Investigation of the importance of trophic interaction and microbial food webs in aquifers for the natural attenuation potential of groundwater

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Abstract: Heterotrophic protists represent a very important trophic link within food chains in different ecosystems, as they shuttle energy and organic matter from prokaryotes to higher trophic levels. In groundwater, they may even themselves represent the most relevant higher trophic level due to the scarcity of higher organisms. But their functional relevance in groundwater ecosystems and their influence on groundwater natural attenuation is only poorly understood. There is a severe lack of knowledge on the biodiversity and activity of natural protozoan assemblages in aquifers, most of all in anaerobic and contaminated zones. Furthermore, also the importance of other microeukaryotes such as fungi as potential contaminant degraders has not been systematically addressed yet. Partly, this can be attributed to a lack of adequate PCR and fingerprinting approaches for protists in aquifers.

Within this thesis, essential tools for the molecular characterisation of microeukaryotic communities in aquifers were successfully established. The suitability of distinct eukaryote-targeted primer pairs were validated and found to differ widely in their ability to detect over-all protistan diversity. By *in silico* predictions, fingerprinting and sequencing of microeukaryote amplicons from hydrocarbon-contaminated aquifer sediment DNA, I could show that the Euk20f/Euk516r primer set in combination with *Bsh*1236I digestion is best suited for the recovery of diverse protistan 18S rDNA lineages with T-RFLP analysis.

These newly developed molecular assays were used to assess protistan communities in distinct redox-zones of a tar-oil contaminated aquifer. A diverse microeukaryote community was detected with profound depth-related changes in diversity and composition hinting at a coupling to local biogeochemical and microbial parameters. Ciliates and cercozoa were found predominantly in the upper, highly BTEX contaminated part of the aquifer, where prokaryotic prey abundances were highest. Kinetoplastid nanoflagellates dominated protozoan communities in the sulphidogenic gradient zone, the 'hot-spot' of bacterial BTEX degradation, and the PAH contaminated strata below. Therefore, detected protozoan groups hint at an active microbial loop, which may influence biodegradation *in situ* to differing extents (quantitatively and qualitatively) in respective zones. Additionally, the detection of a diverse fungal community dominated by yeasts may also indicate a direct involvement of microeukaryotes in contaminant turnover, most of all, as the majority of fungi identified was related to isolates known for aerobic degradation of aromatic hydrocarbons. Their activity and utilisation of substrates under anaerobic conditions, however, remains to be elucidated.

The applicability of DNA-SIP to groundwater protists was evaluated by feeding of ^{13}C labelled prey to a protistan predator, revealing critical detection limits. My established assays were tested on DNA extracts of a SIP experiment addressing toluene degradation under sulphate reduction. These first results show that SIP can be applied to anaerobic contaminant degrading food chains and make further approaches in SIP very promising. The cultivation and isolation of groundwater protists, a prerequisite to unravel the potential role and relevance of distinct groups in groundwater ecosystem functioning, proved to be problematic for contaminated sediment samples due to excessive growth of fungi. Aerobic and anaerobic enrichment cultivations further emphasised the need of refined anaerobic cultivation techniques to be established.

To summarise, molecular tools for the characterisation of protistan communities in contaminated aquifers were successfully established. At an exemplary site, I show that a protistan diversity far greater than previously recognised resides even in contaminated groundwater sediments, and that local protistan populations display similarly significant spatial distinctions in correlation to redox and contaminant scenarios as previously observed

for *Bacteria*. The prospective further application of such molecular tools to study food webs and carbon flow in groundwater ecosystems may further add to an understanding of factors enhancing or limiting biodegradation *in situ*.

Untersuchung der Bedeutung trophischer Interaktionen und mikrobieller Nahrungsnetze in Grundwasserleitern für das Selbstreinigungspotential von Grundwasser

Zusammenfassung: Protozoen gelten in Nahrungsketten verschiedener Ökosysteme als wichtige trophische Verbindung, da sie Energie und organische Verbindungen von Prokaryoten in höhere Trophieebenen übertragen. Da höhere Organismen im Grundwasser selten sind, stellen sie Protozoen dort möglicherweise die bedeutendste höhere trophische Ebene dar. Ihre funktionelle Bedeutung in Grundwasserökosystemen und ihr Einfluss auf das Selbstreinigungspotential von Grundwasser wurden jedoch bislang noch unzureichend erforscht. Eine gravierende Wissenslücke existiert bezüglich der Biodiversität und Aktivität natürlicher Protozoengemeinschaften in Grundwasserleitern. Dies gilt insbesondere für anaerobe und verunreinigte Bereiche. Des Weiteren wurde die Bedeutung mikroskopischer Eukaryoten, wie etwa Pilzen, als potentielle Schadstoffabbauer bislang nicht systematisch erfasst. Dies kann zum Teil auf fehlende PCR- und Fingerprintingmethoden, die für Protisten des Grundwassers geeignet wären, zurückgeführt werden.

Im Zuge dieser Arbeit wurden daher notwendige Verfahren entwickelt, mit deren Hilfe Gemeinschaften von einzelligen Eukaryoten in Grundwasserleitern auf molekularer Ebene beschrieben werden können. Die Eignung verschiedener 18S rDNA spezifischer Primerpaare wurde überprüft, wobei gravierende Unterschiede gefunden wurden bezüglich ihrer Fähigkeit, die gesamte Vielfalt von Protisten zu erfassen. Die Primerkombination Euk20f/Euk516r in Verbindung mit Restriktionsenzym *Bsh*1236I erwies sich am geeignetsten, um 18S rDNA unterschiedlichster Abstammung mittels T-RFLP Analyse zu erfassen. Dies wurde durch *in silico* Vorhersage, sowie Fingerprinting und Sequenzierung protistischer PCR-Amplikons aus Nukleinsäureextrakten gezeigt, die aus kontaminierten Grundwassersedimenten stammten. Mit Hilfe der neu entwickelten molekularen Methoden konnten die Protistengemeinschaften in unterschiedlichen Redoxzonen eines mit Teeröl kontaminierten Grundwasserleiters beschrieben werden. Die so nachgewiesene vielfältige Gemeinschaft mikroskopischer Eukaryoten zeigte deutliche tiefenabhängige Unterschiede bezüglich ihrer Vielfalt und Zusammensetzung. Dies könnte auf eine Kopplung an lokale biochemische und mikrobielle Faktoren hinweisen.

Ciliaten und Cercozoen wurden vor allem im oberen, stark mit BTEX verunreinigten Teil des Grundwasserleiters entdeckt, wo auch bakterielle Abundanzen am höchsten waren. Dagegen dominierten Nanoflagellaten der *Kinetoplastida* die Protozoengemeinschaft der sulfidogenen Zone, wo sich der Hotspot des bakteriellen BTEX Abbaus befindet, aber auch der PAK kontaminierten Bereiche darunter. Die entdeckten Protozoengruppen lassen auf eine aktive mikrobielle Schleife schließen, die möglicherweise den biologischen Abbau der Schadstoffe *in situ* beeinflusst, wobei sich das Ausmaß in verschiedenen Zonen quantitativ und qualitativ unterscheiden dürfte. Des Weiteren deutet die diverse Pilzgemeinschaft, die von Hefen dominiert wurde, eine direkte Beteiligung mikroskopischer Eukaryoten an dem Umsatz von Schadstoffen an, da ein Großteil der identifizierten Pilze sich als Verwandte von Stämmen erwiesen, die für den aeroben Abbau von aromatischen Kohlenwasserstoffverbindungen bekannt sind. Allerdings muss ihre Aktivität und die Verwertbarkeit unterschiedlicher Substrate unter anaeroben Bedingungen noch gezeigt werden.

Die Anwendbarkeit von DNA-SIP auf Grundwasserprotisten wurde untersucht, indem ^{13}C markierte Beute an einen protistischen Räuber verfüttert wurde. Dabei wurden kritische Schwellenwerte in der Detektierbarkeit festgestellt. Meine entwickelten Verfahren wurden abschließend an DNA Extrakten getestet, die aus einem Experiment stammten, das sich mit

dem anaeroben Abbau von Toluol unter Reduktion von Sulfat beschäftigte. Erste Ergebnisse zeigten, dass SIP angewandt werden kann, um Nahrungsketten während des anaeroben Abbaus von Schadstoffen zu untersuchen. Weitere Forschungsansätze von SIP erscheinen daher äußerst viel versprechend.

Die Kultivierung und Isolierung von Grundwasserprotisten ist eine Vorrausetzung, um die potentielle Rolle und Bedeutung verschiedener Gruppen für die Funktionsfähigkeit des Ökosystems Grundwasser zu erfassen. Allerdings erwies sich dies während meiner Arbeit mit verunreinigten Grundwassersedimenten als äußerst problematisch, vor allem bedingt durch das übermäßige Wachstum von Pilzen. Unterschiede zwischen aeroben und anaeroben Anreicherungskultivierungen betonten jedoch die Notwendigkeit, vor allem verfeinerte anaerobe Kultivierungsmethoden zu entwickeln.

Zusammenfassend wurden in meiner Arbeit molekulare Verfahren entwickelt, die die Charakterisierung protistischer Gemeinschaften in kontaminierten Grundwasserleitern ermöglichen. An einem beispielhaften Standort konnte ich eine weitaus größere Diversität von eukaryotischen Einzellern sogar in verunreinigtem Grundwassersediment nachweisen, als sie bislang festgestellt werden konnte. Außerdem zeigten die jeweiligen Populationen ähnlich deutliche räumliche Unterschiede und Korrelation an Redoxzonen und Kontaminationsbereiche, wie sie auch schon für Bakterien beobachtet wurden. Die zukünftige Anwendung solcher molekularer Methoden zur Untersuchung von Nahrungsnetzen und Kohlenstoffflüssen in Grundwasserökosystemen, wird womöglich weiterhelfen die Faktoren zu verstehen, die den biologischen Abbau von Schadstoffen *in situ* verstärken oder limitieren.

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1 General introduction

1.1 Groundwater – endangered resource and its bioremediation

Groundwater is known to be the largest freshwater reservoir of the world (~ 30% of total global freshwater) and serves as humankind's most important potable water resource (Shiklomanov, 1998). Its constant availability in space and time, stable quality and a better protection against contamination have been some of the advantages of groundwater as a supply source compared to surface water (Zektser and Margat, 2003). Major advances in geological knowledge and hydrogeological technology have led to heavy exploitation of aquifers since the 19th century to satisfy the increasing needs of a growing human population. Current global withdrawal rates are estimated to be in a region of up to 700 billion m^3 $year^{-1}$, used in three main sectors: drinking water (65%), irrigation and livestock (20%), industry and mining (15%). Concern about maintaining freshwater quantity and quality is making sustainable resource management of groundwater a major topic of global policy today. Increasing world population and ongoing industrialisation is causing excessive groundwater exploitation and anthropogenic pollution leading to aquifer degradation (Foster and Chilton, 2003). Aquifer salinisation and dropping water tables, but also contamination of groundwater with xenobiotic compounds are some problems that have to be faced. The latter received growing attention since the 1980s and different remediation strategies have been developed up today. Mainly caused by high costs, for example for excavation and burning of contaminated sediments, bioremediation and natural attenuation are spotlighted nowadays (Jørgensen et al., 2007).

