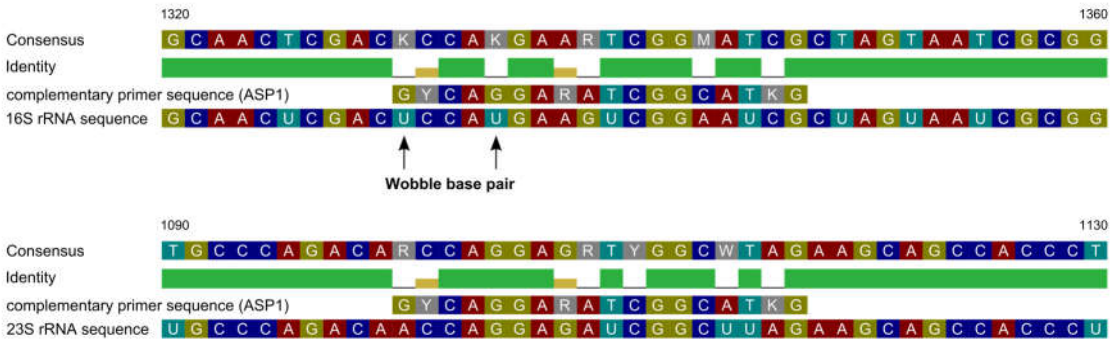


Figure 2-8: ***Alignment of the complementary primer sequence of ASP1 with 16S and 23S rRNA sequences, respectively, of* Geobacter metallireducens*.***

2.3.4 Conclusions

The general applicability of the BACTRAP approach for the *in situ* assessment of microbial BTEX degradation at contaminated field sites was already shown in previous studies (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006). There, contaminant degradation by the communities colonizing the BACTRAP was demonstrated via PLFA-SIP. Yet the PLFA analyses provided only limited taxonomic resolution, and further molecular biological fingerprinting tools such as SSCP were not connected to an analysis of ^{13}C incorporation into nucleic acids. Consequently, key microbial players could not be identified in those studies.

In the framework of this study, biodegradation of benzene on BACTRAPs placed within the constructed wetland systems was demonstrated by the incorporation of ^{13}C into rRNA. RNA-SIP coupled to a metagenomic approach provided a deep insight into the microbial community structures on the BACTRAP carrier material and the surrounding wetland. High-throughput sequencing, which had up until this study not been applied with *in situ* microcosms for benzene-degrading systems, turned out to be a very fast method for analysing whole microbial communities based on RNA sequences, without the necessity of cloning. It allowed for a more comprehensive taxonomic assignment and interpretation than conceivable with T-RFLP, DGGE or SSCP.

Analysis by Illumina sequencing showed that the representative wetland community profiles strongly differed from the BACTRAPs. In the constructed wetland few known benzene-degrading microbes were detected, and when present they were basically in very low abundance.

Furthermore, it indicated that *Dechloromonas* and *Zoogloea* played a key role in benzene metabolism on the BACTRAPs incubated in the wetland. These genera contain species that previously have been reported as being capable of or are associated with benzene degradation (Coates et al., 2001; Chakraborty and Coates, 2005; Weelink et al., 2007; Jechalke et al., 2013). The constructed wetland showed a declining redox potential in the flow direction from around -200 mV to around -340 mV and therefore decreasing level of already limited oxygen. As plant roots in CWs have been shown to deliver oxygen (Armstrong and Armstrong, 1990), and because *Zoogloea* spp. was published to degrade benzene under aerobic conditions (Jechalke et al., 2013), it is suggested that the BACTRAPs in the rhizosphere were exposed to some oxygen and *Dechloromonas* spp. here grew aerobically too.

Irrespective of the comprehensive analysis by Illumina sequencing, some conclusions concerning the BACTRAP approach for benzene degradation, the investigated system, and the identification of microbes active *in situ* can be drawn.

Success of SIP on BACTRAPs relies in part on the habitat parameters, especially on the concentrations and fluxes of unlabelled carbon sources. The presence of high concentrations of benzene with natural isotope abundances as well as other labile organic compounds at the site may have contributed to growth on the BACTRAP carrier material, and hence reduced incorporation of ^{13}C. Some microbes degrading BACTRAP-derived benzene as well as benzene and potentially other substrates from the surrounding environment might have been present but overlooked in the nucleic acid-based approach.

The BACTRAPs served as an *in situ* enrichment cultivation system. In principle, this approach is preferable over the frequently applied lab microcosms, since the incubation takes place directly in the habitat under complex environmental conditions. Nevertheless, it should be kept in mind that the surface properties of the activated carbon are different from the available surfaces in the constructed wetlands and might have led to preferential colonization on the BACTRAPs, as shown for *Geobacter*. While *Geobacter* readily colonized the BACTRAPs, no evidence was obtained for benzene degradation by this genus. However, in a recent study analyzing oxic groundwater contaminated with polycyclic aromatic hydrocarbon and using Biosep® beads (75 % activated carbon) as BACTRAP carrier material, microbes other than those identified in our experiment were enriched (Herbst et al., 2013). Together with our data, this finding can be interpreted as such that BACTRAPs are indeed colonized by specific microbes in different habitats. Thus, it remains uncertain whether the detected benzene degraders are indeed of high relevance in the habitat or whether those are the best suited ones among all *in situ* degraders to reach and colonize the BACTRAPs. *Dechloromonas* and *Zoogloea* were strongly enriched and significantly labelled with ^{13}C, but were of low abundance in the wetland. In order to avoid misinterpretation further characterisation and quantification of relevant catabolic genes in the wetland system will be required.

3 PART A – Stable Isotope Probing
A2: Evaluation of SIP with *in situ* microcosms for Bisphenol A

3.1 Introduction

Bisphenol A (BPA) is a widespread persistent environmental contaminant. It may negatively affect the endocrine system (reproductive system) and development of organisms (Kang and Kondo, 2002; Coors et al., 2003; vom Saal and Hughes, 2005). BPA is used in the synthesis of polycarbonate plastics, epoxy resins and flame retardants, and is one of the highest-volume and best-selling chemicals produced (total annual production of 3.8 tonnes in 2006, (Hansen, 2013)). Mass-use of BPA-containing products together with leaching of BPA from the materials (Albarran and Schuler, 2005) lead to its accumulation in surface waters and soils (Cousins et al., 2002; vom Saal and Hughes, 2005; Hansen, 2013). Effluents from wastewater treatment plants are one major source of environmental BPA (Crain et al., 2007; Corrales et al., 2015). The high potential risk for humans and the environment, even at low doses, of this and other so-called Endocrine Disrupting Chemical (EDC) have made it a public health concern and strongly increased the interest in its (bio)remediation over the last decade (vom Saal and Hughes, 2005; Hansen, 2013). Unfortunately, low pollutant concentrations are not likely to sustain growth of microorganisms (Collado et al., 2013).

Under aerobic conditions BPA can be degraded by various microorganisms including various *sphingomonads* (Lobos et al., 1992; Ronen and Abeliovich, 2000; Ike et al., 2002; Sasaki et al., 2005; Kang et al., 2006; Gabriel et al., 2007; Oshiman et al., 2007; Kolvenbach et al., 2011). Anaerobic microbial degradation is not known – all catabolic pathways identified to date start with the aerobic oxidation of BPA (Kolvenbach et al., 2007).

Due to increasing awareness of the presence and impact of such ‘micropollutants’ the focus of this research was with immobilization-based intensification and stabilisation of biodegradation and bioremediation processes. This was carried out within the framework of the MINOTAURUS EU-project. One specific goal was to assess to what extent bioremediation of EDCs like BPA can be applied directly within natural or engineered environments (e.g. contaminated groundwater, shallow aquifers and constructed wetlands). With the objective of specifically recording and characterising the microbial BPA transformation directly on-site, the aim of this study was to evaluate the applicability of the BACTRAP approach to BPA.

3.2 Material and Methods

3.2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany) unless otherwise specified, and were of analytical grade quality. [U-*ring*-^{13}C]-labelled bisphenol A (chemical purity 99 % (GC-MS)) was synthesised and provided by Prof. Rong Ji (State Key Laboratory of Pollution Control and Resource Reuse, School of Environment, Nanjing University, China). [U-*ring*-^{14}C]-labelled bisphenol A with a specific radioactivity of 7.4 GBq mmol^{-1} was obtained from Hartmann Analytic GmbH (Braunschweig, Germany) and dissolved in methanol at a concentration of 50 µM (≡ activity concentration of 370 Bq µL^{-1}).

3.2.2 Studies on sorption of bisphenol A to carrier material and its microbial transformation

For assessing the applicability of the BACTRAP-system to follow the biodegradation of bisphenol A (BPA) at contaminated field sites, sorption characteristics were determined and microbial transformation was investigated. Using [U-*ring*-^{14}C]-labelled BPA ('^{14}C-BPA'), allowed easy and rapid detection of BPA and its transformation products including biomass. Therefore adequate volumes of the samples containing radioactively labelled BPA were added to Ultima Gold™ scintillation cocktail (PerkinElmer, Waltham, MA, USA), mixed and measured using a Wallac 1414 WinSpectral™ Liquid Scintillation Counter (PerkinElmer).

Adsorption

Each adsorption experiment was carried out with 1 g of the carrier material, granulated activated carbon ('AC') (AUF 540, Adako GmbH, Düsseldorf, Germany), in duplicates. 50, 25 or 10 mg ^{12}C-BPA including ^{14}C-BPA with an activity of 12 500 Bq was dissolved in 180 ml of 75 mM NaOH in distilled water and added to the carrier material. Samples were shaken for one week at 200 rpm at room temperature and afterwards filtered. The amount of adsorbed ^{14}C-BPA was determined indirectly by measuring the non-adsorbed part in the filtrate by liquid scintillation.

Desorption

Desorption kinetics of BPA, loaded to the carrier material (as described above), was investigated by shaking at 400 rpm in 25 ml of distilled water that was renewed after equilibration every 84 hours. The amount of desorbed BPA was determined by measuring the ^{14}C-BPA in the removed water phase by liquid scintillation.

Transformation of BPA

The transformation of BPA was evaluated in a pure culture of the extensively studied *Sphingomonas* sp. strain TTNP3 and with samples of a BPA transforming MBR-Reactor. *Sphingomonas* sp. strain TTNP3 was obtained from Prof. Philippe Corvini (School of Life Sciences, Institute for Ecopreneurship, University of Applied Sciences Northwestern Switzerland) and grown on Standard I Medium as described previously (Corvini et al., 2004). Samples of the BPA transforming membrane bioreactor (MBR) also were obtained from Prof. Philippe Corvini. The permeate of the MBR was used as negative, non BPA-transforming control.

Transformation of BPA was determined in a medium of synthetic wastewater, modified from the OECD Guidelines for testing of chemicals (OECD, 2010), containing 1.9 mg L^{-1} peptone, 1.3 mg L^{-1} meat extract, 3.2 mg L^{-1} Glucose, 7 mg L^{-1} NaCl, 4 mg L^{-1} $CaCl_2$ x $2H_2O$, 1.96 mg L^{-1} $MgSO_4$ x $7H_2O$ and 2.8 mg L^{-1} K_2HPO_4. 100, 60 or 30 µg L^{-1} ^{12}C-BPA including analogue ^{14}C-BPA (500 Bq) dissolved in acetone were submitted to serum bottles. The solvent was allowed to evaporate completely. 9 mL of the growth medium and 1 ml of the reactor sample, the culture of *Sphingomonas* sp. strain TTNP3 (10^9 cells) or the permeate were added. Each bottle was provided with a tube containing 0.75 ml of 1 M NaOH to collect the CO_2 produced. The bottles were closed and incubated for 15 days, while carefully shaken from time to time. Afterwards the bottles were opened and the NaOH was analysed by liquid scintillation. For obtaining more information about the fate of the BPA in the MBR-sample and the culture of *Sphingomonas* sp. strain TTNP3, each culture was centrifuged in order to separate the aqueous medium from the biomass. To dissolve non-incorporated BPA, the biomass was extracted with ethyl acetate and each fraction was measured by liquid scintillation.

3.2.3 Field site

First tests of BPA-loaded BACTRAPs were carried out at a Shallow Aquifer Rhizodegradation pilot plant at the Laboratory of Biochemical Engineering and Environmental Biotechnology, Department of Environmental Engineering at the Technical University of Crete in Chania (TUC). The system of 1 m^3 total volume (Figure 3-1) was planted with two *Juncus acutus* rushes and consisted of a rhizodegradation zone, containing 80 % soil, from which 30 % was sand and one layer of 110 L small-sized gravel at the bottom and one of 55 L medium-sized gravel above. The pilot plant was flooded in recirculating mode with wastewater, supplied from an inlet at the bottom. From a plastic tank of 60 L capacity, water was pumped (24 h d^{-1} with a flow rate of 2 L h^{-1}) from the lower zone to the upper surface. When spiked with 160 mg of BPA in the external tank (initial concentration of 2.667 µg L^{-1} (ppb) groundwater) after less than 20 days, no BPA could be detected anymore via HPLC (personal correspondence with Prof. Nicolas Kalogerakis (Laboratory of Biochemical Engineering and Environmental Biotechnology, Department of Environmental Engineering at Technical University of Crete). Endophytic isolates that degraded BPA in lab-scale experiments belonged to the genera *Sphingomonas* and *Cupriavidus* [Prof. Nicolas Kalogerakis, pers. comm., Laboratory of Biochemical Engineering and Environmental Biotechnology, Department of Environmental Engineering at the Technical University of Crete in Chania (TUC)].

3.2.4 Experimental design

To evaluate its applicability in the system described above, the BACTRAP technology was tested for BPA. Recently developed Direct-Push probes (Schurig et al., 2014) were applied for the exposition. The carrier material, AC, was prepared as described in PART A1. For loading, 33 mg per g_{AC} either ^{13}C-labelled or unlabelled BPA was dissolved in 1 mL acetone. In a crystallizing dish, AC was completely covered with acetone, dissolved BPA was added and the solvent was allowed to evaporate to dryness. The empty glass dish was rinsed with acetone to dissolve BPA again and this solution was added drop-wise back onto the granules (2 times). To remove residual acetone, BPA-loaded beads were evacuated at 50 mbar and held under these conditions for 1 h. AC for the ‘blank’-microcosm was treated similarly, but without any substrate. ‘Blank’-, ^{12}C- and ^{13}C-labelled carrier material (3 g each) was transferred in separate segments into the Direct-Push Probe, which was subsequently exposed in the root zone of the two rushes for 38 days.

After incubation, all samples were immediately frozen and stored at -80°C until analysis.

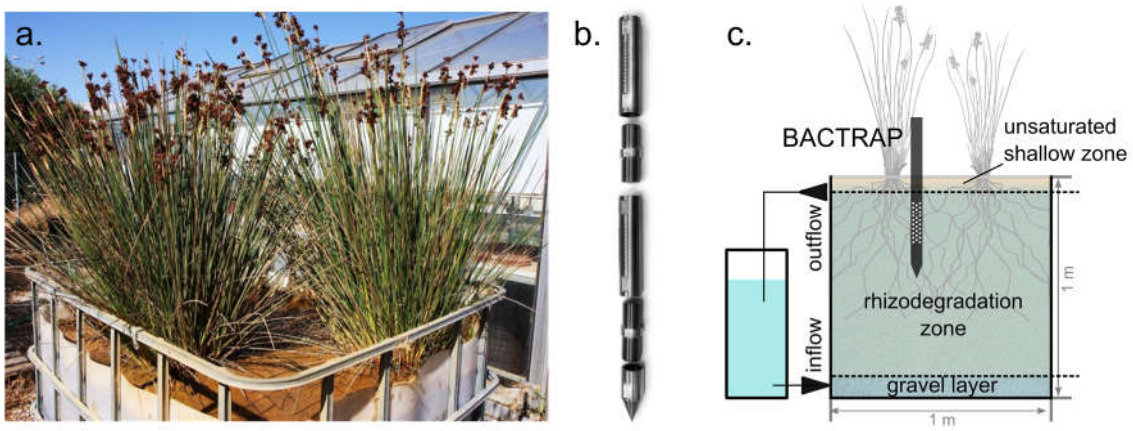


Figure 3-1: ***Experimental setup of the SIP-BACTRAP test in a Shallow Aquifer Rhizodegradation pilot-plant in Chania. a. Photography of the investigated system. b. Direct-push BACTRAP (threefold segmented for parallel exposition of blank, ^{12}C and ^{13}C) for insertion into overgrown rhizosphere. c. Schematic drawing of the rhizodegradation system and BACTRAP placement.***

3.2.5 Extraction of phospholipid fatty acids

Phospholipid fatty acids (PLFA), as biomarkers for metabolic activity, were extracted with a protocol modified from (Bligh and Dyer, 1959), as described in (Schurig et al., 2012). First, 1 g AC was extracted with 5 mL dichloromethane overnight at 30 °C on a horizontal shaker. The dichloromethane phase was evaporated under an N_2 stream and the remaining AC and sediments were again extracted with a mixture of 3 mL phosphate buffer (0.05 M K_2HPO_4 in nanopure water, pH 7.4), 10 mL methanol and 5 mL chloroform overnight at 30 °C. The extraction assay was continuously shaken and treated once with ultrasound for 6 min. For subsequent phase separation, 5 mL of nanopure water and 5 mL of chloroform were added, briefly vortexed and centrifuged for 20 min at 400 rcf and 4 °C. The hydrophobic fatty acids contained in the lower organic phase were united with the dichloromethane extract and dried by evaporating under a stream of N_2. The extract was dissolved in 500 µL chloroform and PLFA were separated via chromatography on preconditioned silica gel columns. Silica gel (Unisil, Clarkson Chromatography Products, South Williamsport, PA, USA) in glass columns was activated by adding 5 mL of 0.02 M ammonium acetate in methanol and subsequently conditioning with 5 mL of acetone and 5 mL of chloroform. The extract was loaded onto the columns and neutral lipids were eluted with 5 mL chloroform and glycol lipids with 5 mL acetone. PLFA were collected separately with 10 mL of methanol, and dried by evaporating under a stream of N_2. They were methylated by 1 h incubation at 37 °C with 2 mL of 0.2 M KOH in methanol and were esterified by subsequent addition of 200 µL of methanol-glacial acetic acid (9:1, v/v).

Fatty acid methyl esters (FAMEs) were extracted three times by partitioning with 3 mL hexane, whereby extraction and phase separation were supported by mixing and centrifugation at 400 rcf and 4 °C for 10 min.

The hexane phase was evaporated to dryness under a N_2 stream and FAMEs were dissolved in 100 µL of an internal standard (10 µg mL^{-1} of 21:0 FAME in hexane). PLFA were quantified and identified by means of gas chromatography-mass spectrometry (GC-MS; Agilent GC 7890A and MS 5975C, Agilent, Santa Clara, CA, USA) 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 x 0.25 mm x 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.

3.3 Results and Discussion

The BACTRAP-technology was evaluated for its applicability for tracking *in situ* BPA transformation. Tests were carried out to determine useable concentration ranges and bioavailability of the substrate during the exposition as well as incorporation rates of the carbon-label into the microbial biomass. Those tests were performed with ^{14}C-labelled BPA, which was chosen over ^{13}C-labelled BPA due to the ease of detection of ^{14}C-labelled transformation products and residual BPA without compromising sorption kinetics or transformation rates.