Groundwater systems have the capability to resist contamination to a certain degree due to natural attenuation mechanisms like dilution or hydrolysis of contaminants and transformation or mineralisation caused by microbial activities (Andreoni and Gianfreda, 2007). Until recently, the main attention of research concerning the role of microorganisms in biodegradation was paid on contaminant degrading bacteria. A variety of strains has been detected that are capable of degrading pollutants like nitroaromatics, chloroaromatics, polycyclic aromatics and petroleum compounds (Atlas, 1981, Holliger et al., 1997) and bioaugmentation strategies are even using catabolically-relevant organisms to enhance remediation but with differing success (Thompson et al., 2005). With failure of bioremediation on some sites the awareness arose that there are abiotic, but also biotic factors that are influencing microbial communities and may favour or limit biodegradation *in situ* (Bouchez et al., 2000, Goldstein et al., 1985). Obviously, hydrogeological and geochemical parameters are relevant for groundwater dwelling organisms and microbial communities have been shown to be adapted to different conditions even on small scales (Haack and Bekins, 2000, Roling et al., 2001). The importance of biological interactions such as competition and predation for the natural attenuation of aquifers has been addressed to even lesser extents and research on groundwater ecology is gaining momentum nowadays.

1.2 Groundwater - a hidden ecosystem

As a unique ecosystem without phototrophic primary production, prokaryotic microorganisms dependent on chemical energy resources are considered the basis of food webs in the subsurface (Bachofen et al., 1998, Ghiorse, 1997). Prerequisite for microbial growth is the presence of carbon sources, electron donors and acceptors. In general, pristine aquifers are oligotrophic environments as substrates are quickly oxidised, in case they have not been removed already in the unsaturated zone (Goldscheider et al., 2006). Due to this, the abundance and activity of groundwater organisms often depend on an exogenous input of organic carbon and prokaryotic cell numbers may range between 10^4 and 10^8 g^{-1} aquifer sediment, the majority being attached on particles (Griebler and Lueders, in press). The transport of organic matter, nutrients and oxygen are crucial factors influencing not only microbial life in the subsurface. Depending on specific hydrological and hydrogeochemical parameters of an aquifer, the composition of indigenous biocenoses and structure of a food web in groundwater habitats can vary widely. In sandy aquifers with small pore sizes for example, predators will be limited to protists (unicellular eukaryotes) and small invertebrates, whereas in karst systems stygobiontic fauna dominated by crustaceans can be found (Sket, 1999).

In general, the food web is based on the microbial loop, a concept based on Pomeroy's hypothesis (Pomeroy, 1974) about heterotrophic protists (so called protozoa) shuttling organic matter from primary producers to higher trophic levels (Fig.1). Preying upon prokaryotes and microeukaryotes (e.g. saprophytic fungi), protozoa also remineralise organic carbon and recycle inorganic nutrients like nitrogen and phosphorous, while serving as food source for higher organisms (Ekelund and Ronn, 1994, Sherr and Sherr, 2002). The microbial loop may be essential in pristine and contaminated aquifers to overcome nutrient limitations suggesting a major role of protists in the groundwater ecosystem function.

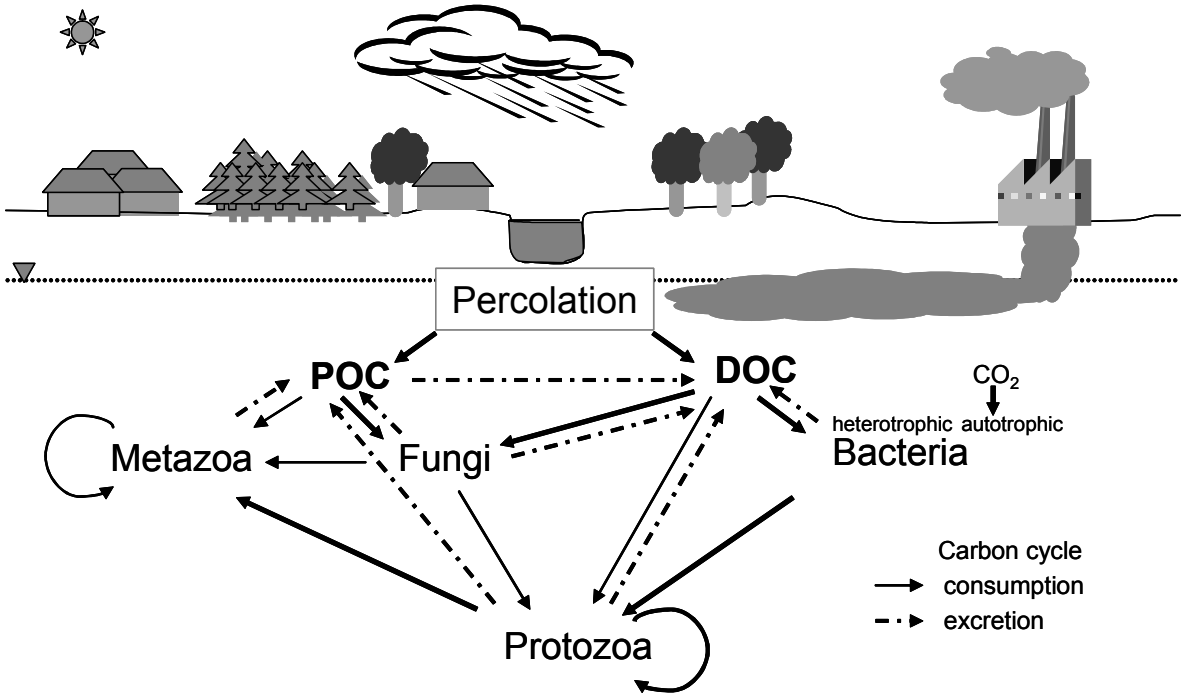

Fig. 1. Food web diagram showing trophic relationships in a subsurface ecosystem. With organic carbon, also inorganic nutrients are recycled by egestion and excretion.

Nowadays the 'loop' concept is known to oversimplify the real complexity in microbial food webs being aware of e.g. species-specific differences of features affecting predator- prey relationships (as size-selective feeding or inducible defences in prey species) and competition (Boenigk and Arndt, 2002, Pernthaler, 2005, Thelaus et al., 2008). Recently, the microbial loop has been extended by the additional consideration of viral interactions (viral loop) (Hornak et al., 2005, Jardillier et al., 2005, Weinbauer and Peduzzi, 1995, Winter et al., 2004). And an ongoing discussion is concerning the question whether top-down vs. bottom-up control of the bacterial prey depends on the trophic state of the environment (Gasol et al., 2002, Pernthaler, 2005). This would be an important aspect comparing pristine and contaminated groundwater habitats. Despite the knowledge that heterotrophic protists are the main consumers of bacteria in diverse ecosystems (e.g. surface waters, waste water or topsoils) (Finlay and Esteban, 1998, Hobbie et al., 1999, Ratsak et al., 1996, Sherr and Sherr, 2002), only little is known about their distribution and diversity, and even less about their functional importance in aquifers.

1.3 Ecology and taxonomy of protozoa

Like prokaryotes, protozoa are ubiquitous organisms in all kinds of habitats, where the only requirement is sufficient moisture and an adequate food source. They are tolerant to a wide range of physical and chemical environmental factors like salinity, oxygen concentration or temperature (Hausmann et al., 2003). Specialized species also occur under anoxic conditions (Bernard et al., 2000, Dawson and Pace, 2002, Fenchel and Finlay, 1995) and stratification horizons in benthic systems coupled to environmental gradients have been detected (Guecker and Fischer, 2003). Diversity studies in oxygen-depleted habitats gained momentum very recently and are revealing a yet undetected biodiversity in various ecosystems (Bernard, et al., 2000, Murase et al., 2006, Takishita et al., 2007). Anaerobic mitochondria, hydrogenosomes and algal or prokaryotic endosymbionts are some mechanisms that were detected supporting protozoan life in anoxia (Embley, 2006, Fenchel and Finlay, 1995, Finlay et al., 1996, Hackstein and Vogels, 1997), where protozoa can stimulate microbial activities by nutrient cycling (Biagini et al., 1998).

Protozoa have an enormous variation in size, ranging from nanoflagellates (~ 2 μm) to the largest amoeba or foraminifera in the range of centimeters, suggesting a high diversity in their functional roles in the environment (Corliss, 2002, Finlay and Esteban, 1998). Bacterivores have a similar size as their prey and possess a comparable growth potential (Ekelund and Ronn, 1994). Their high metabolism facilitates energy and carbon fluxes through ecosystems (Fenchel, 1975). Having a reproduction time of only a few hours they can make rapid use of resources and the wide-spread ability to encyst in a short time makes them resistant to unfavourable conditions (Hausmann, et al., 2003). Bacterivorous protozoa are affecting microbial communities by size-selective feeding, shifting the size structure of prey populations to smaller or larger cells, or by changing community composition by selective feeding, also influenced by possible microbial anti-predator defenses (Hahn and Hofle, 1999, Jurgens and Matz, 2002, Pernthaler, 2005, Posch et al., 2001). Carnivore protozoa are preying upon smaller protists and some species are even known to be specialised on fungal prey (Petz et al., 1986).

Phagotrophy is wide-spread among phylogenetic branches of the eukaryotic tree and therefore protozoa are forming a paraphyletic group, i.e. neither protists (unicellular eukaryotes) nor protozoa represent an evolutionary lineage in a phylogenetic sense (Cavalier-Smith, 2002, Cavalier-Smith, 2003). In former times, protozoa were categorized after

morphological features and grouped into functional groups: amoeba, ciliates and flagellates. An overview summing up the development of classification systems and the history of advances in protistology is given in (Hausmann, et al., 2003). Till now the fundamental issue of how to define the group taxonomically is difficult and rather controversial. To avoid confusions, I will give a brief definition of terms used in this thesis.

- **Protozoa** are heterotrophic protists, i.e unicellular eukaryotes digesting food in vacuoles. Concerning their food source, protozoa may display a range of nutritional modes such as bacterivores, carnivores, osmotrophs or mixotrophic organisms (e.g. *Euglena*).
- **Protists** are unicellular eukaryotes without differentiation of tissues. Cell differentiation may occur by forming cysts or alternate morphologies like mycelium growth in yeast- like fungi. With this definition, protists consist of protozoa, unicellular algae and microfungi and a detailed overview is given by (Adl et al., 2005).
- **Microeukaryote** is used as a more detailed term for microorganism (= microscopic organism), i.e. without the consideration of prokaryotes. In most of the cases, it may be a synonym for protist, despite the fact, that there may be e.g. protozoan ciliates or foraminifera reaching body sizes visible by human eye.

Advances in molecular methods have just started shedding light on complex phylogenetic relationships of protists. Especially the databases of 18S rRNA gene sequences are growing fast and are publicly accessible (e.g. the NCBI database: www.ncbi.nlm.nih.gov). Additionally, amplifying DNA sequences directly from environmental samples allows the detection of not yet cultivable microorganisms (Amann et al., 1995, Moreira and Lopez-Garcia, 2002).

1.4 Protistan diversity and activity in groundwater

Only few studies are available dealing with the diversity of protozoa in groundwater. Due to different sampling techniques, e.g. the use of water from wells or springs instead of drill cores of aquifer material, results are often not comparable (Novarino et al., 1997). Additionally, in most of the studies protozoan species were identified after cultivation and/or affiliated merely to functional groups like ‘flagellates’ without further identification (Fusconi and Godinho, 1999, Sinclair et al., 1993, Strauss and Dodds, 1997, Zarda et al., 1998). Still, identified groundwater dwelling protozoa have been found to belong to diverse phyla, suggesting a large diversity in aquifers (Fig. 2). But it also has to be taken into account that the majority of protozoan species may not to be cultivable under conditions used. Methods like MPN (most probable number) counts to estimate total protozoan numbers in samples also rely on the enumeration of protozoa, which may be very selective. It is also not possible to discern active trophozoits and resting cysts after enumeration.