3.3.1 Adsorption and desorption of BPA on activated carbon

Growth of a number of labelled BPA-degraders to be evaluated on the BACTRAP requires that the ^{13}C-substrate is available at sufficiently high concentration over the whole exposition time. Consequently, a certain amount must be adsorbed on the carrier material prior to *in situ* exposure, which then desorbs to support growth of the attached microbes over a longer period.

Adsorption

Up to now *in situ* microcosm studies were carried out with volatile contaminants that were loaded on the carrier material via the gas phase (Bombach et al., 2010a). This is not feasible for BPA due to its high boiling point (Bp. 360°C at ambient pressure or 220°C at 5 mbar). As an alternative option, sorption via the aqueous liquid phase was tested (Figure 3-2). As expected, the adsorbed absolute amount of BPA increased with increasing BPA amount in the aqueous phase while the percentage of adsorbed BPA increased with lower starting concentrations. There seemed to be a decreasing capacity for loading a specific surface of activated carbon with BPA, determined by the volume of the water phase and the equilibrium of dissolved and adsorbed BPA.
Although the overall amount of BPA per g carrier material provided in this alternative loading procedure was approximately three times lower than that used in previous experiments with other organic compounds (e.g. about 100 mg benzene in Geyer et al. (2005)), it should be high enough to perform an *in situ* microcosm experiment.

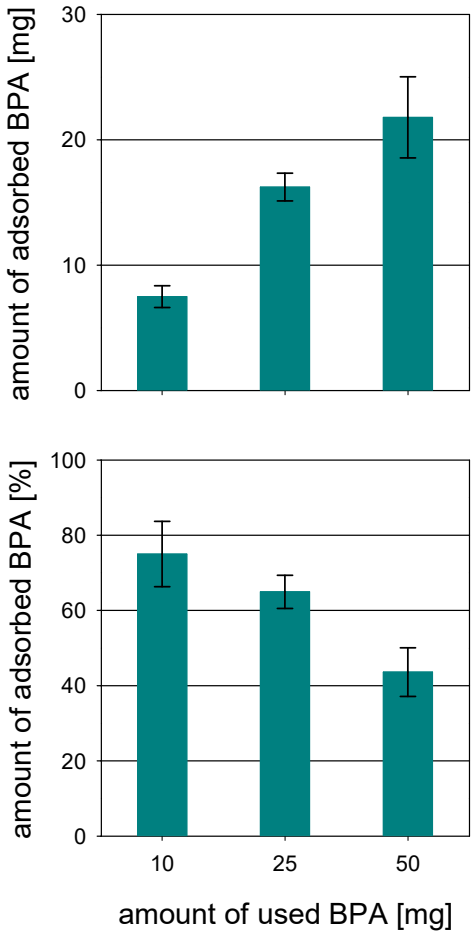


Figure 3-2: Adsorption of BPA on granulated activated carbon out of the aqueous phase.

Desorption

A desorption kinetic analysis of BPA loaded onto the carrier material (as described above) was investigated by shaking the material in distilled water, which was renewed after equilibration every 3.5 days.

Figure 3-3 shows the desorption kinetics of BPA from activated carbon. The actual desorption, exemplarily illustrated for one example, was many times smaller than initially supposed, because each volume of removed water contained powder of activated carbon. After filtration the measured radioactivity was significantly smaller. BPA strongly adheres to the carrier.

According to these results desorption of BPA might be at least as slow as necessary to ensure the availability of BPA over the *in situ* incubation period during an *in situ* microcosm experiment. Whether enough BPA desorbed during the period of incubation will be considered in sections 3.3.2 to 3.3.4.

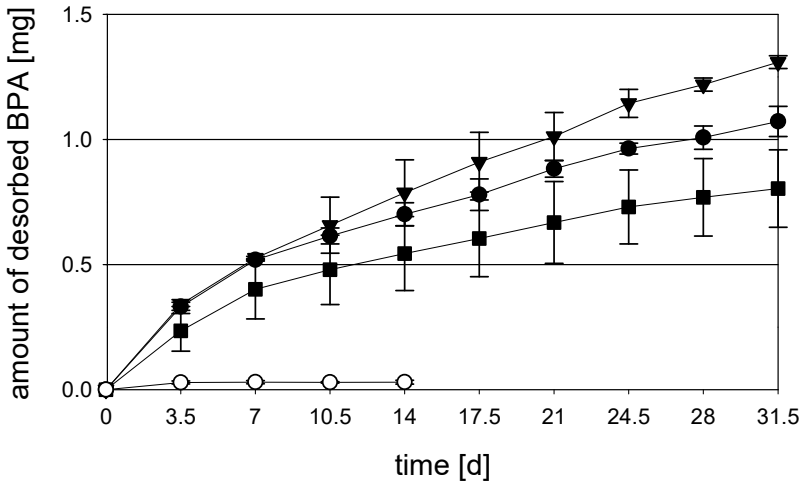


Figure 3-3: ***Desorption of BPA from granulated activated carbon into the aqueous phase. Shown are the desorption kinetics of the carrier material, loaded with 50 mg (unfiltered supernatant ●; filtered ○), 25 mg (unfiltered ▼) and 10 mg (unfiltered ■) substrate.***

3.3.2 Mineralisation of BPA by *Sphingomonas* sp. strain TTNP3 and in the MBR-Reactor

Information about the rate of incorporation of carbon from the offered substrate is critical for evaluating whether the incorporation of a ^{13}C-label is detectable in biomarkers. The success of an *in situ* Stable Isotope Probing experiment using the BACTRAP-technology requires sufficiently high turnover of BPA as well as to a certain extent the incorporation of the carbon-label into the microbial biomass.

A *Sphingomonas* sp. strain TTNP3 was previously applied for treatment of EDC-contaminated wastewater in an MBR system (Cirja et al., 2009) and is relevant to bio-augmentation processes for EDC-contaminated water bodies within the MINOTAURUS project. Here, a pure culture of *Sphingomonas* sp. TTNP3 together with MBR reactor samples were investigated for their capability to degrade BPA adsorbed to activated carbon. Reactor permeate was used as negative control. In the future, other species of the genera *Sphingomonas* and *Cupriavidus* might be of interest in this process, because they were identified in a relevant rhizodegradation pilot plant at the Technical University of Crete in Chania (N. Kalogerakis, pers. comm.) and in a comparable study at the UFZ, respectively (Fischer et al., 2010).

As shown in Figure 3-4 the turnover of BPA to CO_2 was the highest in the MBR-sample with around 40 to 60 %, followed by the *Sphingomonas* sp. TTNP3 with around 25 to 35 %. As expected, there was essentially no CO_2 production from the permeate.

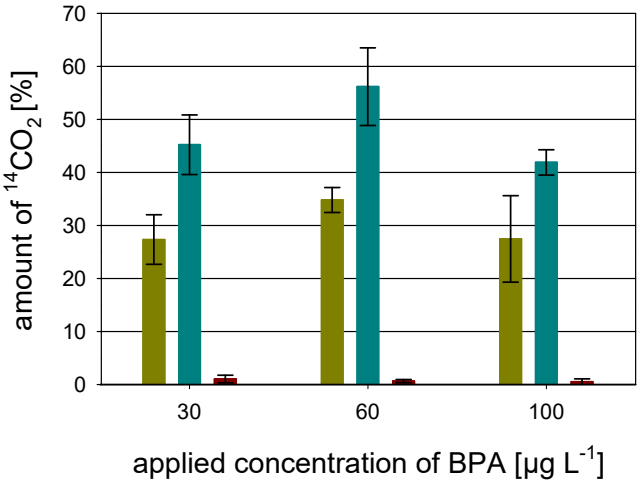


Figure 3-4: ***Production of CO_2 during mineralisation of BPA. Shown are the proportions of the $^{14}CO_2$ radiolabel recovered using three different initial BPA concentrations after 15 days of incubation with a pure culture of* Sphingomonas *sp. strain TTNP3 (■), with samples of an BPA-transforming MBR reactor (■) and with the permeate of the MBR reactor (■).***

The fate of ^{14}C-BPA was determined further in MBR-samples and a culture of *Sphingomonas* TTNP3 (Figure 3-5). Likewise the $^{14}CO_2$ (captured in NaOH) analysis, the relative amount of ^{14}C-atoms in the biomass was higher in the MBR-sample (20 to 30 %) than in the culture of *Sphingomonas* TTNP3 (10 to 15 %). This was apparently due to the lower degradation rate. An extrapolation to total turnover of available BPA would probably lead to comparable labelling of biomass. In that case the incorporation of the BPA into the biomass could be estimated to be about 30 % of the total BPA transformed.

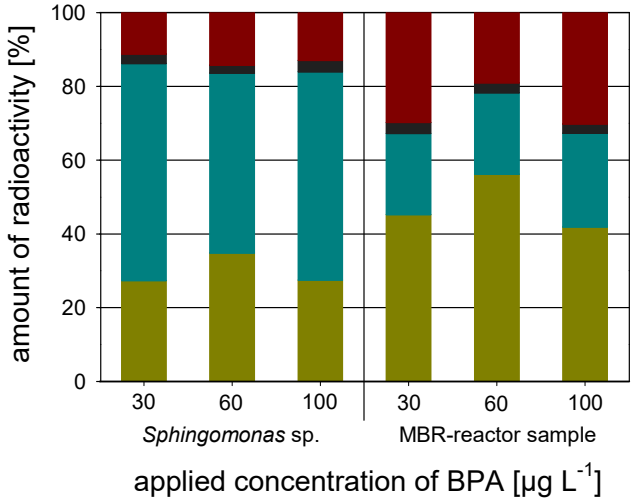


Figure 3-5: ***Fate of BPA in the mineralisation experiment. The distribution of ^{14}C-label in the NaOH reservoir (■), aqueous supernatant of the cultures (■), the biomass (■) and the ethyl acetate washing extract (■) for* Sphingomonas *sp. strain TTNP3 and the sample of the MBR reactor was measured.***

Based on the results regarding adsorption and desorption of BPA to the activated carbon and the determined mineralization of BPA, it seemed reasonable to test whether ^{13}C incorporation from a BPA-loaded BACTRAP is adequate for *in situ* recording and characterising the level of microbial BPA transformation at a real field site.

3.3.3 Experiments on evaluating BACTRAPs for BPA transformation in Shallow Aquifer Rhizodegradation pilot plant at Technical University of Crete

The constructed wetland pilot plant at our MINOTAURUS partner TUC laboratory on Crete was used to test a novel direct push probe (Schurig et al., 2014). The probes were exposed in the soil filter system for 38 days. Preliminary analyses of Phospholipid Fatty Acid (PFLA) extractions, according to the protocol of (Schurig et al., 2014), from the BACTRAPs showed neither unlabelled FAMEs nor accordingly any incorporation of ^{13}C into the biomass. Colonization of BPA degraders on the carrier material or bioavailability of BPA might have been too low for detection of any GC/MS signal.

3.3.4 Conclusions

The aim of this study was to evaluate the applicability of the SIP/BACTRAP approach to BPA, which was never before applied in this context. The data on adsorption and desorption, and mineralisation currently suggest that BPA transformation generally may be assessed via the BACTRAP technology in a variety of habitats such as constructed wetlands and MBRs. However, in a field test of the BACTRAP system in Shallow Aquifer Rhizodegradation pilot plant, no microbial biomarker and ^{13}C-label incorporation could be detected. High sorption of BPA to the carrier material may have led to a low availability of the compound to the biofilm.

Furthermore, up to now the environmental contaminant BPA occurs in low doses, as a ‘micropollutant’. Accordingly, the pollutant was spiked to the pilot plant in very low concentrations (ppb). As incidental carbon source, the microbial community would only be marginally geared for growth on BPA, and relevant degraders might be of low abundance. This, together with the apparently intrinsic low microbial degradation rate, and the scarce availability of BPA would require significantly longer exposure times together with increased amounts of labelled BPA. Ideally, the length of those exposure times would be predicted based on BPA mass balance data from the contaminated site.

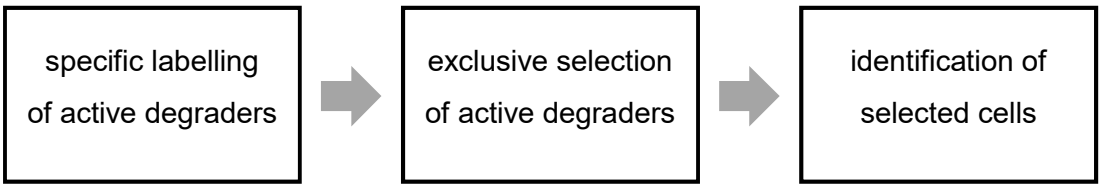
4 PART B: Radioisotope Probing

4.1 Introduction

The identification of specific prokaryotes as biological drivers of organic compound turnover in heterogeneous natural systems directly linked to their *in situ* activity continues to remain one of the greatest challenges in microbial ecology. Procedures and approaches for identification of the degraders, as presented in Chapter 1, depend strongly on cultivation or data-based information or educated guesses on phylogeny or catabolic features of microbes present in the habitat of interest. Nucleic acid SIP, as employed in PART A of this thesis, is a routinely applied methodology that is not reliant on previous phylogenetic knowledge. Due to its requirement for substantial microbial incorporation of label [e.g. >15 at. % for RNA, (Whiteley et al., 2007)] in order to allow for experimental separation of nucleic acids into light and heavy fractions, it is applicable only for detection of microbes that are sufficiently abundant at the habitat and thrive mostly on the labelled compound in the experimental set up. Additionally, the cost-effective realisation of the procedure is restricted to microcosm studies, at best incubated *in situ* (BACTRAPs), which cannot be equated with direct *in situ* activity assessment. A novel and efficient strategy for identification of degraders active in the field, without *a priori* phylogenetic information, is, therefore, highly desirable.

This chapter deals with the development of a novel screening methodology for microbes with *in situ* activity that: (i) is sensitive enough to permit conclusive *in situ* investigations; (ii) is independent of previous phylogenetic knowledge; and (iii) aims at addressing the actual *in situ* state in the field in a more accurate way than currently possible by other methods.

Based on the present approach for activity assessment, the concept of isotope-labelling of active microorganisms is depicted in the following, simplified scheme:



Those microbes that assimilate at least a fraction of the compound of interest become specifically labelled. Based on the detection of the label, whole degrader cells are then individually selected and identified.

Stable isotopes are due to the required relative amount of label (i.e. high natural background and detection limits) not economically feasible for *in situ* analysis, and analysis of many intact cells is limited by the technically demanding measurement of specifically isolated biomarkers. In comparison, radioisotopes have several advantages: their low natural abundance (e.g. $^{13}C \approx 1.11\ \%$; $^{14}C < 10^{-12}$) and the resulting low background together with considerably more sensitive detection methods for radioactivity significantly enhance sensitivity compared to stable isotope analysis. Thus, labelling time can be strongly decreased; during short incubation times the habitat conditions regarding community structure and activity of key players may be maintained in taken samples. Assays close to authentic *in situ* studies may become possible, without the need of adding radioisotopes to nature.

Once the radioisotopes are implemented to the respective system, an exclusive selection of labelled and therefore active degrader cells requires two prerequisites: (i) Based on the incorporated radio-label a convincing distinguishing criterion is needed; (ii) single cells have to be available in a medium or carrier that allows spatial isolation and separation via use of a distinctive feature.

Microautoradiography [developed in the 1960s by Brock and Brock (1966)] is a powerful approach to follow the fate of radioactively labelled substrates into cells under *in situ* conditions by microscopic visualisation of the active community members and their metabolic activity. It targets whole cells instead of working at the subcellular level such as in the analysis of biomolecules pooled from a community (e.g. SIP). Thereby, identification of microbes is not limited to specific biomarkers and remains independent of where ^{14}C is incorporated into the cell. In the past, MAR was successfully applied in a number of studies in microbial ecology (Hoppe, 1976; Meyer-Reil, 1978; Tabor and Neihof, 1984;

Andreasen and Nielsen, 1997; Karner and Fuhrman, 1997; Gray et al., 1999), targeting the detection and enumeration of microbes capable of assimilating specific organic or inorganic compounds.

The MAR approach starts with the assimilation of a radiolabelled substrate into cells. In microbial ecology typically the soft beta emitters ^{3}H, ^{14}C and ^{33}P, and sometimes the strong beta emitter ^{32}P, are used as radiotracers (Nielsen et al., 2003), which allow high resolutions of only a few µm. The labelling procedure includes simple incubation with the radioactive substrate and in general is optimised for the particular substrate and system. One important aspect is that the radio-labelled substrate needs to be washed out from cells and the entire assay if it is not turned over by metabolism.

During the subsequent autoradiographic procedure, [comprehensively described in Rogers (1979)] the sample, normally fixed to light microscope slides, is coated with a special autoradiographic emulsion. This contains photosensitive crystals of silver halogens embedded and stabilized in a gelatine matrix. During a reasonable exposition period, few beta decays, corresponding to limited incorporated radioisotopes, lead to the formation of elementary silver seeds surrounding labelled cells (= 'latent image'). They are enlarged to visible silver grains based on photographic processes. This visualization of the radioactive label enables easy discernibility of degraders and non-degraders.

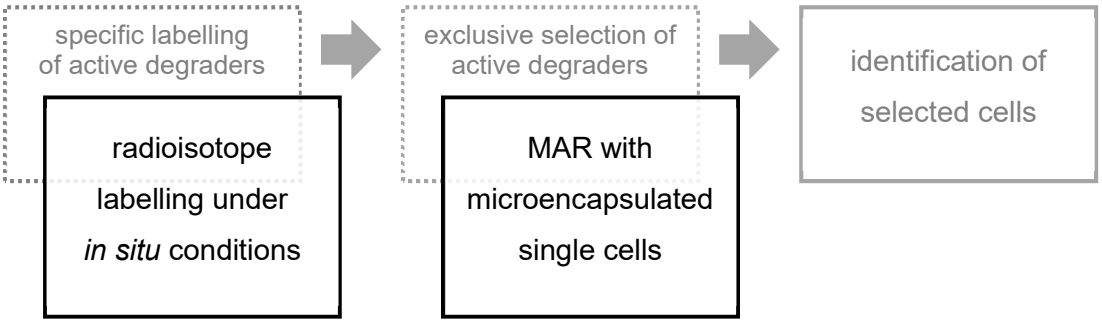
MAR addresses the desired analysis of single cells, but if applied as an individual method assesses only metabolic activity. A meaningful taxonomic identification is only possible for cells with conspicuous morphology. In combination with fluorescent *in situ* hybridization (FISH) (Lee et al., 1999), the range of possibilities is significantly extended, but depends greatly on prior nucleic acid-based information. The need for specific FISH probes limits the identification feasibility to only a few taxa in an active community (Amann et al., 1996). Moreover, the conventional separation and immobilisation of cells on glass slides restricts further analysis of single and active cells apart from FISH. Conceivable selection with a micromanipulator or laser dissector only works for well-separated cells. Either approach requires a high percentage of active degraders in the sample. Otherwise too many microscope slides would have to be prepared and screened. For identification completely independent of any pre-information, single cells are needed in a medium where they can be analysed via MAR and sorted based on signals from MAR.

Microencapsulation of diverse biological material inside of a matrix is frequently used in biomedical and biotechnological applications. The entrapment of whole cells in semipermeable structures allows immobilisation as well as protection of microorganisms from any kind of system-related stress, while using as therapeutic and pharmaceutical resource (Keweloh et al., 1989; Murua et al., 2008) or as technological biocatalyst

(Smidsrod and Skjakbrk, 1990). At the same time the protective barrier does not hinder the transfer of nutrients, oxygen and secreted products and metabolites.

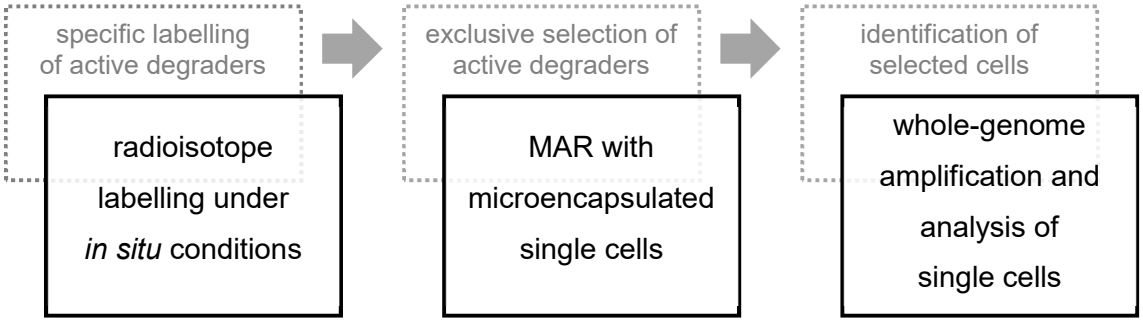
Suitable matrices are special hydrogels, which switch from the liquid solution into a porous and transparent solid gel in the presence of special cross-linking events. These polymer networks are advantageous due to their bio- and cytocompatibility, porosity, hydrophobicity and complete flexibility regarding size and shape of their microscale gel structures (Selimovic et al., 2012). Common cell encapsulation materials are: (i) natural thermosensitive gels like agarose (Wu et al., 2006; Wu et al., 2007), collagen (Wu and Pan, 2009) and chitosan (Yang et al., 2007); (ii) synthetic UV-induced polymers like gelatin methacrylate (Bucke and Brown, 1983; Nichol et al., 2010) or polyethylene glycol-diacrylate (Liu and Bhatia, 2002); and (iii) ion-based cross-linking molecules, including alginate, a copolymer with homopolymeric blocks of β-D-mannuronic acid and α-L-guluronic acid, extracted from marine brown algae. For combination with MAR, the first two groups of materials are unsuitable since commercially available photo emulsions are unstable at higher temperatures and have to be kept away from any kind of light. For development of the current method alginate was chosen. It is the most commonly used hydrogel for producing microencapsulated cells. Beyond its nontoxicity, biocompatibility and biodegradability, its gelling process is mild, fast and simple in the presence of multivalent cations (Draget et al., 2005; Wu and Pan, 2009). The gelation is accomplished instantly by binding guluronic acids of different alginate molecules to added divalent cations such as barium and calcium (Draget et al., 2005).

Small alginate capsules with diameters of few hundred or tens of micrometers are generated via emulsification and internal gelation (Chan et al., 2002), spray drying (Coppi et al., 2002) and co-acervation (Chen et al., 2004). For the present method, the respective operation conditions with high pressures and temperatures or organic solvents are unsuitable since they may destroy encapsulated cells (Wu and Pan, 2009) or hamper photoemulsion. Single cell encapsulation requires beads with diameters below 100 µm, to be generated in a mild, controllable and reproducible way. Several studies describing the principles for the establishment of microfluidic devices for production of small, monodisperse, cell-hosting alginate capsules have been published (Huang et al., 2006; Choi et al., 2007; Hong et al., 2007; Shintaku et al., 2007; Workman et al., 2007; Capretto et al., 2008). They serve as the starting point for the implementation of MAR in microcapsules, which will allow the exclusive selection of active degrader cells.



The visualisation of the radioactive label associated with spatially isolated degraders allows their separation and individual characterisation. The identification of selected cells can be achieved with the help of special, modern amplification strategies. Multiple displacement amplification (MDA), a non-PCR-based DNA amplification technique (Lizardi et al., 1998; Dean et al., 2001) can amplify rapidly and reproducibly the smallest amounts of genomic DNA to quantities that are required for genome analysis. Starting with annealing of random hexamer primers to denatured DNA at a constant temperature the phi29 DNA polymerase continuously synthesises high molecular weight DNA products. Utilizing strand-displacement, rapidly increasing priming events lead to hyper-branched DNA structures of the whole genome.

MDA, as a method for whole genome amplification (WGA), and has been already used on single cells (Handyside et al., 2004; Hellani et al., 2004). Efficient, accurate, simple and fast protocols for single-cell MDA are available (Spits et al., 2006b, a).



Finally, WGA of single cells and appropriate molecular biological analysis complete the MAR-based direct identification of separated active degraders. The resulting task in this study was the development of the novel screening methodology for microbes with *in situ* activity.

The principles and methodological sequence defined above can be accomplished without any previous phylogenetic information or knowledge on catabolic genes. Therefore, it is considered as generally applicable for systems of uncertain composition and unknown

microorganisms, provided that the latter use the offered organic compounds as carbon source. The method is independent of all kinds of genetic probes, and the aim is to analyse active microbes individually, which is enabled by their separation in micro-compartments.

The new approach, '**M**icro**A**uto**R**adiography of **E**ncapsulated **C**ells (MAR-EC)' encompasses the short incubation of an environmental sample with the radioactively labelled substrate. Degrading microorganisms incorporate the label, whereas non-degrading microorganisms do not. It is followed by the encapsulation of cells, ideally on single cell level, together with an autoradiographic emulsion in alginate microbeads. Thus, labelled compound-transforming microbes are spatially isolated from the remaining community. Subsequently, beads are treated in the MAR process. Active and therefore labelled degraders become visually distinguishable and may be separated based on optical differences caused by MAR. To achieve identification, a set of molecular biological methods are available, initiated by powerful multiple displacement amplification (MDA) for single cells.

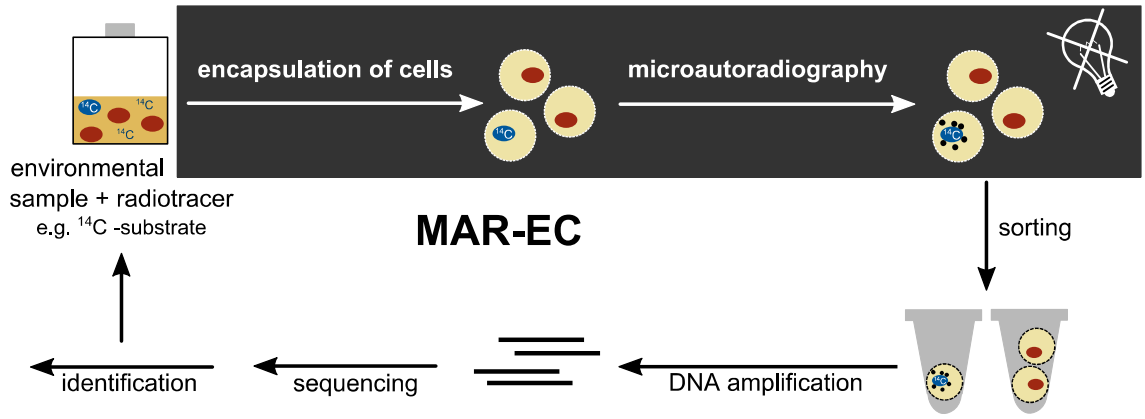


Figure 4-1: ***Workflow for the new identification approach for physiologically active community members.***

Apart from *ad hoc* genome identification and characterization, in principle even short DNA fragments may be sufficient to design new probes or starting points to go back to the original habitat for detailed and integrated analyses.

Several aspects of the basic principle of the overall approach – the incorporation of the label, the encapsulation of cells and their differentiation regarding activity – has been proven on a large bead scale (Mosler, 2011). Cells were incubated with a radioactively labelled substrate (benzene), mixed with the polymerizing capsule material alginate and a

photosensitive emulsion. Comparatively large capsules with a diameter of several hundreds of µm and harbouring thousands of cells were generated using a syringe and cannula by dipping droplets formed into the cross-linking agent. MAR was implemented, depending heavily on standard protocols and chemistry of MAR and MAR-FISH. The whole procedure was carried out in a custom-designed dark box. During microscopic analysis, clear differences of the signals arising from capsules with degraders and with non-degraders were observed.

This study aimed to (i) develop the full process of MAR-EC, as described above, including the conversion of existing principles and methodologies to the demands of MAR-EC at the single-cell level (automation and minimisation down to the single-cell level), and (ii) provide a proof-of-principle by means of a model system. One of the main difficulties in this methodology has been the necessity to work without any kind of light, starting with the introduction of the photosensitive emulsion until fixing the signal at the end of the MAR process. Especially the large-scale production of microcapsules as carriers of single cells will ensure the comprehensive screening of a microbial community from any environmental sample. A technology for high-throughput microencapsulation in the dark had to be established. Additionally, an appropriate way of handling a large number of microbeads during MAR and subsequent sorting was required. Finally, the amplification of DNA from only one cell out of the micro-compartments had to be accomplished.
As a model system, the degradation of benzene was chosen in compliance with the RNA-SIP/BACTRAP study (↗ chapter 2), initially implemented through the simplification of the environmental system into aerobic benzene-degrading and non-degrading microorganisms.

4.2 Material and Methods

4.2.1 Chemicals

All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (Saint Louis, MO, USA), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany) and were of analytical grade quality, including [^{12}C]-benzene (CHROMASOLV® Plus for HPLC, ≥99.9 %, Sigma Aldrich). [U-^{14}C]-benzene with a specific activity of 4.403 GBq mmol^{-1} was obtained as liquid in a break-seal from American Radiolabelled Chemicals (Saint Louis, MO, USA). It was dissolved in 2,2,4,4,6,8,8-Heptamethylnonan (Sigma Aldrich) at an activity concentration of 60 kBq µL^{-1} (i.e. concentration of 13.63 mM).
Alginic acid sodium salt from brown algae in medium viscosity and Sunflower seed oil from *Helianthus annuus* were purchased from Sigma-Aldrich. Nuclear emulsion (ILFORD nuclear emulsion K5) for autoradiographic applications was received from HARMAN technology (Knutsford, Great Britain).

4.2.2 Test organisms and culture conditions

To provide a proof-of-principle two model microorganisms were chosen based on their ability to degrade benzene aerobically: (i) *Pseudomonas veronii* B560 (strain collection UFZ-UMB, Dr. Lukas Wick) was a positive control and (ii) *Escherichia coli* DH5α (strain collection UFZ-UMB) was the negative control. Both were cultivated on mineral medium (7.0 g L^{-1} $Na_2HPO_4 \cdot H_2O$, 2.8 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl, 0.2 g L^{-1} NH_4Cl in H_2O_{dest}) with 2 mL L^{-1} trace element solution (50 g L^{-1} $MgSO_4 \cdot 7H_2O$, 5.0 g L^{-1} $FeSO_4 \cdot 7H_2O$, 2.5 g L^{-1} $MnSO_4 \cdot H_2O$, 3.2 g L^{-1} $ZnCl_2$, 0.5 g L^{-1} $CaCl_2 \cdot 6H_2O$, 0.3 g L^{-1} $BaCl_2$, 0.18 g L^{-1} $CoSO_4 \cdot 7H_2O$, 0.18 g L^{-1} $CuSO_4 \cdot 5H_2O$, 3.25 g L^{-1} H_3BO_4, 5.0 g L^{-1} EDTA and 73 mL L^{-1} 37 % HCl in H_2O_{dest} [slightly modified from Hartmans et al. (1989)], 5 mlL^{-1} vitamin solution (0.05 g L^{-1} vitamin B_{12}, 0.05 g L^{-1} pantothenic acid, 0.01 g L^{-1} pyridoxamine-HCl, 0.02 g L^{-1} biotin, 0.02 g L^{-1} folic acid, 0.025 g L^{-1} nicotinic acid, 0.025 g L^{-1} nicotinamide, 0.05 g L^{-1} α-lipoic acid, 0.05 g L^{-1}4-aminobenzoic acid, 0.05 g L^{-1} thiamine-HCl $\cdot 2H_2O$ and 0.05 g L^{-1} riboflavin in H_2O_{dest} [modified from Genthner et al. (1981)] and 0.5 mg L^{-1} yeast extract (in H_2O_{dest}). Carbon sources were in the case of *P. veronii* benzene at a nominal concentration of 4 mM (1 M stock solution in 2,2,4,4,6,8,8-Heptamethylnonan (HMN) and for *E. coli* it was 22 mM glucose. Both strains were grown in serum bottles sealed with butyl rubber stoppers at 30°C to approximately mid-exponential phase, determined by optical density measurement at 560 nm.

4.2.3 Radioisotope labelling of cells

A defined number of cells were harvested by 10 min centrifugation at 10 000 rcf and 4°C. Cells were suspended in mineral medium with trace elements, vitamins, and yeast extract (see above). For *P. veronii* no additional carbon source besides benzene was used, but for *E. coli* additionally 22 mM glucose was added. Labelling of the cultures was carried out with 0.44 mM ^{12}C-benzene (from the 0.1 M stock solution in HMN) including 1500 kBq mL^{-1} radioactive labelled ^{14}C-benzene and incubated for 60 min at room temperature in serum bottles sealed with a butyl rubber stopper, shaken constantly at 100 rpm.
Subsequently, for determining the incorporation of benzene via liquid scintillation counting, volatile benzene and CO_2 (^{12}C and ^{14}C) were collected for 20 min by changing the gaseous phase in the incubation bottle and washing it in a series of two traps containing ethylenglycolmonomethylether and two traps containing 2 M NaOH (Lehr et al., 1996). Cells were harvested by centrifuging for 5 min at 16 000 rcf, the cell pellet was washed in phosphate-buffered saline (PBS) (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4 in H_2O_{dest}, pH 7.4), centrifuged again and finally suspended in 50 µl of PBS. Adequate volumes of the traps, washing solutions and cell suspensions were added to Ultima Gold™ scintillation cocktail (PerkinElmer, Waltham, MA, USA), mixed and measured with a Packard Tri-Carb liquid scintillation Counter 1600 TR (Packard BioScience, now PerkinElmer).

4.2.4 Encapsulation of cells

Microfluidic devices for the production of small alginate capsules followed the same ‘flow focusing’ principle: a hydrophilic alginate solution containing cells was slowly pumped into a faster continuous flow of a hydrophobic phase, forming microdroplets.

Figure 4-2 demonstrates two common principles of microfluidic applications, whereby the flow-focussing channel is preferable, which delivers smaller droplet sizes (Huang et al., 2006; Hong et al., 2007; Shintaku et al., 2007; Workman et al., 2007; Capretto et al., 2008). By introducing divalent cations to this kind of controlled water-in-oil emulsion, the polymer immediately hardens by forming insoluble gel beads. In the simplest scenario, the gelation process is started by discharging the dispersion into a solution of the cross-linking agent.

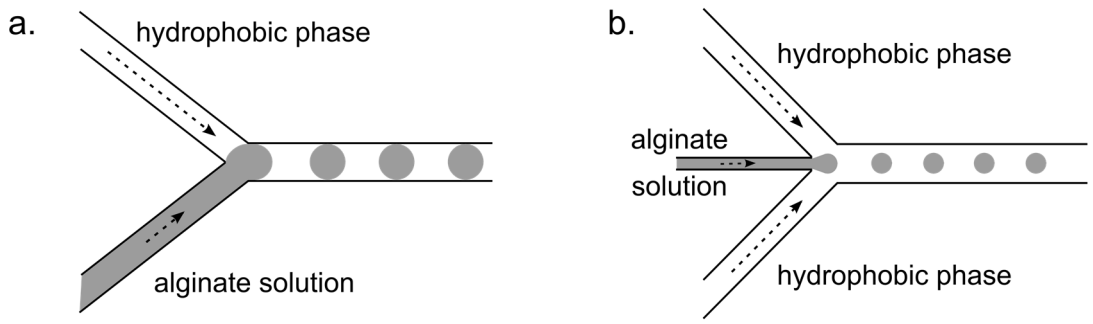


Figure 4-2: ***Common schematic geometries of microfluidic devices for the production of alginate microcapsules: a. 'Y' junction squeezing channel and b. flow focussing channel.***

For the MAR-EC approach the capsule material, comprising alginate (1 – 4 % (w/v) in H_2O_{dest}) and cells (*P. veronii* or *E. coli* in PBS) was supplemented with the photosensitive emulsion in a ratio of 1:1.

In the following, this alginate-cell-photoemulsion mixture is abbreviated as ACPM or designated as functional phase. In accordance with most of the applied systems (Hong et al., 2007; Workman et al., 2007; Capretto et al., 2008; Workman et al., 2008), sunflower seed oil was used as the continuous hydrophobic phase. For gelation, barium ions were used (Haug et al., 1970), provided as an aqueous solution of $BaCl_2$.

By using an appropriate capillary system and adjusted flow rates, droplets with diameters much smaller than 1 mm can be produced. Thus, different systems were tested and the different process parameters of the most promising system were optimized for single-cell application.

With the objective of applying MAR procedure to the cells, the encapsulation process with the photosensitive material used required the strict absence of light.

4.2.5 Microautoradiography

The autoradiographic procedure was described by various authors (a detailed theoretical discourse is given by Rogers (1979)). In general, it comprises the coating of the sample, normally on glass slides, with the special autoradiographic emulsion. It contains finely dispersed, photosensitive crystals of silver bromide and partly silver iodide, embedded and stabilized in a gelatine matrix. Before use, it has to be melted at 43°C, which is followed by dipping the slides with the sample into the emulsion. When cooling down it polymerises again. During the subsequent exposition time, beta radiation leads to the formation of latent silver seeds around labelled cells (reduction of few silver ions to elementary silver atoms). In the following time-controlled development in a slightly alkaline

medium (standard photographic procedure), silver ions are reduced to elementary silver ($Ag^+ + e^- \rightarrow Ag^0$). The invisible 'latent signal' is converted into a visible 'true signal'. Since exposed crystals, catalysed by the latent silver seed, react relatively quickly, silver ion reduction in unexposed crystals is slower by several orders of magnitude.

The development is stopped by optional exchanging of the developer with a stop solution and/or continuing directly with the fixation step to freeze the 'true image'. Therefore, fixation solutions contain complexing agents e.g. sodium thiosulphate, which bind and wash away all the remaining non-reduced silver ions ($2\ [S_2O_3]^{2-} + Ag^+ \rightarrow [Ag(S_2O_3)_2]^3$). The procedure is completed by several washing steps with water.
In the present study, MAR was transferred and adjusted from working on glass slides to applying it to alginate microcapsules.

4.2.6 FISH with encapsulated cells

For the visualization of the spatial distribution of cells inside of the alginate microcapsules down to the single-cell level FISH was applied to beads that passed through the encapsulation process and MAR treatment. Therefore, *P. veronii* as well as *E. coli* were cultivated as described before but not labelled with the radioactive substrate. A defined number of cells (here 100 cells per 100 µm capsule) was fixed with 4 % paraformaldehyde (PFA) in PBS overnight at 4°C and subsequently washed twice with 500 µl PBS using 5 min centrifugation steps at 16 000 rcf and 4°C. Finally cells were suspended in 50 µl PBS and encapsulated following essentially the procedure described above with the omission of the development step since the respective washing step would interfere in microscopic analysis.