Lately, efforts were made to use molecular methods to directly identify microeukaryotes in groundwater samples and the use of such culture-independent tools may display a yet undetected protistan diversity in groundwater, as for a number of other recently investigated ecosystems (Epstein and López-García, 2008).

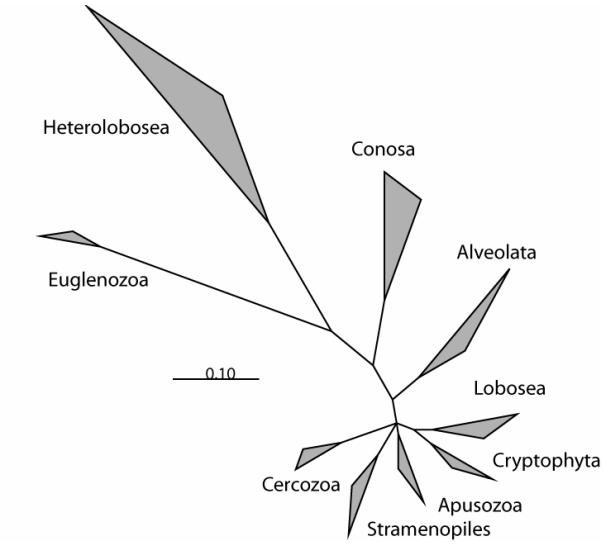

Fig. 2. Phylogenetic tree displaying protozoan phyla detected in groundwater studies so far. The scale bar indicates estimated sequence divergence.

Similar to the identity of groundwater dwelling microeukaryotes, also knowledge about their potential role for the ecosystem functioning and the natural attenuation potential is limited. Most of the studies, which investigated the influence of protozoa on processes in groundwater, reported or at least suspected positive effects of their presence in contaminated aquifers and an overview is given in the following Table 1.

Table 1 Overview of studies dealing with possible effects of protozoa in aquifers.

contaminant	effect of protozoa	citation
BTEX	enhanced bioremediation no effect	(Mattison and Harayama, 2001) (Kota et al., 1999)
DOC	enhanced bacterial growth rate assumed positive effect	(Kinner et al., 2002) (Kinner et al., 1997)
alkylbenzenes	enhanced bioremediation	(Mattison et al., 2005)
fuel	assumed reduced bioclogging	(Sinclair, et al., 1993)
monoaromatic hydrocarbons	assumed positive effect	(Zarda, et al., 1998)
---	assumed reduced bioclogging	(Baveye et al., 1998)

Increasing input of carbon into aquifers often results in anaerobic conditions due to limited oxygen availability. Here, microbial degradation of organic contaminants proceeds via anaerobic pathways (Lovley, 2000). But the knowledge about protistan diversity and particularly their activity in anaerobic environments is still very limited, which especially

includes anoxic zones of the groundwater. Extensive research is still required, as only one study (Kota, et al., 1999) reported no detectable effect on biodegradation of BTEX using different protozoan inhibitors under anaerobic conditions.

Besides protozoa, also members of the *Fungi* represent a protistan group that may play a role in groundwater ecosystems. Only little is known about their activities in aquifers, but the detection of yeast- like fungi in recent studies may hint at an involvement in saturated subsurface nutrient cycles (Brad, et al., 2008, Ekendahl et al., 2003, Luo, et al., 2005).

1.5 Description of the site of investigation: the Flingern site

The majority of the work described in this thesis was done by analysing sediment samples derived by well drillings on a former gaswork site in Düsseldorf-Flingern. Heavily contaminated with BTEX and PAH compounds the site has been subject to extensive research on contaminant degradation since the 1990s. In June 2005, sediment cores were obtained during the drilling of a multi- level well (Anneser et al., 2008). Due to the work of Bettina Anneser and Dr. Christian Winderl (Winderl, 2007), a precise description of biogeochemical and microbial parameters are available for this site. To facilitate reading and to avoid recurrences, the most important informations concerning the site of investigation are summarised in Fig. 3.

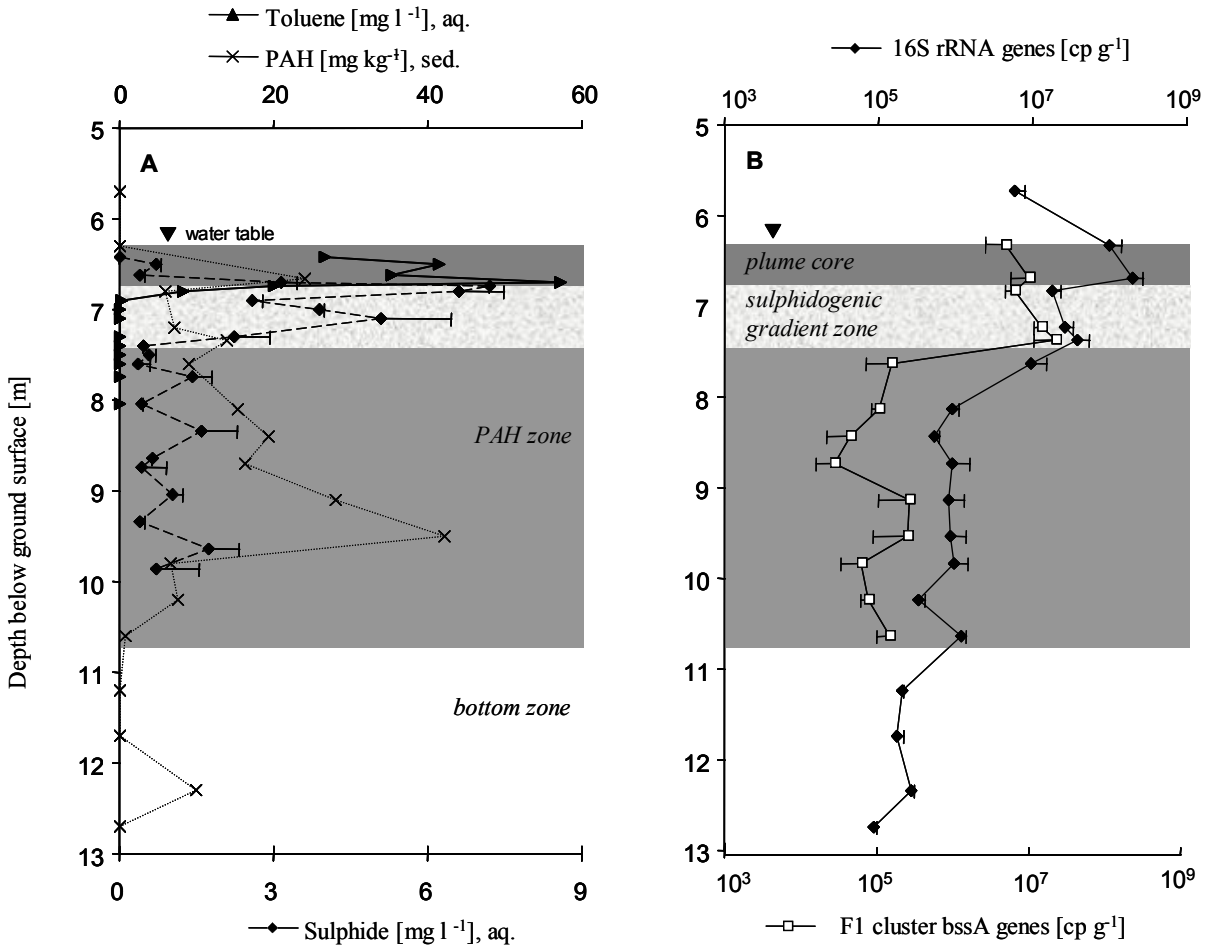

Fig. 3. (A) Biogeochemical parameters of the Flingern site concerning contamination gradients and sulphide concentrations. (B) Quantitative distribution of total bacterial 16S rRNA and bssA genes, the latter being an indicator for biodegradation. Data by courtesy of Bettina Anneser (Anneser, 2008) and Christian Winderl (Winderl, 2007).

1.6 Motivation and outline of the thesis

There is a remarkable lack of knowledge concerning the diversity and role of protists in aquifers, thus characterising the biodiversity of natural protistan assemblages and their functional relevance is urgently needed for gaining insights in the functionality of groundwater ecosystems. Being the most important predators of bacteria, known to influence microbial abundance and activities in other ecosystems, protozoan species might also play a key- role in the groundwater ecosystem. Furthermore, the role of other microeukaryotes such as fungi as potential contaminant degraders has not been addressed yet.

The main subjects of the present thesis were therefore the development and application of molecular methods for the characterisation of protistan communities in aquifers and to gain insights in their role for groundwater ecosystem functioning. In more detail, the following topics should be assessed:

- Establishment and verification of molecular tools to characterise microeukaryote assemblages in groundwater habitats.
- Composition and structure of protistan communities in contaminated aquifers.
- Coupling of protistan communities to biogeochemical and microbial parameters.
- Influence of protists on biodegradation of contaminants.

The first part of the thesis is dealing with molecular analyses, starting with the development of molecular assays (PCR and T-RFLP) suitable for the investigation of protistan diversity in aquifer sediment samples (Chapter 2). The established methods were applied for a comparative diversity analysis through the depth-profile of a contaminated aquifer to assess microeukaryote species distribution and their potential coupling to abiotic and biotic parameters (Chapter 3). The site is briefly described above. The third part describes investigations towards functional aspects of protozoan populations, including cultivation and isolation efforts, a comparison of aerobic and anaerobic enrichment and MPN counts (Chapter 5), as well as first steps towards the application of DNA-stable isotope probing for aquifer protistan communities (Chapter 4).

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2 An optimised PCR/T-RFLP fingerprinting approach for the investigation of protistan communities in groundwater environments

2.1 Introduction

Since Pomeroy's hypothesis on the key-role of microbes in ocean productivity (Pomeroy, 1974) and the establishment of the microbial loop concept (Azam et al., 1983), much importance has been attributed to the diversity and role of phagotrophic protists in natural ecosystems. Their relevance for the remineralisation of nutrients in aquatic and terrestrial environments is now widely accepted (Corliss, 2002, Finlay and Esteban, 1998). Although the study of natural protistan communities, as for that of prokaryotes, has been hampered by the limitations of cultivation-based methods and often a lack of morphological features for species identification, advances in molecular methods have also accelerated the progress of cultivation-independent approaches to study microeukaryotes (Caron et al., 1999).

The amplification of 18S rRNA genes with eukaryote-specific primers followed by cloning and sequencing has been employed to assess protistan diversity in various environments ranging from soil and sediments to freshwater and marine plankton, and even to anoxic or acidic systems (for a recent review, see (Epstein and López-García, 2008)). Due to the laborious nature of cloning and sequencing approaches, a number of PCR-based fingerprinting strategies such as terminal restriction fragment length polymorphism (T-RFLP, (Liu et al., 1997), denaturing gradient gel electrophoresis (DGGE, (Muyzer et al., 1993), or single strand conformational polymorphism (SSCP) (Schwieger and Tebbe, 1998) analyses have also been adapted to microeukaryote communities (Casamayor et al., 2002, Countway et al., 2005, Diez et al., 2001, Medlin et al., 2006, Murase et al., 2006). In general, fingerprinting allows for the processing of samples in higher throughput, and to monitor temporal or spatial dynamics and distinctions in the structure of microbial communities.