Alginate beads with PFA-fixed cells were mixed with 1 mL MilliQ water, filtered through a 0.2 µm pore size, polycarbonate filter (Sartorius, Göttingen, Germany) and washed with 80 % ethanol. The filter with alginate beads was dried in a petri dish.

The subsequent hybridisation was performed in a ScanVIT®-Reactor (Vermicon AG, München, Germany). An absorbant pad (Millipore, Eschborn, Germany) with a diameter of 25 mm was prepared with 500 µL hybridisation buffer according to Manz et al. (1992) [35 % v/v formamide, 5 M NaCl, 1 M Tris (pH 8), 10 % SDS, 10 % Roche Blocking (Roche, Penzberg, Germany) in MilliQ water]. The polycarbonate filter with alginate beads was applied and 120 µL gene probe solution (hybridisation buffer with 35 % formamide and 100 ng/µL oligonucleotide probe) was added. For all eubacteria *EUB338* gene probe [5'-GCT GCC TCC CGT AGG AGT-3', (Amann et al., 1990)], for *E. coli Eco* gene probe (Vermicon AG) was used. Hybridisation took place for 90 min at 46°C in the

ScanVIT-Reactor. Following addition of 2 mL of a washing buffer [1 M Tris (pH 8), 5 M NaCl, 10 % SDS, 0.5 M EDTA, 10 % Roche-Blocking in MilliQ water (Manz et al., 1992)] the incubation was continued for a further 15 min at 46°C. Afterwards the solutions were removed with a water suction pump and the reactor was flushed with 2 mL sterile MilliQ water. The filter with alginate beads was again dried in a petri dish.

Directly before fluorescence microscopic analysis, the filter was covered with 1 droplet of DAPI-Finisher (anti-fading reagent with 1 µg mL^{-1} 4′,6-Diamidin-2-phenylindol, Vermicon AG).

4.2.7 Microscopy and documentation

For documentation of silver grains formed through microautoradiographic processing, the transmission light mode of the Axioskop50 (Carl Zeiss, Oberkochen, Germany) combined to a AxioCam MR (Carl Zeiss) was used. Images were processed with AxioVision Rel. 4.8 software (Carl Zeiss). Capsule diameters were measured after scaling with an external standard.

Fluorescence microscopy of FISH analysis was performed with an Epifluorescence microcope Axiostar plus (Carl Zeiss) and the filter sets for Cy3 (549 nm/562 nm), FAM (492 nm/518 nm) and DAPI (358 nm/463 nm). For documentation the VAS software (vermicon AG) was used.

4.2.8 DNA amplification tests

With the objective of final identification of compound-transforming single cells, in this study it was tested whether DNA can be successfully amplified from encapsulated and chemically and physically treated cells.

A first simplified approach used amplification of the model organism *E. coli* via conventional PCR to check whether the chemicals and solutions (alginate, photoemulsion (Ag^+ or Ag^0), developer, fixer, other optional solutions in MAR process and sodium pyrophosphate used for capsule dissolution) interfered with the PCR.
The amplification mix of 25 µL, with 10x QIAGEN PCR Buffer (Qiagen, Hilden, Germany), contained each deoxynucleoside triphosphate at a concentration of 0.2 mM, the universal bacteria 16S primers 27F, 5'-AGA GTT TGA TCM TGG CTC AG-3' (Suzuki and Giovannoni, 1996) and 1492R, 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Lane, 1991) at a concentration of 0.2 µM each and 2.5 units of HotStarTaqDNA Polymerase (Qiagen).

The reaction was carried out with the following PCR parameters: initial denaturation step of 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s and extension at 72 °C for 80 s, and a final extension step at 72 °C for 10 min. A positive control contained genomic DNA of *E. coli*, and a negative control did not include any template. PCR amplicons were verified via agarose gel electrophoresis.
Finally, the optimised protocol for sample preparation was applied to alginate capsules harbouring *E. coli* cells in the scale of 500 cells per capsule. To simulate high Ag content in capsules, they were exposed to light and underwent microautoradiography including development and fixation. Capsules were dissolved by treatment with 20 µL of 1 mM sodium pyrophosphate (PP_i) for 15 min while slightly shaken.

A second approach tested amplification via MDA. Briefly, the developed sample preparation was used in the MDA protocol and tested whether MDA / phi29 polymerase was working with encapsulated and treated cells. The amplification reaction was applied to capsules harbouring *E. coli* at the scale of 30 cells per capsule. To test Ag-containing capsules, they were treated with light and microautoradiography as well. Again microorganisms were released by dissolving capsules with 1 mM PP_i for 15 min. Cells were disrupted by alkaline lysis, modified from Dean et al. (2002). To this end, 4 µL of cell suspension were mixed with 4 µL of alkaline lysis solution (400 mM KOH, 10 mM EDTA) and incubated at room temperature for 3 min. Subsequently, 4 µL of neutralisation solution (400 mM HCl, 600 mM Tris HCl buffer (pH 7.5), final pH approximately 0.6) were added. Then, 2 µL of the lysed cells were mixed with 3 µL of exonuclease-resistant, thiophosphate-modified random hexamers (5'-NpNpNpNpNp^{S}Np^{S}N-3', 0.5 mM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) and used directly as template in MDA reactions. For the positive control 2 µL of genomic DNA of *Geobacter metallireducens* was heat-denatured at 95°C for 3 min and used instead of lysed cells. A negative control did not contain template DNA. 30 µL amplification mix with 10x phi29 DNA Polymerase Reaction Buffer (New England Biolabs GmbH, Frankfurt/Main, Germany) contained 50 mM KCl, 200 mM trehalose (Pan et al., 2008), each deoxynucleoside triphosphate at a concentration of 1 mM, 0.2x SYBR Green and 3.5 µL of phi29 polymerase (produced and provided as published in Blainey and Quake (2011) from Dr. Anne-Kristin Kaster, Junior Research Group “Single Cell Genomics”, DSMZ, Braunschweig, Germany). MDA was incubated constantly at 30 °C for 15 h followed by final inactivation of the enzyme at 65°C for 3 min. Reactions were performed and synthesised DNA measured with a StepOnePlus™ Real-Time PCR System (Applied Biosystems).

The 16S rRNA gene used was reamplified by PCR essentially as described above, checked by agarose gel electrophoresis and analysed by Sanger sequencing (GATC Biotech, Konstanz, Germany).

4.3 Results and Discussion

The present project pursued two main aspects of the new MAR-EC approach.

It started with the development of each complex, methodological aspect. This comprised (a) the incubation of microbial cells with a radioactively labelled substrate (^{14}C-benzene). The general experimental implementation was slightly adapted from various former MAR and MAR-FISH studies [e.g. (Andreasen and Nielsen, 1997; Lee et al., 1999; Ouverney and Fuhrman, 1999; Gray et al., 2000; Nielsen et al., 2002)]. The more elaborate work addressed the establishment of (b) a high-throughput encapsulation process for single cells and (c) the processing of the microbeads with MAR. The finally intended identification furthermore required (d) an effective amplification of DNA of encapsulated cells. Initial method development work except for (a) was performed without the use of radioisotopes. If necessary, two different kinds of alginate beads were simulated by exposing capsules to light for generating 'black signals', or by omitting the development step during MAR for generation of clear capsules without a silver signal. For few general or comparative methodological issues, clear capsules were produced by the exclusion of photoemulsion.

Once this work had been completed, the overall approach of realisation of MAR with microencapsulated cells was confirmed in a proof-of-principle workflow employing model organisms.

4.3.1 Development of MAR-EC: Encapsulation

Automation of the encapsulation process for cells

The first important aspect for developing the encapsulation process was to advance from handmade, comparably large-scale alginate capsules to automated high-throughput microencapsulation. Frequently, innovative microfluidic chip technology is used for that purpose. They may be individually fabricated of polydimethylsiloxane (PDMS) based on photolithographic procedures (Choi et al., 2007; Hong et al., 2007; Shintaku et al., 2007; Workman et al., 2008). Since the production requires significant know-how and technical effort, commercially available chips in two different but common designs and materials were tested. Their usage was accompanied by substantial problems regarding chemical resistance and resulting leakage, which is exemplarily documented in Figure 4-3.

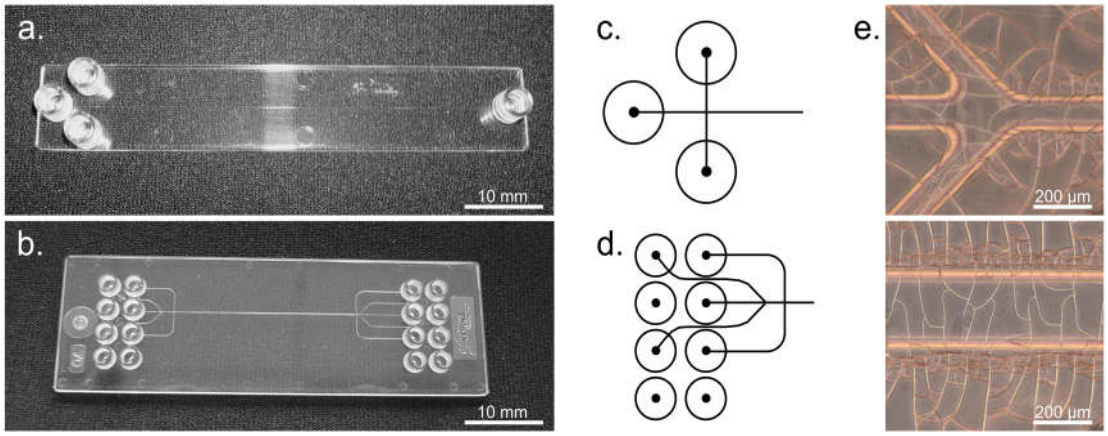


Figure 4-3: ***Tested commercial microfluidic chips (microfluidic ChipShop GmbH, Jena, Germany): a. Cross-shaped channel chip (PMMA) with channel diameters of 50 µm and b. Droplet generator chip (PC) with channel diameters of 70 µm. c. and d. Schematic drawing of the geometry of the respective microfluidic application for droplet generation. e. Micro cracks, which arise through excessive use.***

Due to the associated high costs and inestimable complexity for problem-solving regarding the chip material and finding feasible and available chip geometry, a different microfluidic system was established. It used polyetheretherketone (PEEK) tubing. Because of the excellent mechanical and chemical robustness even at high temperatures and high pressures, the organic thermoplastic polymer is customarily processed for tubing and accessories in liquid handling in, e.g., high performance liquid chromatography. Through a set of available tubes, adaptors and connectors in different sizes, it is also attractive in microfluidic applications (↗ Figure 4-4).

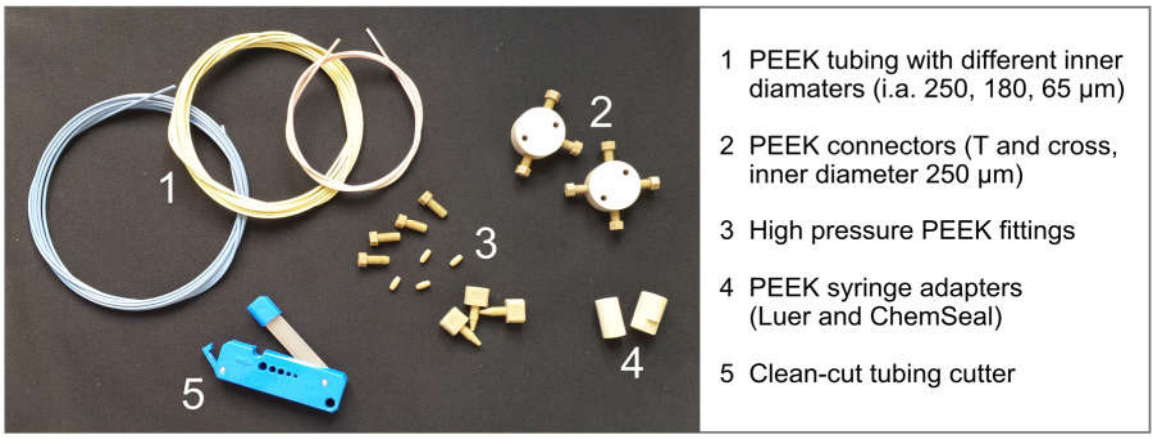


Figure 4-4: ***Set of PEEK accessories used for microencapsulation of cells into alginate matrix.***

A special device for automated, high-throughput encapsulation in the dark based on PEEK technology was designed (↗ Figure 4-5 and Figure 4-6). It was composed of a set of variable tubes with different inner diameters and matching cross- as well as T-junctions and supplied by a pump hosting two syringes (KDS Gemini 88, KD Scientific Inc., Holliston, USA). One syringe contained the functional phase of alginate, photoemulsion (including gelatine) and cells (=ACPM). The other syringe provided the continuous oil flow. The oil phase was first split into two flow-paths, which at a cross-junction came together and due to pressure and viscous stresses forced the capsule material into a narrow thread. Droplets regularly 'teared off', were transported through the tubing and collected in a $BaCl_2$-solution. To avoid early gelation of the gelatine, it was kept at about 40°C by two kinds of water baths (pumped and stationary). To protect the photoemulsion from any kind of light, the whole process was performed in a dark box.

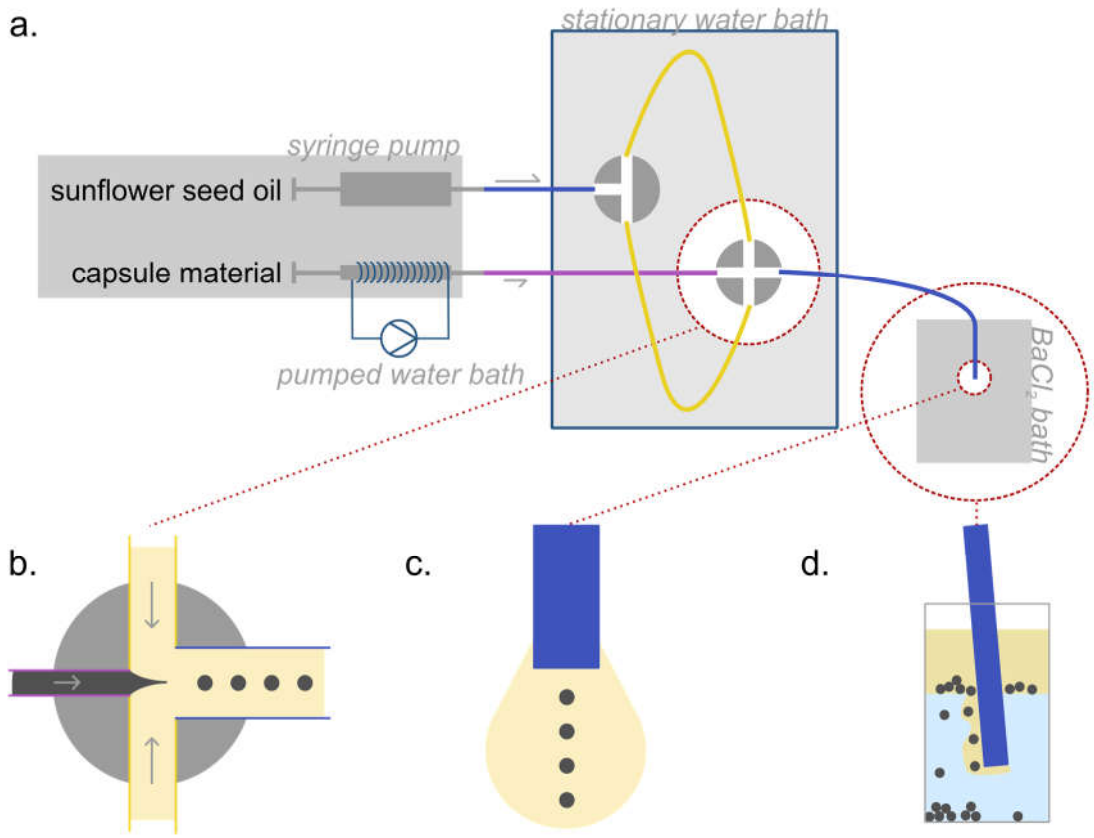


Figure 4-5: ***Flow scheme of the encapsulation process and system. a. General structure of the system composed of the syringe pump providing the functional and continuous phase, the stationary waterbath including the microfluidic part, where microdroplets were formed and the $BaCl_2$ bath for stabilizing and collecting the capsules. b. Presumed flow-focussing process of droplet formation inside of the cross-junction, c., which could be verified by the observed regular appearance of droplets at the end of the tubing. d. In the $BaCl_2$ bath the capsules arrange at the rising oil/water-interphase and after a while sink down.***

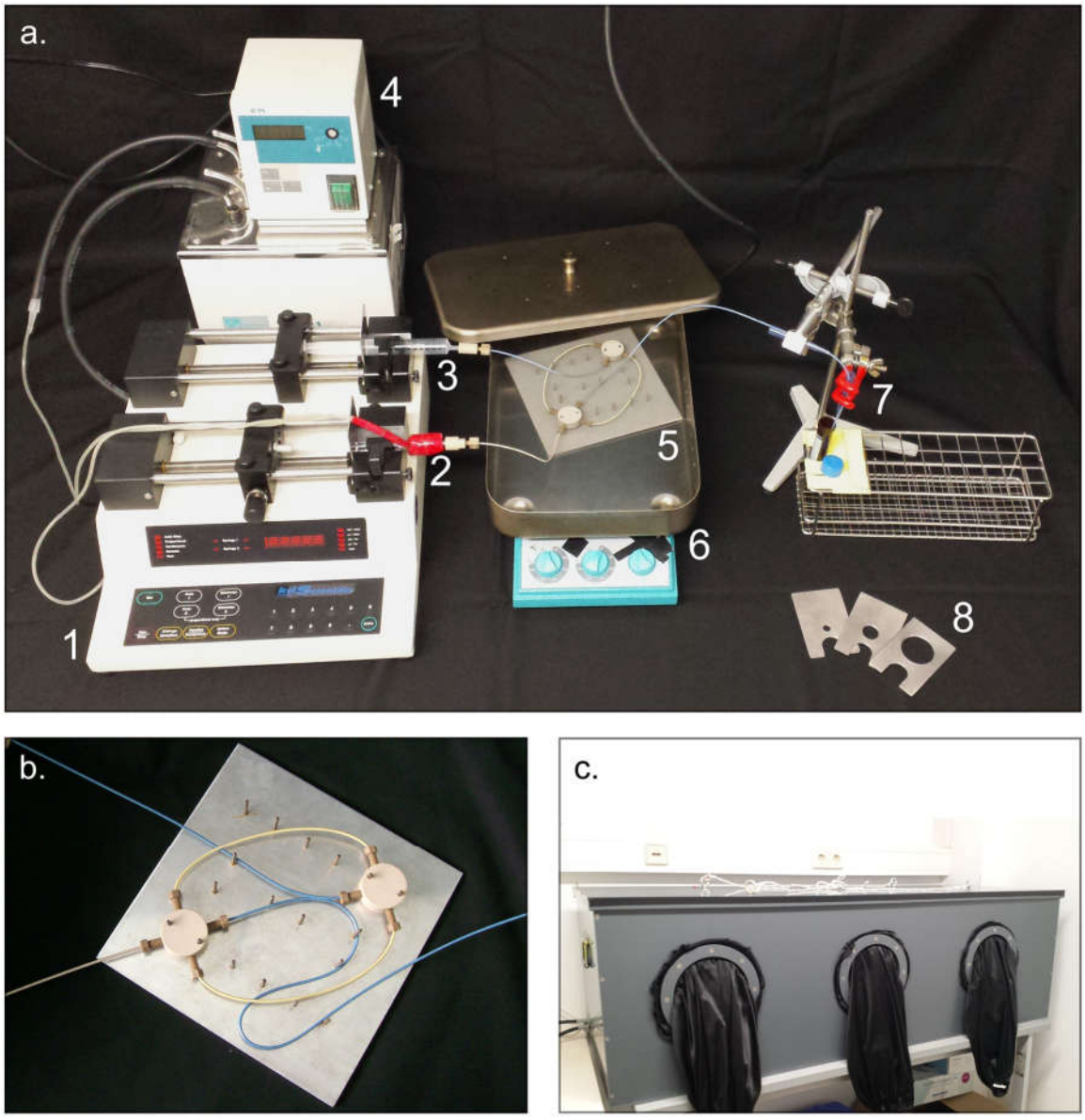


Figure 4-6: ***The encapsulation system. a. Complete setup including (1) a dual-rate pump with two syringes, (2) a Hamilton glass syringe containing previously mixed alginate, photoemulsion and cells with a syringe heating, driven by (4) a pumped waterbath, (3) a PP syringe with sunflower seed oil. Both syringes are stabilised by special washers of stainless steel (8). The PEEK-tubing for capsule forming is fixed on a plug-in plate of stainless steel (5) and maintained at constant temperature in a water bath (6). Capsules are collected in $BaCl_2$ in black, opaque falcon tubes. b. Zoom on the droplet generation device. c. The system is located in a light-proof dark box and reachable via sleeves.***

Minimisation of microcapsules for single-cell application

The second important consideration about developing the encapsulation process was the miniaturisation of the microcapsules as much as possible, to ideally reach single-cell level. Therefore, a selection of system-related parameters were tested and optimized.