However, the specificity and efficiency of the employed PCR primers are crucial points for the success of rRNA gene fingerprinting. For protists, especially, the co-amplification of non-target genes cannot be excluded without concurrent sequencing. The most widely used eukaryote-specific primer pair for the amplification of 18S rRNA genes has been published two decades ago (Medlin et al., 1988) and various modifications, e.g. the shortened forward primer Euk1A (Diez et al., 2001), have been applied in countless environmental surveys since then. Interestingly, identical or very similar forward primers have also been used in studies targeting archaeal rRNA genes (Snell-Castro et al., 2005, Wilms et al., 2006). Thus, when investigating protistan diversity and community structure, the possibility of erroneously covered prokaryotic community components, but also the ratio of detectable fungal vs. protistan 18S rRNA genes have to be taken into account. The successful fingerprinting of protistan populations can be considered as problematic, especially in samples low in protistan or high in non-target rRNA gene copy numbers.

Both characteristics are likely to occur in groundwater environments. Thus, despite a recently increased awareness towards the putative importance of groundwater protists in aquifer food webs and groundwater natural attenuation (Kinner et al., 2002, Novarino et al., 1997, Zarda et al., 1998), reports on the successful application of community fingerprinting approaches for protists in this ecosystem are rare. Only very recently, DGGE has been applied to monitor eukaryote community distribution in a landfill leachate plume (Brad, 2007). To promote the application of microeukaryote and protistan community fingerprinting in

aquifers, a rigid comparative evaluation of the potential of different available primers and the selection of an optimised resolution strategy may be very helpful.

To address these issues, I have validated the suitability of different eukaryote-specific 18S rRNA gene primers in combination with different restriction endonucleases for T-RFLP fingerprinting of protistan and microeukaryote communities in a contaminated aquifer. In a first step, primer specificity and the potential of different restriction enzymes to assess the diversity of protistan populations in groundwater was predicted *in silico*. Later, the applicability of different primer sets for fingerprinting was verified with DNA extracts over a depth-profile of an anoxic, tar-oil contaminated aquifer characterised by decreasing total bacterial abundances (Winderl et al., 2008). Cloning and sequencing allowed scrutinising the detectability of protistan templates in the different DNA extracts and to pinpoint complications caused by high backgrounds of fungal and prokaryote templates. In summary, this chapter describes an optimised PCR/T-RFLP fingerprinting approach for microeukaryote communities successfully applied to characterise protistan populations in a contaminated groundwater environment. Specific information about phylogenetic analysis of eukaryote communities detected in this chapter is given in chapter 3.

2.2 Material and methods

2.2.1 Specificity of eukaryote 18S rRNA gene primers

The specificity of a set of frequently used 18S rRNA gene-specific primers (Table 2) was investigated *in silico* using an ARB database (Ludwig et al., 2004).

Table 2 Eukaryote 18S rRNA gene-specific PCR primers applied in this study.

Primer	5' – 3' sequence	Position (*E. coli*)	Reference (mod. after)
Euk9f [a,b]	CTG GTT GAT CCT GCC AGT AG	9-28	(Diez et al., 2001)
Euk20f [b]	TGC CAG TAG TCA TAT GCT TGT	20-41	(Kowalchuk et al., 1997)
Euk516r [c]	ACC AGA CTT GYC CTC C	502-516	(Amann et al., 1990)
Euk1179r [c]	CCC GTG TTG AGT CRA ATT	958-975	(Hendriks et al., 1989)
Euk1383r [c]	GCG GTG TGT ACA AAK GGC AG	1383-1402	(van Hannen et al., 1998)
Euk1509r [c]	CYG CAG GTT CAC CTA CGG	1509-1527	(Medlin et al., 1988)

[a] Euk9f equals Euk1A (Diez et al., 2001), but is elongated by a 3'-TAG.

[b] Forward primers were fluorescently labelled for T-RFLP fingerprinting.

[c] Reverse primers in 5' – 3' correspond to the reverse complement of the target site.

I used a modified version of the "Jan04" release with a total of 17357 bacterial, 936 archaeal and 9125 eukaryotic aligned 18S rRNA genes. Primer length was partially modified from the original references to minimise PCR artifacts. Primer hairpin formation, self-dimerisation and other thermodynamic properties were predicted using the PrimerSelect module of the Lasergene Software (DNAStar). Single degenerate positions were inserted into reverse primers Euk516r and Euk1383r to account for observed sequence variability between relevant protistan lineages. The forward primer Euk1A (Diez et al., 2001), being identical to a primer recently used for archaeal-specific rRNA gene amplification (Wilms et al., 2006), was modified by a 3 bp extension at the 3'- end to increase eukaryote specificity. The primer is

further referred to as Euk9f. For each primer, all aligned sequences were checked for the presence of unambiguous sequence information at the target site. The matching of primers to non-eukaryote sequences was tested with increasing numbers of allowed mismatches (0-3). Based on the number of predicted hits, the ratio of possible non-target sequences recovered by the primers was calculated for the domains *Archaea* and *Bacteria*.

2.2.2 Sediment sampling and DNA extraction

DNA was extracted from sediment cores of 4 different depths of the contaminated aquifer in Düsseldorf Flingern, Germany. The site and sampling procedures have been described in detail elsewhere (Anneser, 2008). The samples originated from the unsaturated zone (5.7 m below ground) and increasing water-saturated depths (7.4, 7.6 and 8.1 m) at a tar-oil contaminant plume characterised by decreasing bacterial 16S rRNA gene copy numbers (Winderl et al., 2008). See figure 3 for overview. DNA was extracted from freshly thawed ~2-4 g sediment using a previously described protocol (Winderl et al., 2008). Briefly, the procedure included sediment treatment with lysozyme, proteinase K and SDS, subsequent bead beating, extraction with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol, and precipitation with polyethylene-glycol. For each depth, 2-5 replicate extractions (~1 g sediment each) were pooled in 30 µl of elution buffer (Qiagen) and stored frozen (-20°C) until further analyses. The yield of extracted nucleic acids was routinely checked by UV quantification (ND-1000 Spectrophotometer, NanoDrop Technologies) and usually in a range between 2 – 20 ng μl^{-1}.

2.2.3 PCR, cloning and sequencing

Eukaryotic 18S rRNA gene amplicons were generated by PCR from 1 µl template DNA using the primer sets Euk20f/Euk516r for short ("S", 5.7 m, 7.4 m and 8.1 m), Euk20f/Euk1179r for medium-sized ("M", 7.4 m and 8.1 m) and Euk9f/Euk1509r (5.7 m) or Euk9f/Euk1383r (7.6 m) for nearly full-length ("L") amplicons (Table 2). Each PCR reaction (50 µl) contained 1x PCR buffer, 1.5 mM $MgCl_2$, 1 U Taq polymerase, 0.1 mM dNTPs (all Fermentas), 0.5 µM of each primer (Biomers), 10 µg BSA (Roche) and nuclease-free water (Promega). The initial PCR denaturation (94°C, 5 min) was followed by up to 35 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and elongation (70°C, 1 min for "S", 1.5 min for "M" and 2 min for "L" amplicons). All PCRs were performed on a Mastercycler ep thermal cycler (Eppendorf). After PCR, generated amplicons were checked by standard agarose gel electrophoresis and ethidium bromide staining and purified using MinElute PCR purification columns (Qiagen). Amplicons were cloned and sequenced as previously described (Winderl et al., 2007). Sequences were identified using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), integrated into my ARB database (Ludwig et al., 2004), and grouped into major phylogenetic lineages.

At least 50 clones per library were analysed for the "S"- and "M"-sized amplicons, but substantially less clones (12 in average) were sequenced for the low-diversity libraries with "L"-sized inserts. The generated sequence information was used to predict corresponding T-RF lengths [bp] representative for the clones and lineages. In the case of discrepancies of > 2 bp between predicted T-RF lengths and those directly observed in fingerprints of environmental DNA, the affiliation of T-RFs was verified by direct T-RFLP analysis of cloned amplicons. All clone sequences of this study were deposited with GenBank under accession numbers EU646901 to EU647181.

2.2.4 Restriction enzyme selection for T-RFLP

Different restriction endonucleases were evaluated for their utility in T-RFLP analysis with a selection of 18S rRNA gene sequences covering major protistan lineages expected to be present in saturated subsurface environments (Novarino et al., 1997), or clones detected therein. These were sequences amongst the *Cercozoa, Alveolata, Lobosa, Heterolobosa, Chlorophyta, Euglenozoa, Choanozoa*, and different fungi. Again, I used the ARB database and screened the aligned sequences for the presence of unambiguous sequence information at the target sites of the primers Euk20f and Euk516r. Finally, 243 18S rRNA gene sequences were selected as a representative set (see Table A.1 for specific information, accession numbers and affiliation). These were then digested *in silico* with 6 different restriction endonucleases (*Bsh*1236I, *Hae*III, *Hha*I, *Mnl*I, *Msp*I and *Rsa*I) that have been tested on a bacterial dataset before (Engebretson and Moyer, 2003), or have already been applied in eukaryotic T-RFLP studies (Casamayor et al., 2002, Countway et al., 2005, Diez et al., 2001, Lepere et al., 2006).

To assess the suitability of each distinct enzyme for resolving the selected eukaryotic populations, the total number of different operational taxonomic units (OTUs) (= distinct T-RFs or lengths of sequences with no cleavage site between f- and r-primer) as well as the ratio of unrestricted OTUs were recorded. The resolution power was calculated using Margalef's index $D_M = (S\text{-}1)/\ln(n)$, where S is the number of OTUs and n the total number of sequences (Engebretson and Moyer, 2003). Possible values of D_M range from zero, when all T-RFs are of same length to $(n\text{-}1)/\ln(n)$, when every T-RF is unique and therefore representing one OTU. The percentage of lineage-specific OTUs was calculated as proportion of OTUs mainly (>80%) formed by T-RFs of one taxonomic group. T-RFs that occurred only once were excluded from this analysis, otherwise every singleton would have resulted in one lineage-specific OTU. For two selected enzymes, the effective resolution power for the natural eukaryote diversity detected in the clone libraries was visualised by *in silico* digestions of the summarised clone sequences and plotting of deduced OTU abundances.

2.2.5 T-RFLP fingerprinting

T-RFLP analysis of eukaryote 18S rRNA gene amplicons was done with primer pairs Euk20f-FAM/Euk516r ("S"), Euk20f-FAM/Euk1179r ("M") and Euk9f-FAM/Euk1509r ("L") (all from Biomers) and *Bsh*1236I (Fermentas) digestion of 10-20 ng purified amplicons. Electrophoresis and electropherogram evaluation was performed as described before (Lueders et al., 2006) using an ABI 3730 DNA analyser and the GeneMapper 5.1 software (Applied Biosystems). T-RF frequencies were inferred from peak heights (Lueders and Friedrich, 2003). The reproducibility of my entire T-RFLP workflow was exemplarily verified for the "S" primer set and one sediment sample, by extracting duplicate DNAs and generating four replicate fingerprints per extract.

2.2.6 Quantitative PCR of archaeal 16S rRNA genes

For four sediment samples (6.7 m, 7.4 m, 7.6 m and 8.1 m), three independent DNA extracts (used for bacterial 16S rRNA gene qPCR in (Winderl et al., 2008)), were used to additionally quantify total archaeal 16S rRNA gene copy numbers. The qPCR was done with primer set Arch109f/Arch912rt and a standardisation with a full-length *Methanosarcina barkeri* 16S rRNA gene amplicon as previously described (Chan et al., 2005), using a slightly modified qPCR chemistry and a Stratagene MX3000P qPCR cycler (Winderl et al., 2008).

2.3 Results

2.3.1 *In silico* evaluation of the potential of different eukaryote-specific primer pairs for recovering groundwater protistan communities

Different primers published as eukaryote specific (Table 2) were tested for their potential recovery of non-target prokaryotic rRNA genes at decreasing PCR specificity (increasing allowance of mismatches, MM). Caused by different quantities (> 17,000 bacterial and ~ 900 archaeal sequences in the database) and lengths of sequence entries, the numbers of potentially matched non-target hits differed greatly between the primers and were mostly dependent on the presence of sequence information at the target site. Therefore, ratios of potential non-target hits are shown in Table 3.

Table 3 Mismatch-dependent ratios of potential non-target hits within the prokaryotic domains predicted *in silico* for the eukaryote-specific primers used in this study.

	Primers and ratios of non-target prokaryotic 16S rRNA genes covered						
Domain *Mismatches*	**Euk1A**[b]	**Euk9f**	**Euk20f**	**Euk516r**	**Euk1179r**	**Euk1383r**	**Euk1509r**
Archaea [a]	**[386]**	**[386]**	**[462]**	**[887]**	**[874]**	**[696]**	**[204]**
0	0.13	0	0	0	0	0	0
1	0.26	0	0	0	0	0.01	0
2	0.79	0.12	0	0.14	0	0.06	0.19
3	0.91	0.25	0	0.21	0.29	0.93	0.85
Bacteria [a]	**[4756]**	**[4756]**	**[6883]**	**[16923]**	**[16269]**	**[15032]**	**[3321]**
0	0	0	0	0	0	0	0.01
1	0	0	0	0	0	0	0.29
2	0	0	0	0	0	0	0.33
3	0	0	0	0.01	0	0.01	0.39

[a] Total numbers of archaeal and bacterial sequences in the database with unambiguous sequence information at the target site are given in brackets.

[b] Only evaluated *in silico*, not used in PCR in this thesis.

Most primers could be confirmed as eukaryote specific at 0 MM, except for Euk1A (Diez et al., 2001) that already matched 13% of possible archaeal non-targets without MM, increasing to 91% at 3 MM. Our extended version Euk9f performed slightly better by mis-targeting only at 2 MM, but still covered 25% of archaeal non-targets with 3 MM. Therefore, of the two permutations, only Euk9f was further evaluated in my analyses. The Euk20f forward primer showed first mis-targeting only if 5 or more MM were allowed.

Of the analysed reverse primers, the Euk516r and Euk1179r sequences hit < 30% of possible archaeal non-targets, even if 3 MM were allowed. This was in contrast to the two "full-length" reverse primers (Euk1383r and Euk1509r), which recovered > 80 % of archaeal non-targets at 3 MM. Most primers showed a much better specificity against bacterial non-target hits and matched bacterial sequences only when 4 or more mismatches were allowed. As an exception, the Euk1509r primer showed a decreased specificity (~ 30% recovery) against bacterial non-targets already at 1 allowed MM.

2.3.2 Application of eukaryote-specific primer sets in natural aquifer samples

To verify the predicted specificities and coverage of the selected primers and some respective combinations, between 1 and 2 clone libraries were generated per aquifer DNA extract and depth. *In situ* sequence information was also indispensable for the interpretation of subsequently generated T-RFLP fingerprints. The phylogenetic composition of the libraries as indicated by the frequency of clones affiliated to major eukaryotic and non-target lineages is summarised in Table 4. Here, I refrain from providing more than kingdom-level information on the affiliation of the detected clones due to the methodological focus of this first chapter. The detailed description of the distinct microeukaryote communities encountered in the different biogeochemical zones of the Flingern aquifer will be subject of chapter 3.

Table 4 Abundance representation of target microeukaryote and non-target phylogenetic lineages detected with different primer combinations via cloning and T-RFLP fingerprinting in aquifer DNA extracts.

	Frequency of clones and detected lineage-specific T-RFs [b,c]							
Sampling depth	**5.7 m**		**7.4 m**		**7.6 m**		**8.1 m**	
Amplicons [a] *- Covered lineages*	**clones**	**T-RFs**	**clones**	**T-RFs**	**clones**	**T-RFs**	**clones**	**T-RFs**
"S"								
- Protists	0.72	0.68	0.34	0.13	–	0.76	0.57	0.55
- Fungi	0.05	0.03	0.46	0.51	–	n.a.	0.27	0.21
- Plantae	0.02	n.a.	n.d.	n.d.	–	n.a.	0.04	0.01
- Metazoa	0.22	0.21	0.20	0.21	–	n.a.	0.12	0.12
- Archaea	n.d.	n.d.	n.d.	n.d.	–	n.a.	n.d.	n.d.
- Not assignable	n.d.	n.d.	n.d.	0.12	–	0.23	n.d.	0.10
"M"								
- Protists	–	0.73	n.d.	n.d.	–	0.81	n.d.	n.d.
- Fungi	–	0.03	0.85	0.76	–	n.a.	0.74	0.47
- Plantae	–	n.a.	n.d.	n.d.	–	n.a.	0.26	0.34
- Metazoa	–	0.08	0.15	0.22	–	n.a.	n.d.	n.d.
- Archaea	–	n.d.	n.d.	n.d.	–	n.a.	n.d.	n.d.
- Not assignable	–	0.10	n.d.	0.02	–	0.17	n.d.	0.19
"L"								
- Protists	0.94	0.52	–	n.d.	0.25	n.d.	–	n.d.
- Fungi	n.d.	0.02	–	0.73	n.d.	n.d.	–	0.63
- Plantae	n.d.	n.a.	–	n.d.	n.d.	n.d.	–	0.09
- Metazoa	n.d.	0.23	–	0.13	n.d.	n.d.	–	n.d.
- Archaea	0.06	0.04	–	n.d.	0.75	0.82	–	n.d.
- Not assignable	n.d.	0.12	–	0.15	n.d.	0.12	–	0.28

[a] The primer combinations used for "S", "M", and "L" amplicons are given in the materials and methods section.

[b] n.a., not applicable (lineage-specific T-RFs were not assignable because of absence of clone data or because clone frequencies contributing to a given T-RF were < 20 %); n.d., not detected in this depth; –, not analysed.

[c] Identified lineage-specific T-RFs for the different depths and lineages are summarised in Table 6

The potential of the primer combinations to recover different eukaryotic community members greatly differed between samples. In the zone above the groundwater table (5.7 m), protists clearly dominated both the "S" and "L" clone libraries. The "S" library recovered genes of other eukaryotic lineages within the fungi, plants and metazoa, while the "L" library also contained 6 % of archaeal non-target clones. This preferred recovery of archaeal non-

target amplicons with the "L" primers was even more pronounced at the depth of 7.6 m, where 75 % of the clones contained archaeal inserts.

At 7.4 m, only 34 % of "S" clones belonged to protistan lineages, showing an increase of especially fungal inserts to 46 %. Interestingly, this increase in fungal abundance was even more pronounced for the "M" amplicons from the same depth, where 85 % of the clones were identified as fungi and protists were not detected at all. The same result was observed in the deepest sample (8.1 m), where again no protistan clones could be identified using the "M" primer combination and the library was dominated by fungi. In contrast, 57 % of the clones amplified with "S" primers were protists in the same depth. Based on these findings, the "S" primer set was regarded as superior for the recovery of protistan community members from aquifer DNA extracts.

2.3.3 Development of a T-RFLP fingerprinting assay

On basis of the evaluation of the different primer sets and primer specificity, I next wanted to find an optimal restriction endonuclease for use in T-RFLP fingerprinting of microeukaryote community amplicons generated from groundwater DNA using primer set "S". This was first done by comparing the resolution power of 6 enzymes that have previously been applied in eukaryotic T-RFLP studies (Casamayor et al., 2002, Countway et al., 2005, Diez et al., 2001, Lepere et al., 2006), or that have been similarly evaluated for use in bacterial T-RFLP (Engebretson and Moyer, 2003). For this, I selected a hypothetical dataset of 243 aligned sequences representing major microeukaryotic lineages expected to be present in saturated subsurface environments (Novarino et al., 1997), or clones detected therein (see Table A.1 for specific details). In general, all 6 enzymes produced a comparable number of *in silico* OTUs ranging from 90 for *Hha*I to 122 with *Hae*III (Table 5).

Table 5 Number of OTUs and Margalef density indices D_M predicted with different *in silico* restriction endonuclease digests for a selection of microeukaryotic 18S rRNA genes expected for groundwater habitats [a].

Restriction endonuclease	Target site	Total no. of OTUs	% of unrestricted OTUs [b]	D_M [c]	% of lineage-specific OTUs [d]
*Bsh*1236I	CG^CG	100	5	18	62
*Hae*III	GG^CC	122	34	22	40
*Hha*I	GC^GC	90	4	16	58
*Mnl*I	CCTCN^	99	54	18	27
*Msp*I	C^CGG	113	5	20	61
*Rsa*I	G^TAC	105	31	19	43

[a] The data set contained 243 aligned sequences representing major microeukaryotic lineages expected to be present in saturated subsurface (Novarino et al., 1997), or clones detected therein. See Table A.1 for details.

[b] Proportion of sequences without cleavage recognition site upon amplicons generated with the "S" primers.

[c] For a community of 243 18S rRNA genes, the maximum value of D_M is 44.

[d] OTUs representing single clones were excluded. OTUs with lineage-specific clone frequencies of > 80 % were defined as a lineage-specific.

The endonucleases formed two groups considering the ratio of sequences without restriction sites. *Msp*I, *Bsh*1236I or *Hha*I were predicted to cleave > 90 % of all selected sequences, whereas > 30 % remained uncut after restriction with *Hae*III, *Rsa*I or *MnI*I, respectively. As shown by the Margalef density index D_M, *Hae*III showed the highest potential to produce fragments varying in size when all OTUs were considered. The ratio of observed lineage-specific OTUs showed a clear advantage for *Msp*I and *Bsh*1236I, where > 60 % of OTUs were characteristic for only one (micro-) eukaryotic lineage.

On this basis, *Msp*I and *Bsh*1236I were identified as most suitable for the T-RFLP fingerprinting of microeukaryote communities in groundwater environments. To further support enzyme selection, I compared hypothetical resolution patterns of *Msp*I and *Bsh*1236I digestion for the summarised clone sequence data within widely applied standardisation limits of T-RFLP analysis (~ 50 – 900 bp fragment length). As displayed in Fig. 4, *Bsh*1236I can be expected to produce fragments over a wide range of detection, i.e. from > 50 to over 700 bp, whereas *Msp*I fragments were predicted to lie mostly between 200 and 400 bp. This can be explained by the fact that the recognition site of *Msp*I is located in a highly conserved region of the eukaryotic 18S rRNA gene, and is actually part of the widely used eukaryote-specific rRNA probe Euk309 (Lim et al., 1996). Therefore, *Bsh*1236I seems to provide a better range of resolution in fingerprinting of protistan communities in groundwater and was selected for subsequent use.