The first parameter to be considered was the size of the tube conducting the functional phase. Kim et al. (2007) and Capretto et al. (2008) both stated that with decreasing tube diameter the bead size decreases. It is an effect of the orifice and the resulting cone. This correlation could be confirmed (↗ Figure 4-7) and consequently the smallest available PEEK tubing of 65 µm was used.

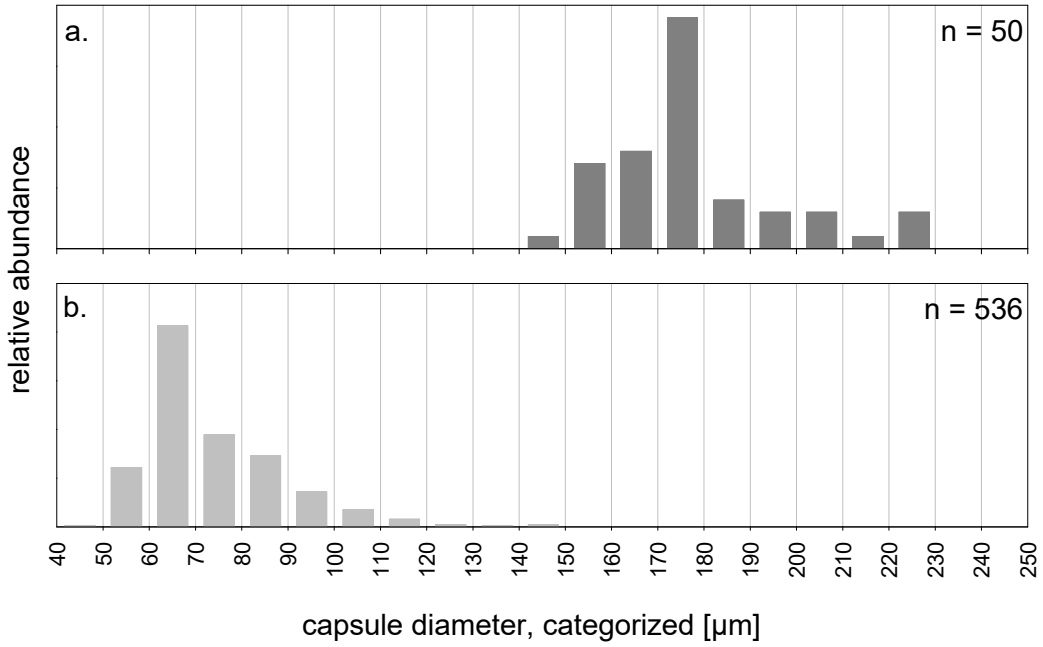


Figure 4-7: ***Relative abundance of capsules within size categories of the capsule diameter depending on two different capillary diameters for the functional phase (ACPM) for the same flow rate. Inner capillary diameter: a. 130 µm, b. 65 µm. n = sample size. Encapsulation parameters: flow rate ACPM = 0.2 µL min^{-1}, flow velocity oil = 2 m min^{-1}.***

It should be noted that when working with tubing of different diameters, there is a clear difference between the flow rate, as moved volume per time unit, and the flow velocity, as mean speed of a phase moving along a cross section. Both are connected by the cross sectional area and therefore the diameter. In considering the functional phase it is appropriate to compare flow rates, since transported volume is directly linked to drop volume and diameter. The sunflower seed oil exerts a pressure on the cone of the ACPM, which depends on its velocity. Due to this fact, stating the flow velocity for the continuous phase is more appropriate, since in contrast to the flow rate it allows a direct comparison of this parameter, independent of the tube diameter. However, the settings of syringe pump were flow rates.

The next investigated parameters were the flow rates or velocities and the flow rate ratio of both phases. Both can crucially influence droplet formation (Anna et al., 2003; Sakai et al., 2004; Huang et al., 2006; Choi et al., 2007; Hong et al., 2007; Kim et al., 2007; Shintaku et al., 2007).

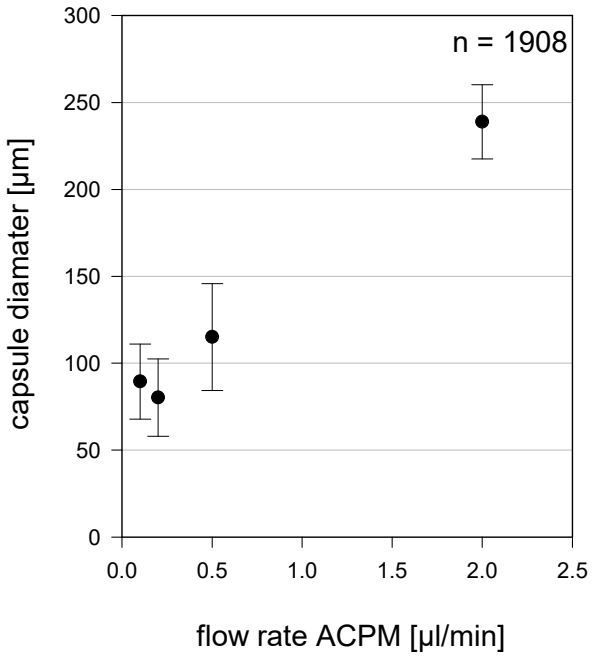


Figure 4-8: ***'Mean capsule diameter' capsules depending on different flow rates of the functional phase (ACPM). Flow velocity for the continuous phase (sunflower seed oil) = 1 m min^{-1}. n = sample size.***

The capsule diameter in general decreases with decreasing flow-rate of the functional phase (↗ Figure 4-8). This correlation could mainly be observed for comparably high flow-rates and if the flow-rate was varied in the range of several fractional digits. For small fluctuations of a few 0.1 µL min^{-1}, the effect was much less pronounced. Moreover, the capsule size seemed to stagnate at a flow-rate of 0.1 µL min^{-1}. Further flow-rate decrease only resulted in a lower generation rate of capsules of similar size as at higher flow-rates.
It could be furthermore proven that the capsule diameter decreases with increasing velocity of the sunflower seed oil as a result of an increasing pressure on the cone of the ACPM (↗ Figure 4-9). Corresponding to an ascending flow-rate ratio, the value for the functional phase should have been set to approximately 0.1 µL min^{-1}, whereas the flow velocity of the continuous phase should have been enlarged as much as possible.

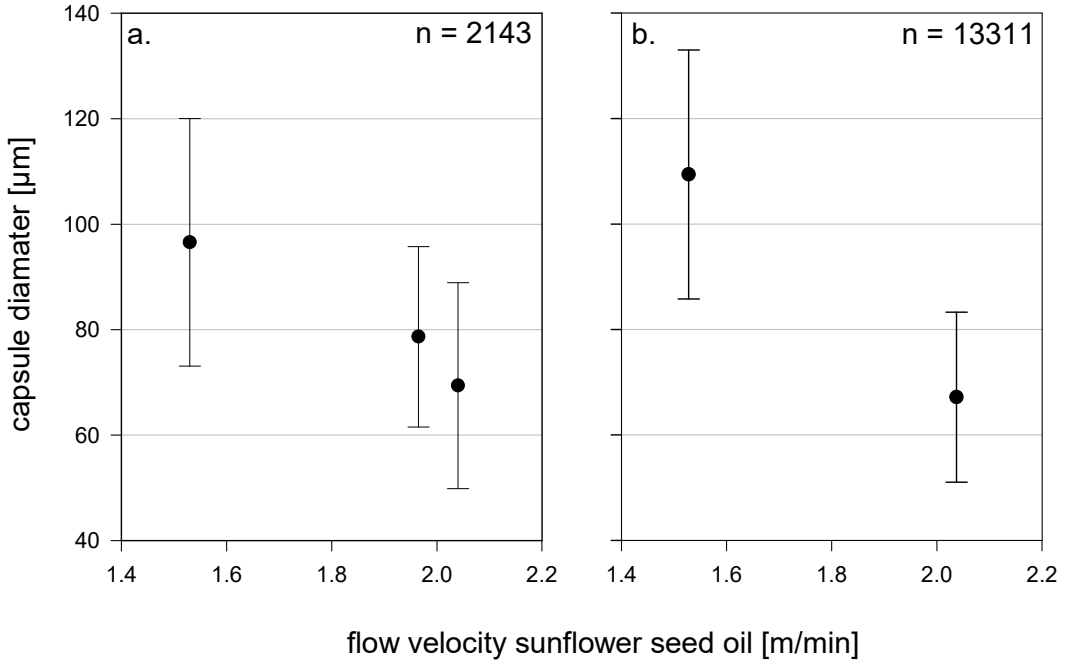


Figure 4-9: ***'Mean capsule diameter' capsules depending on different flow velocities of the continuous phase (sunflower seed oil). Flow-rate for the functional phase (ACPM) = a. 0.2 µL min^{-1} and b. 0.1 µL min^{-1}. n = sample size.***

The practical implementation of the derived ideal parameters to the applied system was restricted in realising a maximum velocity for the oil. Extremely high pressures cause the syringes to split or the pump to stall. To remedy these issues, the syringe was downsized and the capillaries were enlarged. However, the first resulted in a limitation of the volume of the oil reservoir and the latter to problems maintaining the flow velocity in a higher consumption of oil. The running time of the device became limited. In order to sustain high-throughput as much as possible, the final compromise was use of a 10 mL syringe, which was stabilised by special washers. The tube diameter was set to 180 µm respectively 250 µm for the input and output tube. In this configuration the maximum possible flow velocity was 1.5 m min^{-1}, which for this configuration of tubes was a flow-rate of 100 µL min^{-1} and could be kept for one and a half hours until the oil reservoir was empty.

The final parameters to be investigated included the factors influencing the droplet formation and included the phase properties: viscosity of the functional phase and interfacial tension of the two included phases. For variation of the viscosity, different alginate concentrations (1 % - 4 % (w/v)), mixed with an equal volume of photoemulsion were tested. Capsules did not become substantially smaller with decreasing alginate concentration but they lost stability. Alginate solutions of higher viscosity led to

inaccuracies in handling in the dark. The best option for generating stable beads in reproducible sizes was to use an alginate end concentration of 1 % (w/v) in the ACPM.
To influence the interfacial tension, the sunflower seed oil was mixed with the common surfactant, sorbitan monooleate Span 80. With a hydrophilic/lipophilic balance (HLB) of 4.3 this emulsifier stabilizes water-in-oil systems and can improve microdroplet morphology and decreases their size (Anna and Mayer, 2006; Choi et al., 2007; Capretto et al., 2008). The surfactant was tested at concentrations between 0 and 2 % (w/v). With an increasing concentration, a reduction in size could be observed but it was accompanied by loss of the regular form of the capsules. Furthermore, a separation of the alginate and photoemulsion occurred in the presence of sunflower seed oil with Span 80. At a surfactant concentration of 2 % (w/v) the microbeads were no longer able to pass through the interphase of oil and $BaCl_2$ and to sink down. Consequently, further evaluation of the use of a surfactant was discontinued.

4.3.2 Protocol for MAR-EC: Encapsulation

On the basis of automation and optimisation of the encapsulation process and parameters, the following final protocol was established:

In the dark, alginate [2.3 % (w/v)] was slowly stirred at a ratio of 7:1 with the cells in PBS for 10 min at 40°C in a water bath. The suspension was then completed with an equal volume of photoemulsion and stirred for a further 5 min at 40°C. Using a 100 µL Hamilton glass syringe and a syringe pump, the alginate-cell-photoemulsion mixture (ACPM), at a constant temperature of 40°C, was pumped into the custom-made device constructed of PEEK tubing with different inner diameters [see Figure 4-5 and Figure 4-6 (pp. 61-62); tubing: Ø 250 µm (blue), 180 µm (yellow) and 65 µm (violet)]. The flow-rate was set to 0.1 µL min^{-1}. The continuous sunflower seed oil flow was simultaneously provided via the pump through a second 10 ml syringe of PP. It was pumped with a much higher flow-rate of 100 µL min^{-1} (1.5 m min^{-1}). After an initial stabilisation phase of 15 min, the accumulated capsules were collected for 1 h in black, opaque falcon tubes containing $BaCl_2$ (500 mM) and a thin upper oil layer. The tubes were stored upright at room temperature and in the dark.

4.3.3 Development of MAR-EC: Microautoradiography

There is a range of commercially available emulsions, development and fixation agents, optimised for conventional MAR applications. Yet within the scope of this study, MAR was implemented in a new way, embedding in microspheres of alginate, which required a re-development of the generally applied processes of MAR.

Adaptation of the MAR process for alginate capsules

The stability of alginate hydrogels depends mainly on two crucial properties:
First, solutions of alginate selectively bind most di- and trivalent cations to form the characteristic hydrogels. There are different affinities for the different ions. For alkaline earth metals it increases in the order Ba^{2+} > Sr^{2+} > Ca^{2+} >> Mg^{2+}, which affects the strength of the gel (Haug et al., 1970; Draget et al., 2005). Consequently, Ba^{2+} provided as $BaCl_2$ was preferred in the present application. In the presence of increasing concentrations of non-gelling, monovalent cations, the gel starts to swell and becomes destabilised (Martinsen et al., 1989). This effect is based on the exchange of ions and can also be observed when gelling ions are captured by complexing agents like EDTA (Skjak-Braek and Moe, 1992).
Furthermore, alginates are most stable at pH between 5 and 9 (McDowell, 1956). Beyond these values, glycosidic linkages are cleaved by proton catalysed hydrolysis or by beta-alkoxy elimination, and the structure depolymerises (Haug et al., 1963). Both cause problems with the stability of alginate gel structures. These problematic effects increase with decreasing alginate capsule size.

With regard to the MAR procedure, common developers contain reducing substances such as hydroquinone, metol, amidol, and *p*-phenylenediamine. In principle, any reagent with appropriate reducing properties can act as a developer (Rogers, 1979). An alkaline medium that accelerates the development process is maintained by a buffering system. Further additives are typically included for process optimization and preservation of the developer.
All tested solutions dissolved the alginate beads due to their ionic composition, additives or pH. The relatively new ‘Kodak professional Xtol developer’ uses derivatives of ascorbic acid and phenidone as reducing agents (Opitz and Zawadzki, 1998). Although the capsules were destroyed in the commercial developer due to its additives, its composition was promising since it worked at a weakly alkaline pH of 7-9. To circumvent the disadvantages of the additives, a new developer was created based on Xtol. It contained

the two reducing substances sodium isoascorbate and 4-hydroxymethyl-4-methyl-1-phenyl-3-pyrazolidone together with TRIS to maintain a pH of 9 and $BaCl_2$ to additionally stabilise the capsules.

Table 4-1: Composition of the developer for MAR with alginate encapsulated cells.

Ingredient	**Concentration [g L^{-1}]**
Sodium isoascorbate monohydrate	13
4-Hydroxymethyl-4-methyl-1-phenyl-3-pyrazolidone	0.2
Tris (≡ 10 mM)	1.2
Barium chloride monohydrate (≡ 50 mM)	12.21

In a test series the appropriate developing time was adjusted for the microcapsules. Typically in autoradiography, samples are washed with a stop solution of slightly acidic pH to interrupt the development process. Here, distilled water was used to avoid depolymerisation of the alginate capsules due to low pH.

For fixation usually a solution of sodium thiosulphate [30 % (w/v)] is used. The anion binds the non-reduced, ionic silver in soluble complexes but the monovalent cation (Na^+) affects the gel stability. This problem was solved by lowering the sodium thiosulphate concentration and by counteracting the presence of sodium with additional divalent cations. Unfortunately, Ba^{2+} in the relevant, high concentration forms a poorly soluble salt with thiosulphate. This led to swelling or in some cases to the mechanical destruction of the capsules by forming large crystals inside them. Ca^{2+} ($CaCl_2$) was used instead.

Table 4-2: Composition of the fixer for MAR with alginate encapsulated cells.

Ingredient	**Concentration [g L^{-1}]**
Sodium thiosulphate pentahydrate	100
Calcium chloride dihydrate (≡ 50 mM)	7.4

The new solutions were working well when individually applied to the capsules, but when applied in the autoradiographic sequence of developer – H_2O (stop bath) – fixer – H_2O (washing step), swelling and often even the dissolution of most capsules occurred.

Swelling of a polymer network is mainly caused by the osmotic effect of polymer counter-ions unequally distributed between the inside and the outside of the gel (Skjak-Braek and Moe, 1992). Permanent changes in the ionic strength of the applied solutions hence caused osmotic stress to the capsules, resulting in their destruction. Consequently, stop solution and washing solution (WashSol) were adjusted in their salinity to the other solutions with a suitable ratio of NaCl and $CaCl_2$. Again, Ca^{2+} instead of Ba^{2+} was used to avoid reactions with the fixer, when used before and afterwards.

Table 4-3: Composition of the Washing solution for MAR with alginate encapsulated cells.

Ingredient	**Concentration [g L^{-1}]**
Sodium chloride	87.7
Calcium chloride dihydrate (≡ 50 mM)	7.4

For final stabilisation of the capsules in the last step of MAR they were washed with $BaCl_2$.