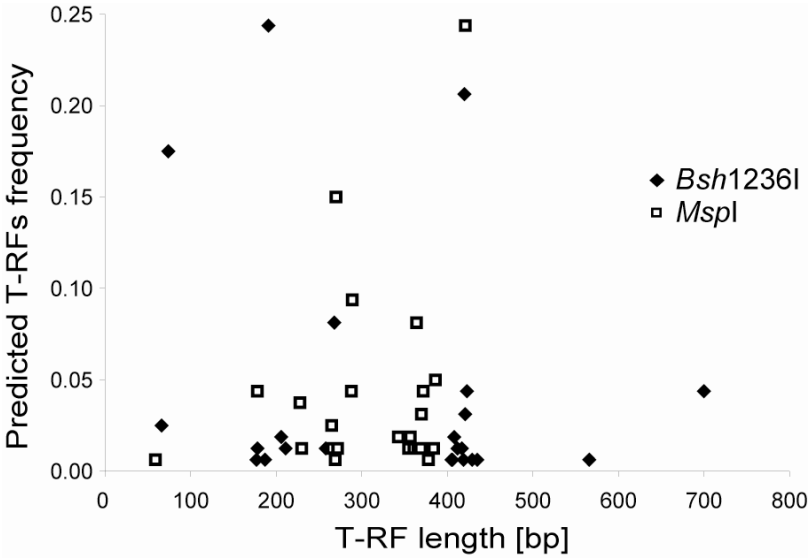

Fig. 4. Theoretical length [bp] and frequency of OTUs as predicted by *in silico* digestion of summarised clone sequence data of the 3 "S" clone libraries (160 clones in total) after restriction with *Bsh*1236I and *Msp*I. Values ar given in Table A.2.

2.3.4 T-RFLP fingerprinting of aquifer microeukaryote communities

After comparing the suitability of the different primer pairs and endonucleases for T-RFLP fingerprinting of environmental microeukaryote communities, I finally verified these predictions by analysis of aquifer sediment DNA extracts with the "S", "M" and "L" primer sets and *Bsh*1236I restriction (Fig. 5).

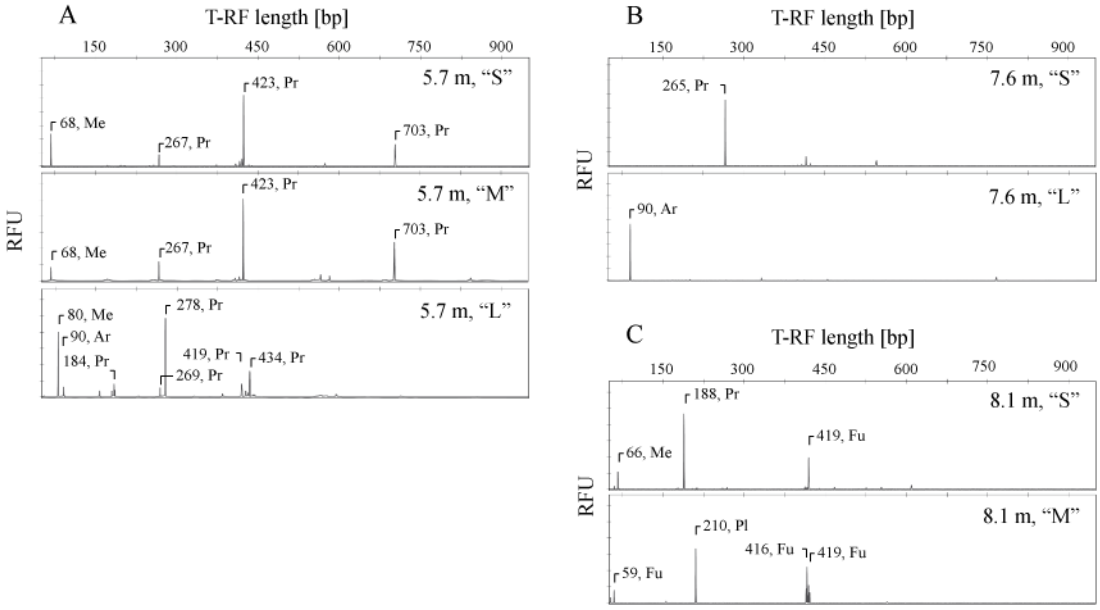

Fig. 5. Exemplary T-RFLP fingerprints of aquifer sediment microeukaryote communities generated with different primer combinations ("S", "M" and "L") and amplicon digestion with *Bsh*1236I. Dominant T-RFs are labelled with lengths [bp] and phylogenetic affiliation (Pr: Protist, Pl: Plantae, Fu: Fungi, Me: Metazoa, Ar: Archaea) as inferred from respective clone sequence data. Fingerprints were from (A) an unsaturated sample above the capillary fringe, (B) a saturated sample close to the zone of active toluene degradation (Winderl et al., 2008), and (C) a deeper saturated sample. RFU: relative fluorescence units.

Wherever observed T-RFs matched fragment sizes predicted by available clone sequence data from the same depth, they were assigned to the corresponding lineages to sum up T-RF frequencies (Table 4). The different lineage-specific T-RFs contributing to summarised T-RF frequencies for the different depths and lineages are listed in Table 6.

Table 6 Identified lineage-specific T-RFs contributing to the summarized T-RF frequencies indicated in Table 4[(a)]

		Assignment of T-RFs (bp) according to corresponding clone sequence data			
	Depth [m]	5.7	7.4	7.6	8.1
"S"	Protists	267, 420*, 423*, 703	188, 206, 412	265	188, 412, 609
	Fungi	416, *(420) (b)	59, 212, 407, 414, 416, 419	n.a. (c)	419
	Plantae	*(423)	n.d.	n.a.	210
	Metazoa	68, 573	66	n.a.	66
	Archaea	n.d.	n.d.	n.a.	n.d.
	not assignable	n.d.	68, 526, 553	407, 415, 423, 545	60, 416, 467, 554
"M"	Protists	267, 408, 423, 703	n.d.	265	n.d.
	Fungi	416	212, 414, 416, 419	n.a.	59, 414, 415, 417, 419
	Plantae	n.a.	n.d.	n.a.	210
	Metazoa	68	67	n.a.	n.d.
	Archaea	n.d.	n.d.	n.a.	n.d.
	not assignable	566, 844, 1144	564	267, 414, 423, 555, 761	52, 421
"L"	Protists	184, 269, 278, 419, 432, 434	n.d.	n.d.	n.d.
	Fungi	427	222, 224, 417, 427, 430	n.d.	427, 430
	Plantae	n.a.	n.d.	n.d.	223
	Metazoa	80	80	n.d.	n.d.
	Archaea	90	n.d.	90	n.d.
	not assignable	157, 179, 185	60, 139, 521	201, 332, 455, 766	62, 69, 414, 547

[(a)] T-RFs with a peak abundance of >1.5 % total peak height were identified in accordance to clone sequence data from the corresponding libraries. Discrepancies between observed and predicted T-RF length were checked by T-RFLP of digested M13 amplicons of clones.

[(b)] *() Lineage contributes < 20 % to peak abundance according to clone library data

[(c)] n.d., not detected; n.a., not assignable due to absence of sequence data

As for clone affiliation, I refrain from providing more detailed information on the microeukaryote lineages represented by specific T-RFs due to the methodological focus of this first chapter. Predicted and measured T-RFs for $OTU_{97\%}$ of clones are summarised in Table 9 (Chapter 3).

Fingerprinting analysis of the unsaturated zone sample (5.7 m) gave almost identical lineage detectability and summarised T-RF frequencies for the “S” and “M” primer sets (Fig. 5A, Table 4). But the electropherogram generated with the “L” primer set reveals a higher diversity of peaks and also shifts in lineage-specific T-RF abundances. Due to the distinct labelled f-primers, ~11 bp must be subtracted to compare “L” T-RFs to those observed in “S” or “M” fingerprints. Thus, especially the 80 bp T-RF characteristic for *Metazoa a*nd the protistan 278 bp peak increased in relative abundance in the “L” fingerprint compared to the respective “S” and “M” counterparts (68 and 267 bp, respectively). Also, a 90 bp T-RF representing the archaeal clones detected in the “L” clone library was identified. Distinctions between the different fingerprints became even more apparent at greater depths. The archaeal 90 bp-peak absolutely dominated the “L” fingerprint at 7.6 m (Fig. 5B), which corresponded to the high frequency of archaeal clones detected with the “L” primer set in this depth. In contrast, the “S” (Fig. 5B) and also the “M” (Table 4) electropherograms from the same depth recovered protistan T-RF frequencies of >75 %. This clearly confirms the preferred

mismatching of the "L" primers to archaeal non-target templates predicted by my database evaluation (see above).

Interestingly, also the "S" and "M" primer sets, for which no distinct microeukaryote-specific performance was predicted by my *in silico* work, behaved different in some depths. Hence, no protistan T-RFs could be identified with amplicons produced by the "M" primer set from depths at 7.4 m and 8.1 m. Here, fungal and unexpectedly also plant-related T-RFs dominated in accordance to clone data (Fig. 5C, Table 4). This was in contrast to the "S" amplicons generated from the same depths allowing for the detection of protistan T-RFs (and clones, respectively), even when they where absent in fingerprints generated with the "M" and "L" primers.

The reproducibility of my "S" T-RFLP assay was exemplarily verified for a sediment sample for the Flingern site, by extracting duplicate DNAs and generating four replicate fingerprints per extract (Fig. 6). T-RFs abundances were adequately reproducible, with an average standard deviation of 2.1 % relative T-RF abundance for each extract, and 2.8 % T-RF abundance for both replicate extracts together. The variability for some specific T-RFs was considerably higher between extracts (up to 6.6 % SD in T-RF abundance, e.g. for the 213 and 419 bp T-RFs in Fig. 6). This may, however, not necessarily indicate a lack of reproducibility for my assay, but could also be connected to extant heterogeneity in microeukaryote communities between duplicate ~3 g sediment aliquots used for DNA extraction.

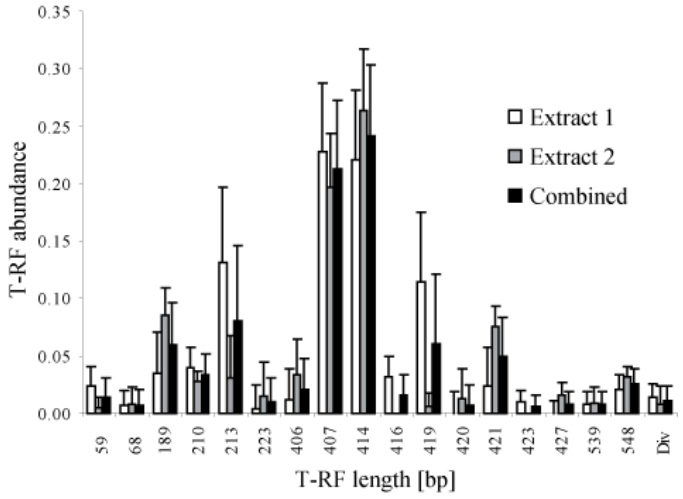

Fig. 6. Reproducibility of eukaryote-targeted T-RFLP fingerprints. Duplicate DNA extracts from an exemplary depth of the Flingern site were investigated with four replicate fingerprints per extract. Average T-RFs abundances are given plus SD for both extracts and for the combined averages. Values are given in Table A.3.