Practical implementation MAR for alginate microcapsules

Another challenge for establishing MAR in the new context was its practical implementation with cells inside of alginate microcapsules instead of immobilised on handy glass slides. A huge number of very small capsules needed to be handled in the dark, incubated with the different solutions in a fixed chronology. Filtration seemed to be the only logical strategy. Simple approaches based on plain filters, pressure or suction, failed due to leakage and irreversible fixing of the beads on the filter material connected to their deformation and destruction. A solution to this problem was found in sample concentrators, traditionally designed for fast, non-denaturing concentration of biological samples by membrane ultrafiltration. They harbour vertical polyether-sulfone membranes, which minimize membrane blockage by alginate capsules. A dead-stop technology prevents samples to be concentrated to complete dryness and therefore additionally reduce mechanical stress on the capsules.

A remaining physical and chemical influence of the different solutions was observed in the formation of aggregates, irreversibly cross-linked and stabilized by added divalent cations. As far as possible, they could be avoided by thorough mixing of the sedimented capsule in each applied solution.

4.3.4 Protocol for MAR-EC: Microautoradiography

Encapsulated cells that had been collected in $BaCl_2$ in conical centrifuge tubes in the dark were used for the development of the MAR procedure. Formation of latent silver seeds in the capsules was allowed to take place over a period of up to 9 days incubation during which capsules sank down to the bottom of the tube. While protecting the beads from any light exposure, they were applied to Vivaspin™ 500 centrifugal filter units (MWCO 100kDa, GE Healthcare Life Sciences, Chalfont St Giles, Great Britain). The co-transfer of oil was avoided as much as possible because it can block the pores of the membrane in the filter unit.

First, the $BaCl_2$ solution was used to wash the capsules and they were subsequently separated by centrifugation for 5 min at 1677 rcf (5000 rpm, Centrifuge MiniSpin®, Eppendorf, Hamburg, Germany). All subsequent solutions were added to the capsules in a volume of 450 µL in the order shown in Figure 4-10. If necessary the spin column was incubated with the solution, which was afterwards removed by centrifugation. Briefly, capsules were washed with H_2O three times. The developer was added and immediately centrifuged (= 5 min incubation). After a subsequent washing step with WashSol, the fixer was added and incubated for 3 minutes before starting centrifugation. Following another two washing steps with WashSol, the capsules were stabilised by washing with 500 mM $BaCl_2$. When necessary, the capsules were stored in $BaCl_2$ at 4°C. For further use they were washed again three times with H_2O.

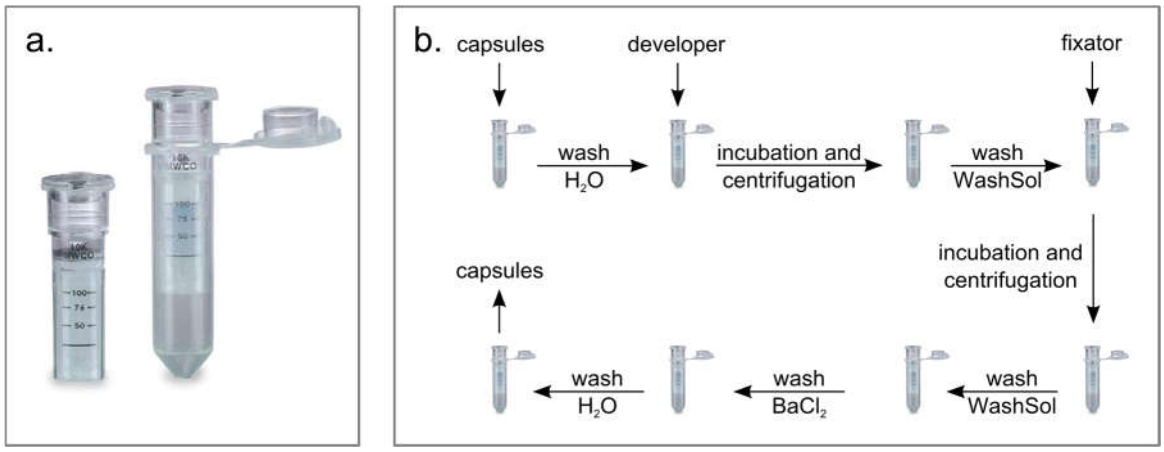


Figure 4-10: ***Microautoradiography with microencapsulated cells. a. Centrifugal filter units with vertical polyethersulfone membranes. b. Applied sequence of different washing, development and fixation steps.***

4.3.5 Development of MAR-EC: Single cell application

It had to be evaluated whether simple, temporally limited mixing by stirring and subsequent encapsulation allows spatial isolation of cells and to allow working on a single-cell level. FISH was applied for visualising the distribution of cells in the microcapsules. Beads were treated similarly to MAR but the development step was omitted to generate optically clear microcapsules in which the microbes were fluorescently labelled.

A defined number of *E. coli*, or *E. coli* plus *P. veronii* cells were processed, total DNA was unspecifically stained with DAPI, and vital bacteria and vital *E. coli* were labelled with appropriate hybridisation probes.

For *E. coli* a spatial separation of single cells could be observed in comparatively large capsules (↗ Figure 4-11 a). In smaller capsules of upto 20 µm the cell density decreased down to as low as one or two cells.
Moreover, FISH analysis demonstrated microbiological purity of the process performance: In tests with *E. coli* congruent signals for both FISH probes, i.e. vital bacteria and *E. coli*, could be observed. Contamination by other bacteria could be excluded. For *P. veronii* no adequate gene probe was available but selective cultivation on benzene and equivalent processing of cells and capsules allowed the conclusion that signals of vital bacteria not assignable to *E. coli* belonged to *P. veronii* (↗ Figure 4-11 b and c). Capsules with a mixture of the two organisms also showed the required spatial separation of single cells.
The distribution of cells and the isolation of individual cells for very small capsule dimensions allowed the prediction that a simple setting of cell concentration in the ACPM was sufficient to generally achieve isolation of single cells in capsules.

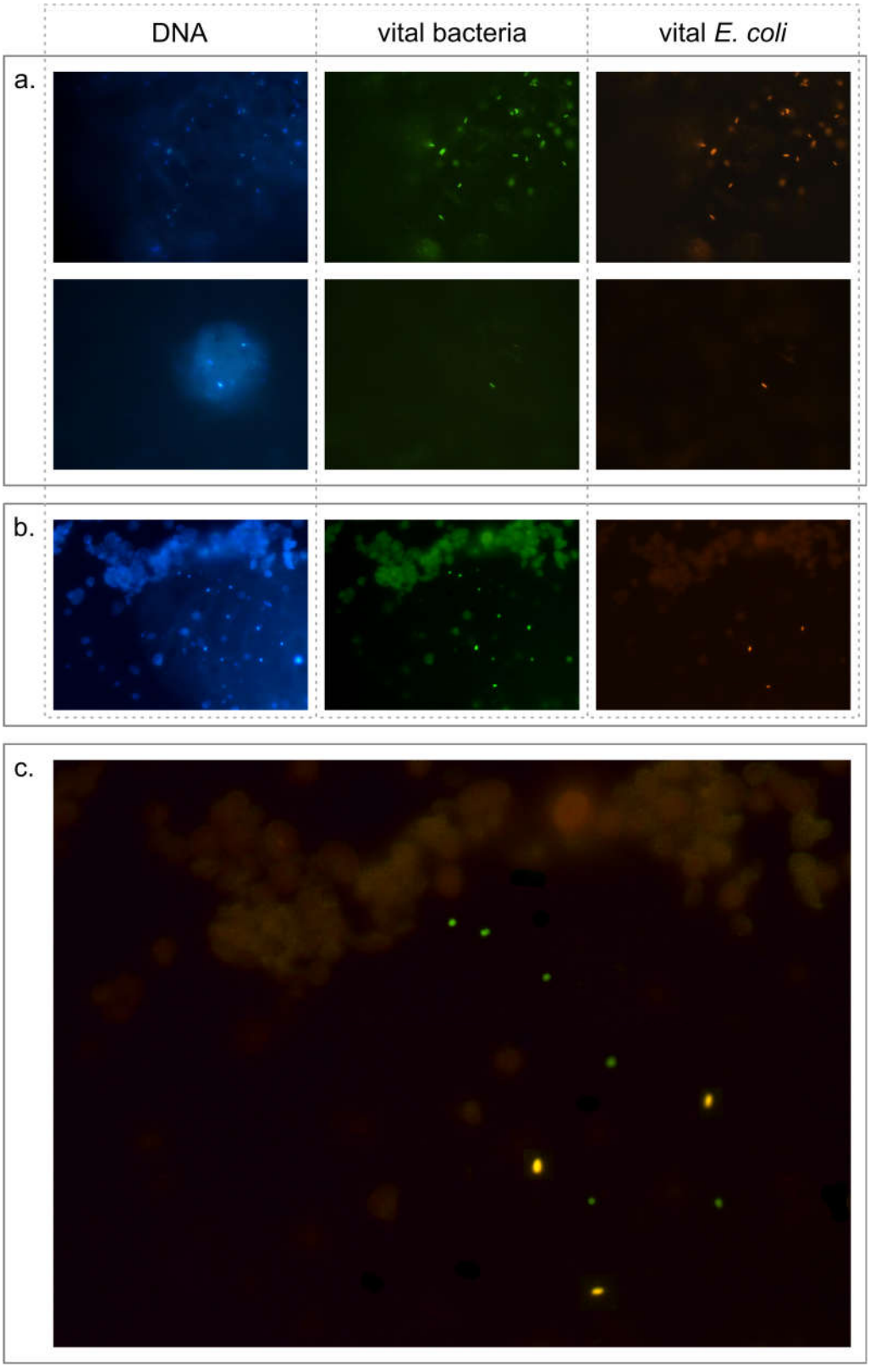


Figure 4-11: ***Visualisation of microencapsulated cells via DAPI (column 1, blue) and specific FISH probes (column 2 and 3, vital bacteria in green, vital*** **E. coli** ***in orange). Capsules contained either a.*** **E. coli** ***or b. an equal mixture of*** **E. coli** ***and*** **P. veronii.** ***In c. the pictures of both FISH probes were overlaid to distinguish between the two microorganisms (*** **E. coli** ***in green and*** **P. veronii** ***in yellow).***

4.3.6 Development of MAR-EC: DNA amplification

Next, it was tested whether DNA amplification from encapsulated and chemically and physically treated cells is possible. First tests focused on conventional amplification via PCR with TaqDNA Polymerase. Using cells or genomic DNA of the model organism *E. coli*, the influence of all of the involved chemicals and solutions [alginate, photoemulsion (Ag^+ or Ag^0), developer, fixer, washing solutions in MAR process and sodium pyrophosphate (PP_i)] was tested. All of them inhibited PCR at the concentration used in the MAR-EC procedure. PP_i was particularly inhibitory for PCR, which has been reported before (Tabor and Richardson, 1990). Consequently, the concentration of all MAR-EC components had to be diluted out prior to DNA amplification to prevent interference with the procedure.

The MAR-treated microcapsules in the test assay contained a large amount of silver, at least equal to real radioactively labelled capsules. First the capsules needed to be dissolved by incubation with PP_i. Afterwards, PP_i and the capsule material including Ag and possible residues of the other chemicals used in MAR had to be separated from the cells or appropriately diluted. The best solution was found in a centrifugation step. Around 15 capsules, each harbouring approximately 500 cells of *E. coli* were treated with PP_i. Subsequently, the mixture was centrifuged at 3000 rcf for 3 min and the supernatant carefully replaced by the PCR Mastermix. By including this procedure, the 16S rRNA gene of *E. coli* could be amplified successfully from alginate microcapsules (↗ Figure 4-12).

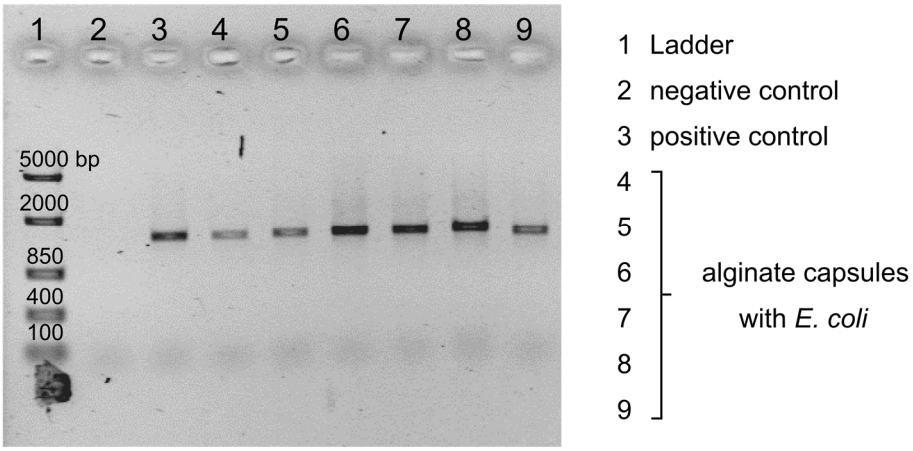


Figure 4-12: ***16S rRNA gene amplicons of* E. coli *in alginate capsules. Positive control: genomic DNA of* E. coli*, negative control: no template. Ladder: FastRuler Middle Range DNA Ladder (Thermo Fisher Scientific).***

In the next step, the applicability of MDA employing phi29 polymerase was tested on alginate capsules. For this purpose the number of cells was drastically reduced to the scale of about 30 cells per capsule. Around 15 capsules per MDA, produced with or without photoemulsion and treated by including or excluding the development step during MAR, were dissolved and centrifuged exactly as described before. Afterwards cells were lysed and used in the amplification reaction.
The reproduction of DNA was measured as increasing fluorescence of SYBR green binding to nucleic acid (↗ Figure 4-13).

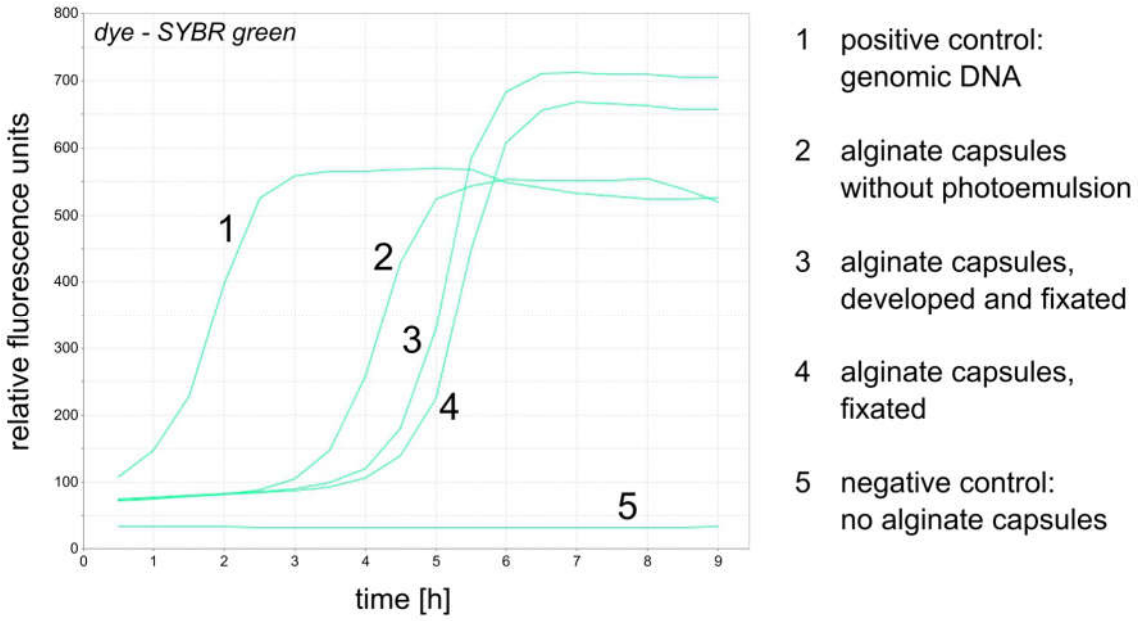


Figure 4-13: ***Results of whole genome amplification via MDA. Alginate capsules were treated differently, used in the reaction and successful amplification was measured by fluorescence of the nucleic acid stain SYBR green.***

The amplification of the genomic DNA of the positive control began immediately, whereas the reaction with DNA from encapsulated cells started after a lag period. The effect was also observable when a cell suspension was tested instead of cells inside of capsules. This might be due to the fact that the genomic DNA was purified after extraction, while the DNA of the cells was not. In the negative control, no noteworthy signal occurred during the whole incubation time. The differences in the curve shape of various treated capsules did not explicitly indicate any inhibition of the MDA. The observed lag time with capsule-derived cells as template could be due to lower cell numbers. In conclusion, MDA was found to be applicable for the desired approach.

The amplified DNA was purified and the 16S rRNA gene was successfully re-amplified and sequenced. Sanger-sequencing suggested that the procedure was robust against contamination, i.e. the obtained sequences appeared pure and specific.

4.3.7 Proof of Principle

Radioisotope labelling of cells

Pure cultures of *E. coli* and *P. veronii* were incubated aerobically for 1 h with a mixture of unlabelled and ^{14}C-labelled benzene. As listed in Table 4-4 most of the label was recovered as starting substance, trapped in ethylenglycolmono-methylether. As expected, the recorded $^{14}CO_2$ ratio, which indicates mineralisation, was negligibly low for the non-degrading *E. coli* whereas in the *P. veronii* culture a transformation of 1.7 % of the ^{14}C benzene to $^{14}CO_2$ was observed. This turnover was also detectable as ^{14}C-incorporation into cells. Although the model substrate benzene is known to accumulate in cell membranes and to change membrane properties (Sikkema et al., 1994), the non-degrader hardly ad- or absorbs the substrate. In contrast to the degrader *P. veronii*, benzene could be removed from the *E. coli* cells by a few washing steps.

Table 4-4: ***Radioactivity balance after 1 h incubation with radioisotope-labelled benzene for two model microorganisms.***

Radioisotope labelling of cells	***E. coli***		***P. veronii***	
	Activity		Activity	
	[Bq]	[%]	[Bq]	[%]
used in labelling	*14700000.00*	*100.00*	*14700000.00*	*100.00*
ether traps	12277987.50	83.524	11890125.00	80.885
NaOH traps	849.10	0.006	249825.00	1.699
washing solutions	678855.21	4.618	735766.33	5.005
cell suspension	**332.21**	**0.002**	**201853.28**	**1.373**
total recovery	*12958024.02*	*88.150*	*13077569.61*	*88.963*

For the model system, a clear labelling of compound-transforming microbes could be achieved within a short incubation time under cultivation conditions. This allows the conclusion that for metabolically active communities similar results can be obtained. Depending on the microorganism, slightly adapted incubation parameters will likely have

to be applied, but equivalent studies close to authentic *in situ* conditions have been successfully conducted before in several MAR and MAR-FISH studies (Andreasen and Nielsen, 1997; Lee et al., 1999; Ouverney and Fuhrman, 1999; Gray et al., 2000; Nielsen et al., 2002).

Encapsulation of radioactively labelled cells combined with MAR

Subsequently it was tested whether the attained incorporation of radioactive benzene enabled the visual differentiation of degrader and non-degrader cells isolated in microcapsules by the new MAR protocol. ^{14}C-benzene-incubated cells of *E. coli* and *P. veronii* were separately encapsulated into microbeads. The process was down-scaled to a few cells per capsule (approximately 5 cells per 50 µm capsule). The average capsule diameters attained per experiment are presented in Figure 4-14.