2.3.5 Exemplary assessment of archaeal non-target abundances

Table 7 Results of archaeal qPCR

Depth [m]	16S rDNA copies g^{-1} sediment	SD
6.7	$1.62*10^4$	$1.49*10^4$
7.4	$1.75*10^4$	$1.42*10^4$
7.6	$7.63*10^4$	$9.81*10^4$
8.1	$1.36*10^4$	$8.20*10^3$

Archaeal qPCR revealed a considerable large population of potential non-target rRNA genes in the investigated depths. Measured archaeal 16S rRNA gene copy numbers are given in Table 7.

2.4 Discussion

I present an optimised microeukaryote-targeted T-RFLP fingerprinting approach suitable for the investigation of intrinsic protistan communities in groundwater habitats. The investigation of protistan communities in aquifers is especially challenging, as these habitats are especially likely to be low in protistan and high in non-target rRNA gene copy numbers. Via *in silico* predictions and by comparing environmental clone libraries and respective fingerprints, I showed that some primer sets widely applied for the characterisation of microeukaryote communities in the environment will fail to recover protistan lineages from aquifer samples.

Thus, especially the "S" and "M" primer sets were predicted and shown to exhibit a superior specificity for eukaryote templates. In contrast, the frequently applied full-length ("L") primer combinations show a tendency to preferentially amplify archaeal non-target templates. Interestingly, the fact that the recovery of *Archaea* was only problematic in some sediment depths (5.7, 7.6 m), seems to support our hypothesis that mismatching is at least partially controlled by high backgrounds of specific non-target templates. While the abundance of bacterial rRNA genes only varied between 6.6 x 10^6 and 1.1 x 10^7 g^{-1} sediment in the two respective depths (Winderl et al., 2008), archaeal rRNA gene-targeted qPCR revealed an almost 6-fold increase in abundance at 7.6 m (7.4 x 10^4 copies g^{-1} sediment) compared to the other depths (Table 7). This may well explain the results obtained with the "L" primer set for this depth.

Considering the high ratio of archaeal non-targets matching the "L" primers at decreased PCR specificity in my ARB database, and also the fact that a very similar f-primer has been used previously in archaeal community studies (Snell-Castro et al., 2005, Wilms et al., 2006), it is obvious that in environments high in archaeal numbers the microeukaryotic community will be especially hard to detect. Archaeal mismatching is more likely to be a crucial point in microeukaryote analyses than bacterial mismatching due to the closer sisterhood of *Archaea* and *Eukaryota* in evolutionary theory (Cavalier-Smith, 2002).

Using the shorter "S" and "M" primer combinations, no prokaryotic mis-targeting was detected at any depth in the clone libraries. However, the "M" amplicons showed increased fungal abundances, most likely connected to the use of the distinct reverse primer (Euk1179r). Although I cannot explain this observation by *in silico* predictions at present (differential matching of protists and fungi was not evident in my database screenings), primer-dependent distinctions in the recovery of specific microeukaryote lineages have been previously described. A recent multiple PCR-primer approach (Stoeck et al., 2006) revealed an overlap of only 4% of eukaryotic OTUs in clone libraries generated with three different eukaryote-specific primer sets. Preferential amplification during PCR can have a variety of reasons (v. Wintzingerode et al., 1997), but the reproducibility of my findings (in cloning and T-RFLP) as well as the generally good reproducibility of our entire T-RFLP workflow (Fig. 6) indicates that systematic factors of PCR selection rather than PCR drift (Polz and Cavanaugh, 1998, Wagner et al., 1994) favoured the amplification of fungal genes with reverse primer Euk1179r. Amplification competition can be caused by different GC contents, primer site affinities, the accessibility of rRNA genes inside genomes and other factors (Fry et al., 1997, Hansen et al., 1998, Hartmann and Widmer, 2008).

The selection of an optimal restriction endonuclease using my *in silico* dataset resulted in comparable OTU numbers, but more than 30 % of OTUs had no restriction site for the enzymes *Hae*III, *Rsa*I or *Mnl*I. Undigested amplicons may be disadvantageous due to various reasons: The resulting T-RFs are limited to smaller size ranges and OTU homoplasy is more likely (Thies, 2007), thus concealing potential eukaryotic diversity. Unrestricted OTUs are

also generally longer and often proximal to each other, thus T-RF drift (Kaplan and Kitts, 2003) is also more likely to occur and complicate the interpretation of T-RFLP patterns. Because of these disadvantages, the high numbers of OTUs observed for *Hae*III, *Rsa*I or *Mnl*I are only of minor use in T-RFLP analysis. Especially considering the ratio of T-RFs directly assignable to taxonomic groups, the use of *Bsh*1236I and *Msp*I seems to be most promising in protistan T-RFLP analysis. For practical reasons as detailed in Fig. 4, I decided to use *Bsh*1236I in this thesis, but optimal enzyme selection may well be worth reconsidering for different systems and habitats.

Comparing the results of the clone libraries with T-RFLP data (Table 4), it is apparent that the fingerprinting approach using the "S" primers is well reflecting eukaryotic community composition as detected in the clone libraries. Of course, one drawback of T-RFLP analysis is the inferior resolution for rare species, leading to an underestimation of diversity. This, however, is a general feature in microbial community fingerprinting which in no way lessens the utility of such approaches for the rapid screening and comparison of community structure in larger sets of samples.

In summary, I have clearly identified the "S" primer pair (Euk20f/Euk516r) as most suitable for the detection and fingerprinting of protistan 18S rRNA genes in the investigated aquifer samples. Intrinsic *Fungi* and other eukaryotes were also recovered by the assay, but this has to be accepted considering the wide phylogenetic dispersal of protists of potential ecological relevance within the eukaryotic domain. The general applicability of this assay for groundwater and also other protistan habitats, however, requires more extensive scientific attention. Nevertheless, this is to my knowledge the first report using T-RFLP analysis for protistan communities in groundwater systems. The described assay may help to shed more light on the structure and function of protistan populations in aquifers and other habitats. The subsequent chapter is dealing with the application of the established assay ("S" primer set) for a depth-resolved characterisation of the Düsseldorf-Flingern site and describes the composition and diversity of detected eukaryotic communities also with the "M" assay selective for fungi.

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3 Depth-resolved molecular analysis of protistan communities through the redox zones of a BTEX contaminated aquifer

3.1 Introduction

Groundwater is humankind's most important source of potable water. Reflected in the idea of ecosystem services and recognizing the benefits obtained from hydrologic ecosystems (Brauman et al., 2007), research on the functioning of groundwater systems is gaining momentum these days, especially due to the growing awareness of anthropogenic depletion and pollution of this essential resource (Foster and Chilton, 2003). Contamination of aquifers with xenobiotic compounds, e.g. mono- and polycyclic hydrocarbons, has led to particular scientific interest in microbial activities contributing to the degradation of such contaminants and the attenuation of environmental pollution. Today, microbial activities leading to the removal of hydrocarbons under both aerobic and anaerobic conditions are fairly well understood (for recent review see (Andreoni and Gianfreda, 2007)) and also fungi and algae are known to be able to degrade BTEX and PAH compounds aerobically (Middelhoven, 1993, Reineke, 2001, Todd et al., 2002). However, biogeochemical factors such as the limitation or depletion of essential nutrients (nitrogen and phosphorous) or electron acceptors (e.g. oxygen, nitrate, sulphate), but also biological interactions such as competition or predation may limit or influence *in situ* biodegradation potentials (Albrechtsen and Christensen, 1994, Van Stempvoort et al., 2007, Wilson and Bouwer, 1997). These controls of intrinsic natural attenuation processes in groundwater are only beginning to be addressed, and in-depth scientific elucidation is certainly essential for a comprehensive understanding of aquifer ecosystem functioning.

Food webs are a prerequisite of the flow of carbon and energy in most ecosystems. In groundwater habitats, food chains are generally less complex due to the scarcity or complete absence of higher organisms. Nevertheless, phagotrophic protists represent an important higher trophic level above prokaryotes and may play an important role in groundwater's natural attenuation potential. Protists are well accepted to influence carbon and nutrient flow and to directly affect the survival and activity of prokaryotes in various environments (Corliss, 2002, Pernthaler, 2005, Sherr and Sherr, 2002). However, only little is known about their distribution and diversity in groundwater, and even less about their functional importance especially in contaminated aquifers (Novarino et al., 1997).

To date, most studies addressing the impact of protists on biodegradation in groundwater performed cell counts for functional groups such as flagellates, ciliates or amoeba (Fusconi and Godinho, 1999, Kinner et al., 2002, Sinclair et al., 1993, Zarda et al., 1998), revealing high protistan abundances even in contaminated and oxygen-depleted zones. Others used protists isolated from aquifer samples for experiments in batch cultures, detecting positive effects on biodegradation of organic pollutants (Mattison and Harayama, 2001, Mattison et al., 2005, Mauclaire et al., 2003, Tso and Taghon, 2006). It has been assumed that protozoa can reduce bio-clogging (Mattison et al., 2002, Sinclair et al., 1993), enhance microbial activities like DOC degradation or nitrification rates (Kinner et al., 2002, Strauss and Dodds, 1997), and also stimulate anaerobic microbial activities (Biagini et al., 1998).

Contrarily, there is a current lack of knowledge of the true diversity and identity of protists and other microeukaryotes to be found *in situ* in contaminated aquifers. As for prokaryotes, advances in molecular methods have recently accelerated insights into protistan diversity in various environments (for review see (Epstein and López-García, 2008)).

Additionally, recent studies also focused on microeukaryote diversity in anoxic (Behnke et al., 2006, Dawson and Pace, 2002, Takishita et al., 2007) as well as hydrocarbon-contaminated environments (Brad, 2007, Lara et al., 2007a). It has been established that microbial degradation and intrinsic bacterial communities can be tightly coupled to specific biogeochemical zones of a contaminated aquifer (Anneser, 2008, Fry et al., 1997, Roling et al., 2001, Winderl et al., 2008). Such concepts may be also true for intrinsic microeukaryote populations in aquifers, and that such zone-specific community distinctions may refer to the putative role of microeukaryotes in biodegradation.

To address these questions, I have assessed microeukaryotic community variation through distinct redox and contamination zones of the tar-oil contaminated aquifer at Düsseldorf-Flingern, for which the biogeochemical conditions and microbial community distribution have been precisely described (Anneser, 2008, Winderl et al., 2008). The site has been shown to harbour a highly contaminated plume core of BTEX compounds, underlain by a sulphidogenic gradient zone most active in sulphate-dependent BTEX degradation. An involved catabolic marker, the benzylsuccinate synthase (*bssA*) gene was shown to be most abundant in the sulphidogenic gradient zone. Both 16S rRNA and *bssA* gene quantities decreased towards deeper layers, where BTEX compounds were no longer detectable, but which still contained considerable PAH contamination (Fig.3).

Nucleic extracts from frozen sediment samples of these different depths were used for the amplification of eukaryotic 18S rRNA genes allowing the phylogenetic identification of indigenious microeukaryotes in clone libraries and the detection of community shifts with microeukaryote-targeted T-RFLP fingerprinting analyses. By application of the optimised PCR/T-RFLP fingerprinting assay established in chapter 2, I provide evidence for surprisingly characteristic distribution patterns of different microeukaryote lineages, and discuss their potential functional relevance in anaerobic contaminant turnover.