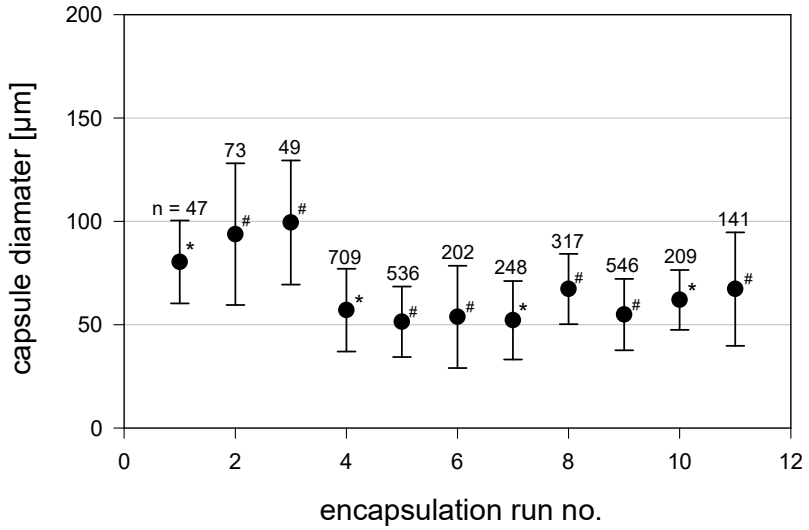


Figure 4-14: ***Reproducibility of the capsule diameter in the microencapsulation process. Measured capsules include either* E. coli *(*) or* P. veronii *(#). n = sample size. The procedure was repeated 11 times.***

Although all 11 experiments were carried out under the same parameters and conditions, a variation of the mean diameter and its standard deviation was recognizable. Especially the first three runs clearly showed higher values. Those fluctuations could also be observed during development of the encapsulation. They possibly arose through variations in mixing alginate and photoemulsion in the dark, when volumes may have varied when pipetting the viscous solutions. It is more likely, however, that the actual geometry of the device connected to the high forces inside of the capillaries is crucial. Due

to costs, PEEK connectors were reused in many experiments. Although they were cleaned and rinsed with different organic solvents, residual sunflower seed oil tends to form resins in the presence of oxygen. These resins can cause poorly soluble deposits and change the conditions in the tubing. In this application implemented with PEEK-technology, the flow-rate ratio, primarily the flow-rate of the continuous phase, for reaching capsule diameters of less than 100 µm was very high compared to other applications (Hong et al., 2007; Shintaku et al., 2007; Workman et al., 2007; Capretto et al., 2008; Workman et al., 2008). The resulting high pressure and small inhomogeneities were probably the reason for the variation of capsule sizes and large standard derivations. However, the approach was deemed sufficiently adequate for proving the principles of MAR-EC.

Capsules with the benzene-degrading *P. veronii* were incubated for up to 3 days and subsequently were treated by the MAR protocol. Although the incorporation of organic carbon (1.4 %) appeared relatively low, after only 1 day exposure clearly visible silver grain signals were visible inside the capsules (↗ Figure 4-15). This underlines the high sensitivity of the approach. Signals did not become significantly larger after 2 or 3 days' exposure. Already exposed photosensitive material seemed to absorb additional radiation and thus limited the range of the radiation detected, consequently hindering the formation of stronger signals. The short exposure time suggests that MAR-EC is indeed an applicable new method.

Cells of the negative control *E. coli* were incubated within capsules for up to 4 days, in one long-term experiment even 9 days. No silver granule signals could be observed (↗ Figure ***4-15***). Capsules remained completely clear even after long exposure times.

Consequently, an unambiguous visual differentiation of microcapsules with degrader and with non-degrader cells became possible.

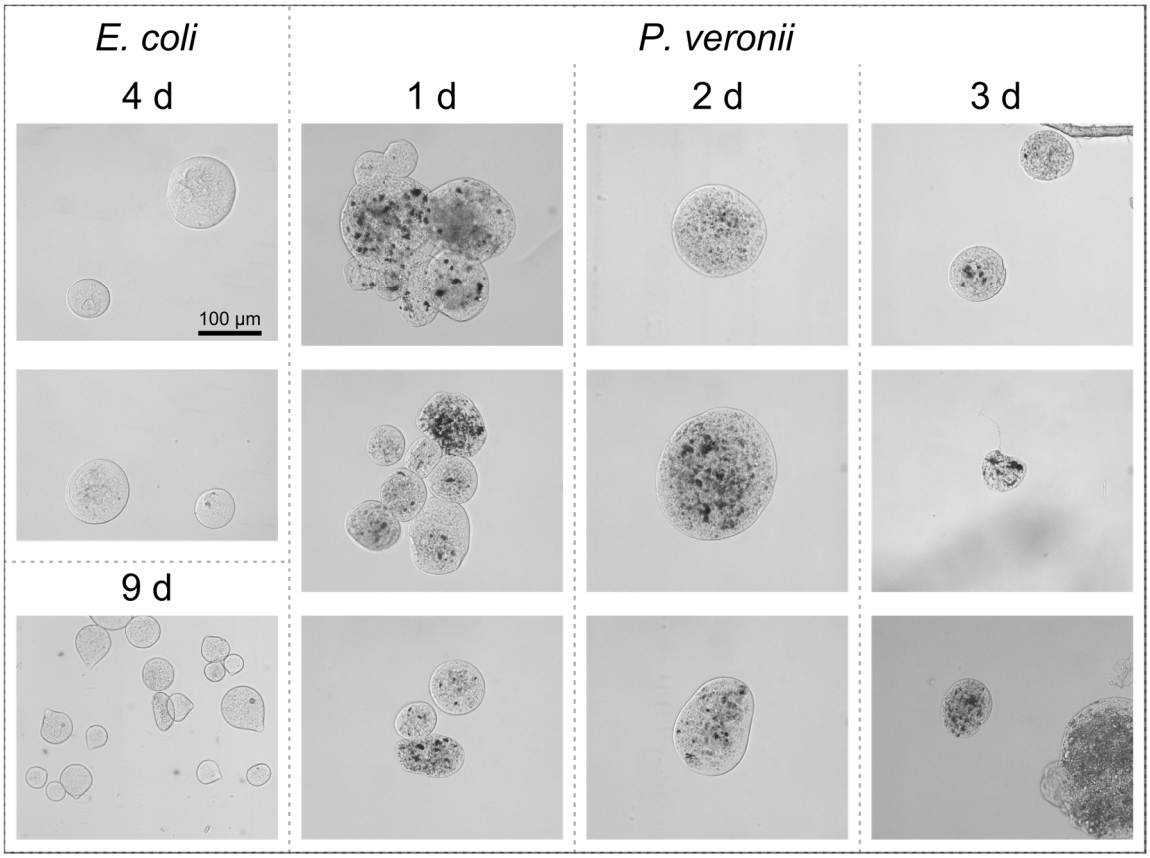


Figure 4-15: ***MAR-processed microcapsules harbouring either non-degrading* E. coli *or degrading* P. veronii*. MAR capsules were exposed for various time periods of up to 9 days.***

4.3.8 Conclusions

Within the framework of this study a new approach for *in situ* identification of active degraders without prior phylogenetic information was developed and evaluated. The proof-of-principle experiment demonstrated the potential of microencapsulation coupled to microautoradiography. Here, the overall approach could be confirmed developing this MAR approach with high sensitivity and with bacterial numbers down to the level of a few cells (in some cases single cells) spatially isolated in microcompartments. Based on first molecular biological investigations, the DNA remained stable and its amplification resisted any inhibition by physical parameters, such as structures, matrices and by the chemicals used in the newly established protocol. Therefore, identification of microbial degradation processes at the single-cell levels using the applied capsules seems to be achievable.

At the first stage an innovative new screening approach, which is intended to identify *in situ* metabolically active microbes without prior genetic information was established. The protocol allows going from sampling to the selection of active degraders and

obtaining their phylogenetic information within a few days. Due to the high-throughput nature of the process options, the screening of whole communities seems to be feasible.

However, the new approach is not yet “ready-to-use” and requires further optimization. One of the primary concerns for the next stage is to reliably achieve single-cell level in a majority of capsules. Due to the size of the silver grain signals, the ratio of the signal to the bead diameter has to be decreased by the production of smaller capsules. Mainly an improved reproducibility and a smaller size variation, but perhaps also the overall reduction of the capsule size, requires switching from the encapsulation via the PEEK device to a more precise and more reliable and reproducible system. In principle, the microfluidic chip technology enables the implementation according to the same parameters, but perhaps provides more flexibility. Even though the use of commercially available chips was not successful at the beginning of this study, their application has been described several times (Huang et al., 2006; Choi et al., 2007; Hong et al., 2007; Shintaku et al., 2007; Capretto et al., 2008; Workman et al., 2008). Based on the empirically obtained knowledge during these experiments and a suitable cooperation with chip producers, a single-cell application should be realisable soon.

Another point that is absolutely necessary to consider is the establishment of a high-throughput sorting of capsules with and without signals. The initially considered separation based on differences in the density of beads with and without silver crystals is not feasible due to in-homogeneities in the density of the alginate beads themselves. A sorting based on light scattering in the style of flow cytometry would be favoured, since it principally can cope with a high number of microcapsules in a very short time. A similar approach has been applied on a trial basis for alginate beads with entrapped and fluorescently stained cells separating them from free cells and empty beads (Borner et al., 2011; Borner, 2013). Preliminary experiments at the DSMZ (Anne-Kristin Kaster) suggest that flow cytometry-based sorting of encapsulated cells is indeed possible.

Finally, a protocol for whole-genome amplification in the form of MDA needs to be developed and optimised for single cells from inside of alginate beads. Then, subsequent molecular-biological analysis will enable the identification of metabolically active cells.

5 General Conclusions

This work targeted in-depth analyses of microbial communities involved in compound turnover in natural and engineered environmental systems. Particular focus was on the *in situ* identification of active microbial key players via isotope labelling strategy. To this end the spectrum of methods, which are not based on previous phylogenetic information, was improved.

Firstly, the **Stable Isotope Probing** approach was combined with *in situ* incubation as a component of the BACTRAP technology.

In PART A1 the linked application of *in situ* microcosms and RNA-SIP with in-depth phylogenetic analysis via high-throughput community sequencing aimed at *in situ* identification of active microbial benzene degraders at a model constructed wetland system. This was an innovative approach as this set of methods had not been applied together before, and not much was known about the *in situ* role of microbes degrading benzene in laboratory cultures. Within this study, two known contaminant-transforming genera, namely *Dechloromonas* spp. and *Zoogloea* spp., were assigned as those responsible for degrading benzene, probably under aerobic conditions, in the *in situ* microcosms.

Beyond this, several shortcomings of this methodological approach were identified. In general, the applied methodological path was an extension, or merger, of separately well-established and routinely used approaches for the *in situ* investigation of microbial communities (Mandelbaum et al., 1997). The BACTRAP technology (Geyer et al., 2005; Kästner et al., 2006; Stelzer et al., 2006) allowed growth of active benzene degraders and labelling of biomarkers with stable isotopes under close to real *in situ* conditions. This is difficult to achieve with any other technology, particularly with regard to time and costs. The metagenomic analysis of fractionated, labelled RNA was a methodological improvement in the study. The taxonomical variety of the samples was demonstrated in a precision and depth, which could not be achieved with T-RFLP, DGGE or SCCP. Unfortunately, the isotope probing itself did not work adequately for the considered system. High concentrations of the unlabelled contaminant and to some extent other carbon sources led to insufficient labelling. In the absence of highly labelled fractions, only a tentative identification of some players could be realised, which might have thrived also on other carbon sources.

A more general deficiency of the SIP concept itself concerns the relevance of detected degraders in the habitat. Due to high costs of the labelled substrate, the SIP approach relies on a form of microcosm – *ex situ* or better, as applied here, *in situ*. Although when

compared with laboratory microcosms, BACTRAPs are strongly preferable for *in situ* monitoring, like laboratory microcosms they can lead to results with restricted information about the habitat. Carrier material for labelled carbon source is not sediment material in the natural system and might be colonized only by certain microbes (Bennett et al., 2000; Griebler et al., 2002). Active microbes on the BACTRAP, even if strongly labelled with stable isotopes, may not play a key role in the habitat (Hendrickx et al., 2005).

In PART A2, the aim was to extend the BACTRAP *in situ* incubation concept to the micropollutant BPA. The results of these experiments were not particularly favourable concerning the applicability of the BACTRAP technology for BPA. Although tests on adsorption, desorption and mineralisation in the laboratory were promising, a BACTRAP field-test showed no microbial growth or ^{13}C-incorporation on the carrier material.
The availability of BPA for microbes might have been severely limited due to an overly strong sorption to the activated carbon. Due to the low BPA concentrations applied, the low incorporation might have been assisted by the dominant use of other carbon sources available in the habitat. In addition, the low concentrations of the micropollutant in the wetland system under investigation might have led to a generally low abundance of BPA degraders. However, based on the experiences gained in the study of benzene degradation in the constructed wetland system (PART A1), at least some microbial biofilm/biomarkers based on other available carbon sources were anticipated for the comparable Shallow Aquifer Rhizodegradation pilot plant (PART A2). The question is whether there was indeed any labelled biomarker present or whether biomarkers could not be extracted properly from the BACTRAP. Longer exposure times with increased BPA concentrations may give an answer and may help to evaluate the use of the SIP/BACTRAP combination for BPA. Nevertheless, the applicability of the SIP/BACTRAP approach for micropollutants may remain restricted to the more sensitive PLFA- and Protein-SIP. In any case, the same caveat described in PART A1 remains.

Secondly, to overcome current limitations on sensitivity and environmental complexities, **Radioisotope Probing** might be a solution for authentic *in situ* identification and activity assessment of relevant key microbial players in contaminated systems. Up to now, this approach has relied on gene probes. PART B of this thesis aimed to optimize conditions to circumvent the dependence on prior phylogenetic or catabolic information with a sophisticated idea of a direct identification for *in situ* active microorganisms: '**M**icro**A**uto**R**adiography of **E**ncapsulated **C**ells (MAR-EC)'. While MAR-EC is not yet applicable to environmental samples, critical steps towards implementing the new methodology were achieved and the potential of the overall approach could be confirmed. It will enable probe-independent identification as with SIP but at the same time will

overcome the main problem of SIP by fast and much more sensitive labelling under authentic *in situ* conditions by using ^{14}C or other radioactive isotopes.
In contrast to MAR, which is often coupled with FISH technology, the high-throughput application of MAR-EC aimed for in this study reduces the impact of misinterpretation of false-positive signals. Additionally, RIP, when compared to developments including Raman and SIMS technologies incurs lower costs and technological effort for encapsulation and subsequent MAR.
However, MAR-EC depends on working with radioactivity, albeit in small doses, which in many countries requires permissions regarding safety regulations (e.g. separate laboratories, equipment, procuring and disposing of radioactive substrates and waste). Furthermore, it is restricted by the availability of expensive labelled substrates. Radioactively labelled substrates might be more expensive than their already costly stable isotope-labelled counterparts, even though lesser amounts are needed due to MAR-EC's sensitivity.

Open questions regarding the SIP and RIP applications mainly concern the type of the environmental system and/or sample to be analyzed. SIP, coupled with the BACTRAP technology, is typically used in aqueous media, whereas recently developed Direct-Push-BACTRAPs have made water-saturated and water-unsaturated zones of soil accessible to analysis (Schurig et al., 2014). The use of soil or sediment samples for MAR-EC will require an effective detaching of biomass from the sample matrix. Since the problem also appears for MAR-FISH, solutions may be found and tested for MAR-EC as have been reported for MAR plus FISH (Rogers et al., 2007). The most important aspect here is to remove and separate intact single cells.
Whereas a realistic quantification of *in situ* degradation via SIP might fail due to an artificial colonisation of the BACTRAP, MAR-EC cannot be used for a quantification of degradation rates. However, the results could be used for primer or probe design and therefore for a variety of further molecular biological analyses, both qualitative and quantitative.
One of the biggest challenges for MAR-EC as a tool for single-cell analysis is the potential contamination with DNA from other sources. In the absence of DNA contamination, however, whole genome amplification via e.g. MDA will allow genome analysis and would therefore deliver more information than 16S rRNA-based identification of actively degrading microorganisms.

Within this thesis, the set of tools for *in situ* identification of key active microbial players that are not based on previously acquired phylogenetic information could be extended using a novel combination of available approaches and by the development a new method that will hopefully soon be readily available for full application.

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Declaration of Independent Work / Eigenständigkeitserklärung

Herewith I, Henrike Nitz, born on the 22.06.1987 in Zeitz, confirm that I am familiar with the promotion regulations of the Faculty I of Natural Science - Biological Science of the Martin-Luther-University Halle-Wittenberg. I further approve that this present doctoral thesis, entitled 'Monitoring tools for microbial *in situ* activity in natural and engineered environmental systems' represents my own work in accordance with the university regulations and no other support than listed were used in this work. This doctoral thesis was only submitted at the faculty board of the Faculty I of Natural Science - Biological Science of the Martin-Luther-University Halle-Wittenberg and nowhere else for scientific evaluation or dissertation.

Hiermit erkläre ich, Henrike Nitz, geboren am 22.06.1987 in Zeitz, dass ich mit der Promotionsordnung der Naturwissenschaftliche Fakultät I – Biowissenschaften der Martin-Luther-Universität Halle-Wittenberg vertraut bin. Außerdem erkläre ich, dass ich die hier vorgelegte Dissertation mit dem Titel "Monitoring tools for microbial *in situ* activity in natural and engineered environmental systems" 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.

25.9.2018 Henrike Nitz

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25.9.2018 Henrike Nitz

Publications and Presentations

Nitz, H., Lünsmann, V., Duarte, M., Jauregui, R., Pieper, D. H., Müller, J. A., Kästner, M. 2017. Investigation of microbial benzene degradation in a constructed wetland using *in situ* microcosms in combination with stable isotope probing and 16S rRNA sequence analysis. in preparation.

Zanaroli, G., Beck, H., Beimfohr, C., Cichocka, D., Corvini, P., Frascari, D., Hofmann, U., Kästner, M., Macek, T., Müller, J. A., Uhlik, O., Schlosser, D. (2015): Chapter 2 – Analytical and monitoring methods. In: Hochstrat, R., Wintgens, T. and Corvini, P. (Eds.) Immobilized Biocatalysts for Bioremediation of Groundwater and Wastewater, IWA Publishing, London, pp. 15-48.

Schurig, Christian; Mueller, Carsten W; Höschen, Carmen; Prager, Andrea; Kothe, Erika; Beck, Henrike; Miltner, Anja; Kästner, Matthias. (2015). Methods for visualising active microbial benzene degraders in *in situ* microcosms. Applied Microbiology and Biotechnology; Heidelberg 99(2): 957-968.

Beck, H., Duarte, M., Jauregui, R., Pieper, D. H., Müller, J. A., Kästner, M. Poster Presentation. Investigation of microbial benzene degradation in a constructed wetland using *in situ* microcosms in combination with stable isotope probing and 16S rRNA sequence analysis. Annual Conference of the Association for General and Applied Microbiology (VAAM), Marburg, Germany, 2015.

Beck, H., Sigrist, R., Mosler, S., Müller, J. A., Kästner, M. Oral Presentation. Hunting for active degraders: Novel screening approach combining substrate-specific radiolabelling of cells and their separation in microcompartments. Annual Conference of the Association for General and Applied Microbiology (VAAM), Dresden, Germany, 2014.

Beck, H., Sigrist, R., Mosler, S., Müller, J. A., Kästner, M. Poster Presentation. Hunting for active degraders: Novel screening approach combining substrate-specific radiolabelling of cells and their separation in microcompartments. The 5th Congress of European Microbiologists (FEMS), Leipzig, Germany, 2013

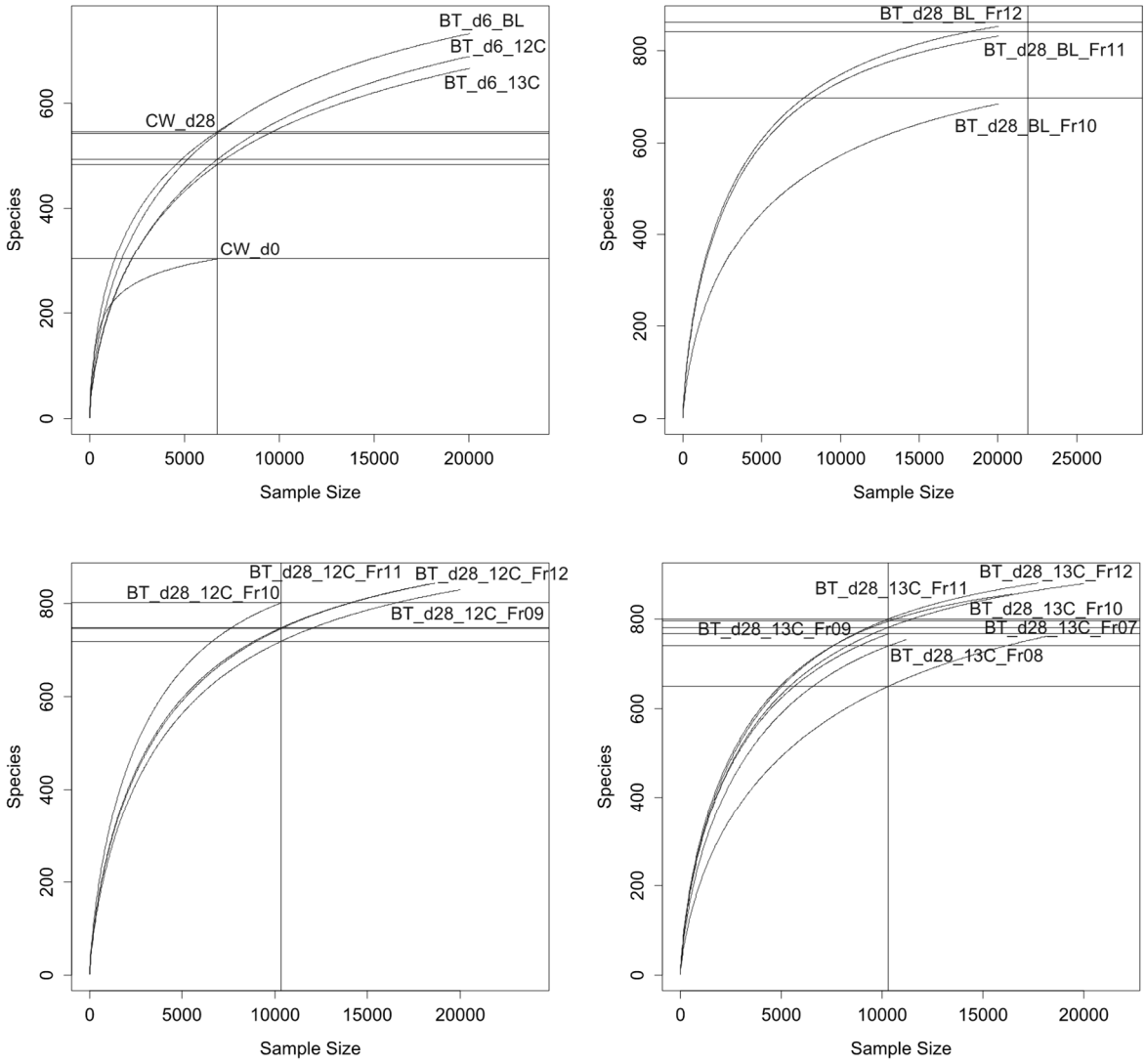
Beck, H., Sigrist, R., Mosler, S., Müller, J. A., Kästner, M. Poster Presentation. Screening for degraders: Novel identification approach using substrate-specific radiolabeling of cells isolated in microcompartments. Annual Conference of the Association for General and Applied Microbiology (VAAM), Bremen, Germany, 2013.

Appendix

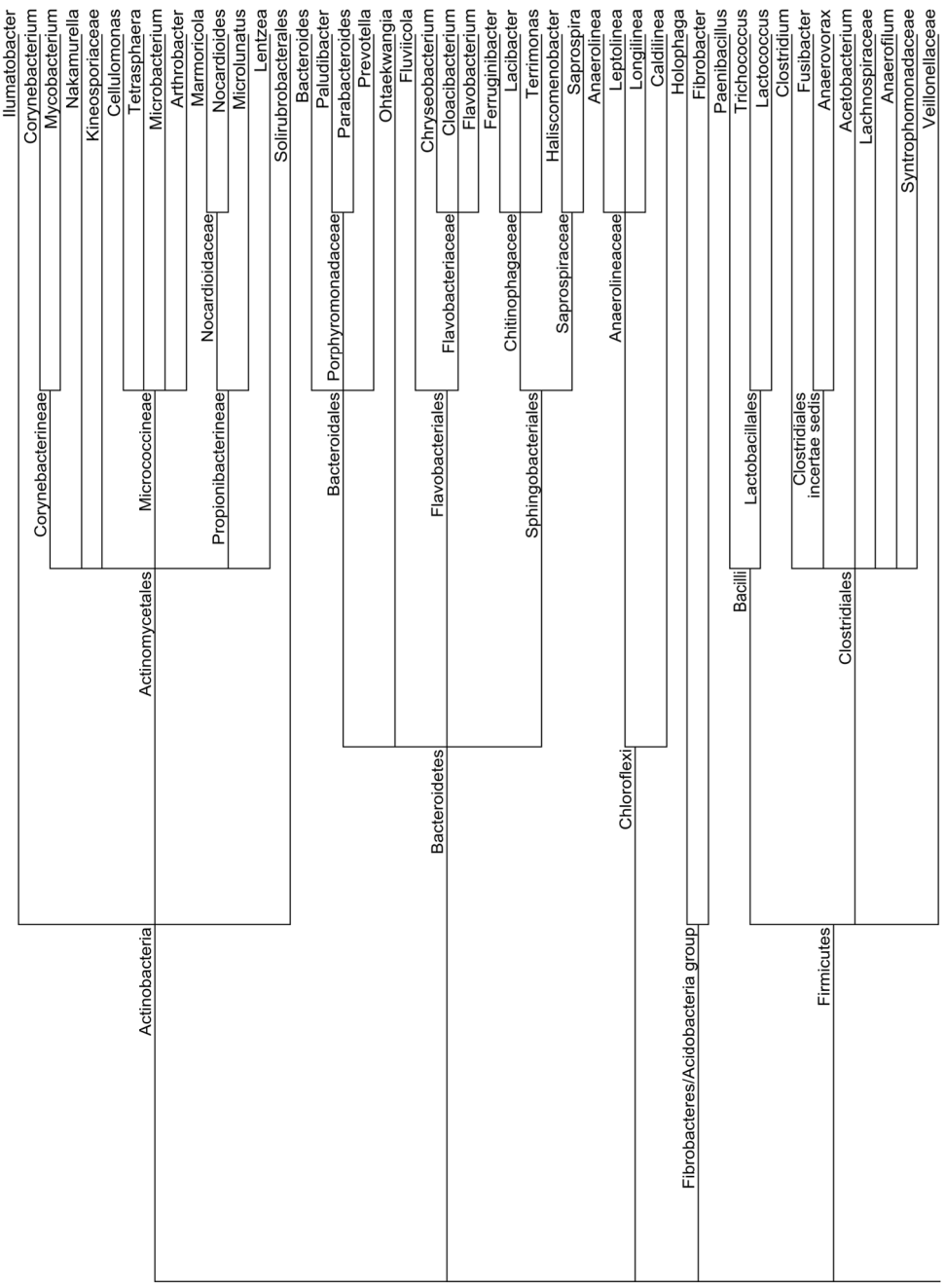
Appendix 1: Illumina primers used in the SIP study.

Primer name	Primer Sequence (5'-3')	Reference
IlluFBC17	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCCGTCAAGAGTTTGATCMTGGCTCAG	Camarinha-Silva et al. (2014)
IlluFBC18	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGCTCCAAGAGTTTGATCMTGGCTCAG	
IlluFBC19	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATAGACCAAGAGTTTGATCMTGGCTCAG	
IlluFBC20	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATATTGCAAGAGTTTGATCMTGGCTCAG	
IlluFBC33	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAAGACAAGAGTTTGATCMTGGCTCAG	
IlluFBC34	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTCCATCAAGAGTTTGATCMTGGCTCAG	
IlluFBC35	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTCGGCCAAGAGTTTGATCMTGGCTCAG	
IlluFBC36	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGCGGCAAGAGTTTGATCMTGGCTCAG	
IlluFBC37	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACCTTCAAGAGTTTGATCMTGGCTCAG	
IlluFBC38	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACGACCAAGAGTTTGATCMTGGCTCAG	
IlluFBC39	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGATTAGCAAGAGTTTGATCMTGGCTCAG	
IlluFBC40	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATCCCAAGAGTTTGATCMTGGCTCAG	
IlluFBC41	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCAGCCAAGAGTTTGATCMTGGCTCAG	
IlluFBC42	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGACTCAAGAGTTTGATCMTGGCTCAG	
IlluFBC43	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTCATCAAGAGTTTGATCMTGGCTCAG	
IlluFBC44	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGCCAACAAGAGTTTGATCMTGGCTCAG	
IlluFBC45	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGCTTCCAAGAGTTTGATCMTGGCTCAG	
IlluFBC46	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTACCCAAGAGTTTGATCMTGGCTCAG	
IlluFBC47	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAATCCAAGAGTTTGATCMTGGCTCAG	
IlluFBC48	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTACGTCAAGAGTTTGATCMTGGCTCAG	
Multiplexing PCR	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	Illumina Inc.
IlluRevAdap	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCTGCCTCCCGTAGGAGT	
Index 5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTC	
Index 8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTC	

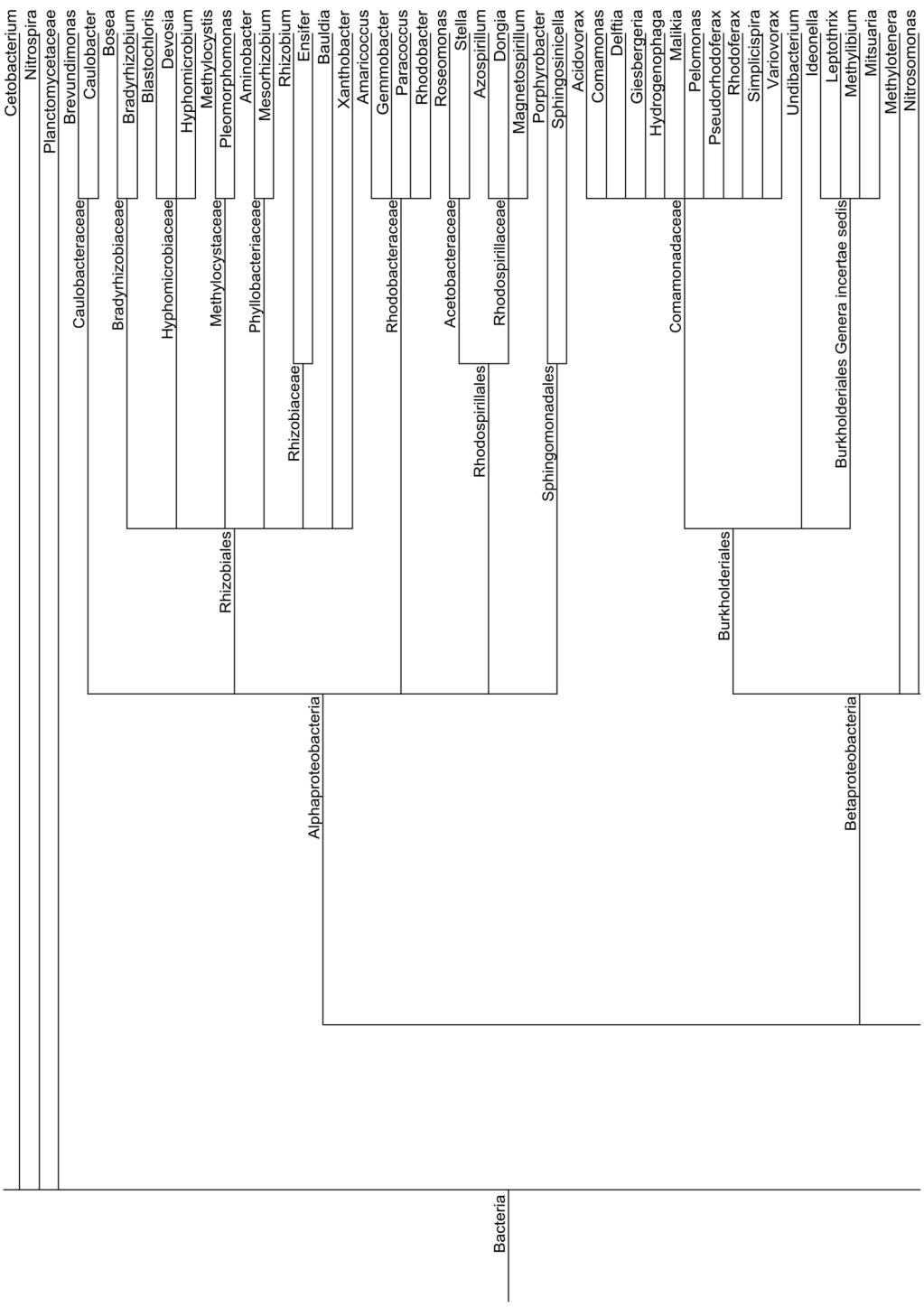
Appendix 2: Rarefaction curves of Illumina sequencing data set.



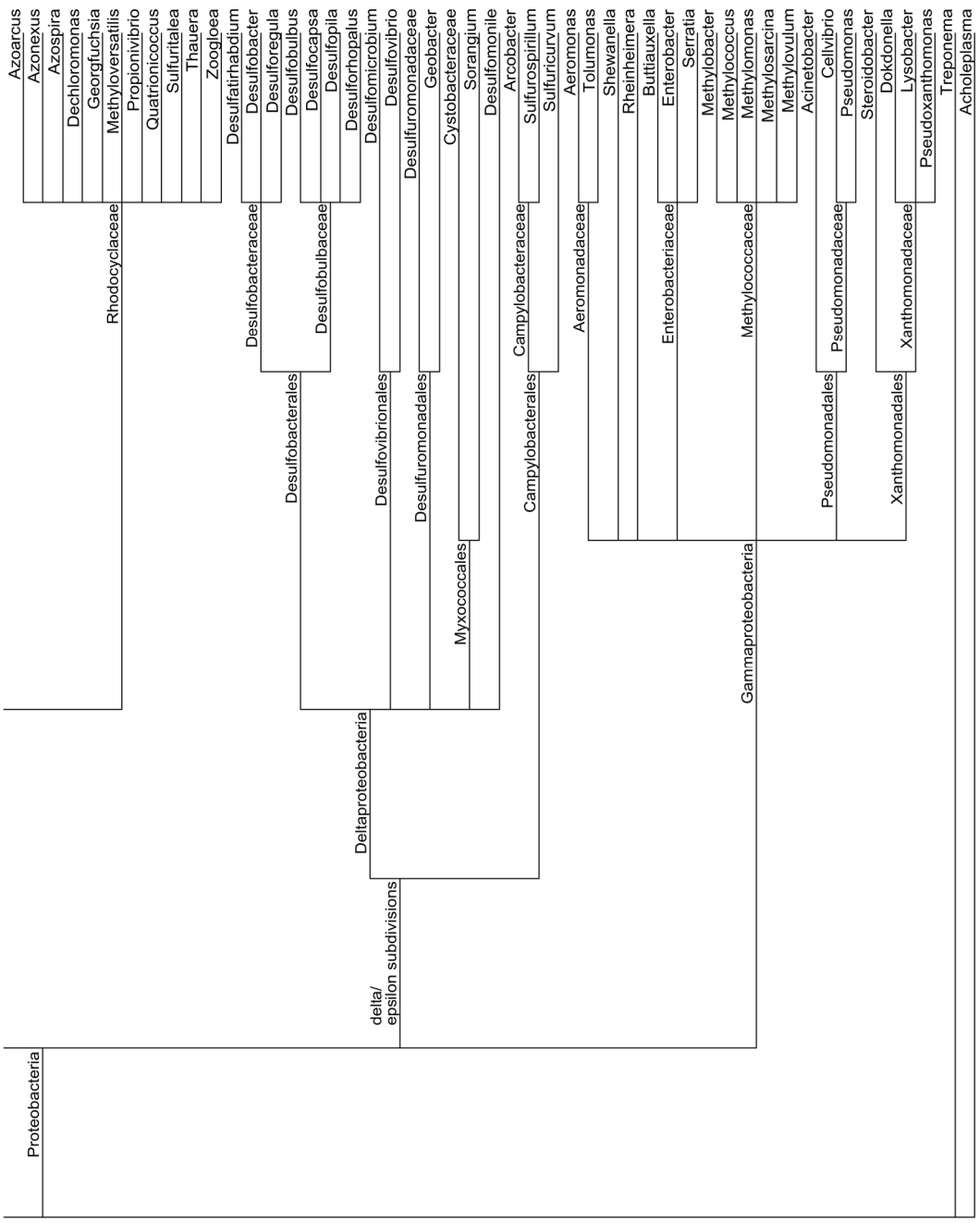
Appendix 3 part 1/3: Assigned sequences to taxonomic groups.



Appendix 3 part 2/3: Assigned sequences to taxonomic groups.



Appendix 3 part 3/3: Assigned sequences to taxonomic groups.



Appendix 4: ***ADONIS: Non-parametric statistical test of the general multivariate hypothesis of differences in the composition and/or relative abundances of organisms of different species in samples from different groups. Samples were grouped, and group means tested of significant dissimilarities by multivariate analysis of variances based on Bray-Curtis distance matrices.***

#	CW, BT_6d, BT_28d	BT_28d	BT_28d_13C
samples group 1	CW_0d, CW_28d	BT_28d_BL_10, BT_28d_BL_11, BT_28d_BL_12	BT_28d_13C_07, BT_28d_13C_08
samples group 2	BT_6d_BL, BT_6d_12C, BT_6d_13C	BT_28d_12C_09, BT_28d_12C_10, BT_28d_12C_11, BT_28d_12C_12	BT_28d_13C_10, BT_28d_13C_11, BT_28d_13C_12
samples group 3	BT_28d_BL, BT_28d_12C, BT_28d_13C	BT_28d_13C_07, BT_28d_13C_08, BT_28d_13C_09, BT_28d_13C_10, BT_28d_13C_11, BT_28d_13C_12	-
p-value (alpha of 0.05)	0.017 (*)	0.091 (.)	0.099 (.)

‘(*)’ significant, ‘(.)’ only a tendency