3.2 Materials and Methods

3.2.1 Sediment sampling and DNA extraction

DNA was extracted from sediment cores of the tar oil contaminated aquifer in Düsseldorf-Flingern (Germany), taken during the installation of a high-resolution multi-level well (HR-MLW 19222) in June 2005. The site, sampling procedure and biogeochemical measurements have been described in detail elsewhere (Anneser, 2008, Winderl et al., 2008). A short overview is given in Fig. 3. The analysed samples covered the unsaturated zone (5.7 m below ground surface), the capillary fringe (6.3 m), the contaminant plume core (6.7 m, >40 mg L^{-1} BTEX, >15 mg L^{-1} naphthalene, >8 mg L^{-1} sulphide in groundwater), the sulphidogenic gradient zone underneath the plume core (7.2 and 7.4 m), a PAH dominated deeper zone (8.1, 9.1, 9.8 and 10.2 m, 10-40 mg kg^{-1} PAH in sediments) and a less contaminated bottom zone (11.7 m).

DNA was extracted from freshly thawed ~2-4 g sediment aliquots by treatment with lyzozyme and proteinase K with subsequent purification by extraction with phenol-chloroform-isoamyl alcohol and chloroform- isoamyl alcohol following a previously described protocol (Winderl et al., 2008). For each depth, 2-5 replicate extractions (~1 g sediment each) were pooled in 30 µl of elution buffer (Qiagen) and stored frozen (-20°C) until further analyses.

3.2.2 Cloning and sequencing, phylogenetic analyses

PCR conditions for eukaryotic 18S rRNA gene amplicons from 1 µl template DNA using the primer set Euk20f/Euk516r ("S" combination, used for all 10 depths) and Euk20f/Euk1179r ("M" combination, used for extracted DNA of 7.4, 8.1 and 10.2 m depth) were as described before in chapter 2. Also the protocol used for cloning and sequencing can be found elsewhere in detail (Winderl et al., 2007). Sequencing reads were assembled and quality checked using the SeqMan II software (DNAStar). Clones were initially affiliated using BLAST (http://www.ncbi.nlm.nih.gov) and subsequently integrated into an ARB database based on the "Jan04" release (Ludwig et al., 2004) updated for groundwater protists using the ARB FastAligner utility and further manual refinements. All sequences were checked for chimeric nature by using Chimera_Check (version 2.7) of the Ribosomal Database Project II (Maidak et al., 2001) and the Bellerophon server (Huber et al., 2004). Potential chimeras were further verified by BLAST analysis of partial sequences. 4 sequences of chimeric origin were detected (1 at 5.7 m and 6.3 m each, 2 at 9.8 m depth) for the Euk20f/Euk516r primer set and 3 in the "M" library of 10.2 m depth and removed. Furthermore, 14 % of all clones (15 % of "M" clones respectively) were excluded from further analysis due to incomplete inserts or low sequence quality.

A core phylogenetic tree for important lineages detected was reconstructed by PUZZLE as implemented in ARB (testing reliability of internal branches by analysing 10'000 intermediate trees) based on a selection of full-length 18S rRNA genes using only conserved alignment positions defined by a > 50% base-frequency filter (1600 columns in total). Afterwards, representative clone sequences from this study as well as relevant closely related partial NCBI GenBank entries were added to the tree using the ARB parsimony interactive sequence adding tool. Finally, a consensus tree of both dendrograms was manually constructed, while puzzling values as well as average branch lengths stemming from major bi- and multifurcations were conserved.

For each clone library, Jukes-Cantor corrected distance matrices were calculated using the ARB Neighbour Joining algorithm and used for rarefaction analysis as a measure of clone library coverage and diversity statistics (non-parametric Chao1 richness estimator, Shannon diversity index *H'*) by DOTUR (Schloss and Handelsman, 2005). Clones were grouped into OTUs on a 3 % distance level. Similarity of clone libraries was statistically evaluated using ∫-LIBSHUFF (Schloss et al., 2004) with an ARB generated Jukes-Cantor corrected distance matrix with 10'000 iterations, taking into account recent insights that this cannot reliably discriminate, which community may be a subset of another (Schloss, 2008). P-values were corrected for multiple comparisons after Bonferroni (Abd El-Latif et al., 2006). Additionally, SONS (shared OTUs and similarity) was used to calculate classical Jaccard (J_{clas}) index as incidence-based measure of community similarity (Schloss and Handelsman, 2006) with $J_{clas} = S_{12}/S_1+S_2-S_{12}$, where S_1, S_2 are the number of observed or estimated OTUs in library 1 and 2 and S_{12} the number of shared OTUs. All sequences from this study will be available at GenBank.

3.2.3 T-RFLP fingerprinting

Terminal restriction fragment length polymorphism (T-RFLP) analysis of eukaryote 18S rRNA gene amplicons was done as described in chapter 2, using eukaryote-specific primer pair Euk20f-FAM/Euk516r and Euk20f-FAM/Euk1179r (Biomers) and *Bsh*1236I (Fermentas) digestion. Eukaryotic diversity in fingerprints was estimated by calculating Shannon *H'* as $H' = -\Sigma\, pi \ln pi$, where *pi* is the relative frequency of a specific T-RF (Hill et al 2003).

3.3 Results

3.3.1 T-RFLP fingerprinting of depth-resolved eukaryotic communities

First, eukaryotic diversity and apparent community shifts over different depths of the contaminated aquifer were evaluated using the optimised T-RFLP fingerprinting assay established in chapter 2. Analyses covered the different zones of the contaminated aquifer characterized by changing biogeochemical and bacterial community patterns, and by decreasing bacterial 16S rRNA gene abundances (Anneser, 2008, Winderl et al., 2008). The resulting electropherograms (shown in Fig. 7A) revealed substantial shifts of eukaryotic T-RF patterns throughout the depth-profile.

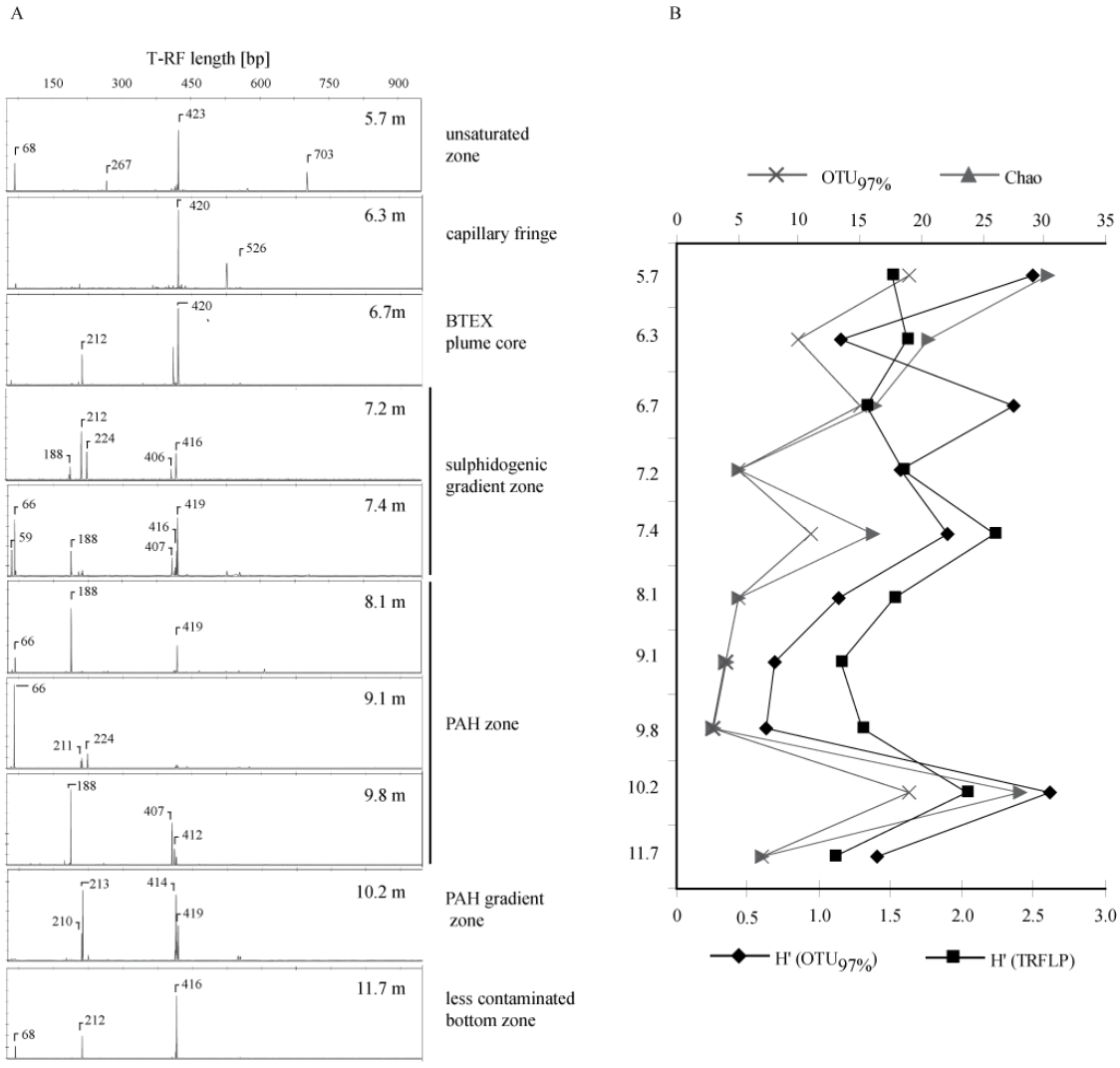

Fig. 7. (A) Depth-resolved eukaryote 18S rRNA gene T-RFLP fingerprinting of the Flingern aquifer. Selected characteristic T-RFs mentioned in the text are labelled by their lengths [bp]. (B) Comparison of detected number of OTUs and estimated richness (Chao1) and of Shannon *H'* diversity indices calculated over T-RF abundances of T-RFLP analysis and OTU abundances of clone libraries. Values are given in Table A.4.

The eukaryote community from an unsaturated soil sample (5.7 m) produced unique T-RFs (267, 423 and 703 bp), not detectable at the capillary fringe (6.3 m) or in any deeper sample. The electropherograms of 6.3 m (with a unique 526 bp peak) and 6.65 m shared a dominant 420 bp peak not to be found in other strata, whereas a 212 bp peak of the latter was

detected also in the sulphidogenic gradient zone at 7.2 m and in the less contaminated bottom zone at 11.7 m depth. Both also shared a 416 bp peak with the 7.4 m sample. A 224 bp peak was detected in 7.2 and 9.1 m depth only. From the sulphidogenic zone on, a 188 bp T-RF appeared, becoming dominant at depth of 8.1 and 9.8 m. It was not detected at 9.1 m. Contrarily, a 66 bp peak also found in the sample of 7.4 and 8.1 m clearly dominated this fingerprint. Another important T-RF was at 419 bp, found at 7.4, 8.1 and 10.2 m depth, the latter displaying also some unique peaks (210, 213 and 414 bp). Thus, as indicated also by the calculated Shannon *H'* (Fig. 7B), T-RFLP analysis detected not only shifts of prominent community components, but also differences of overall diversity between the samples. *H'* increased towards a maximum in the sulphidogenic gradient zone at 7.4 m depth and was lower again in the zones below (except for the sample at 10.2 m).

3.3.2 Sequence analysis of depth-resolved eukaryote clone libraries

In order to identify the eukaryote lineages represented within T-RFLP patterns, 10 clone libraries were constructed from the depth-resolved DNA extracts and 594 clones were sequenced in total. The number of valid (complete, non-chimeric) sequences (518 in total) of each clone library is given in Table 8.

Table 8 Clone library compositions of different depths. In brackets the number of $OTU_{97\%}$ are given for a lineage. Identical OTUs are marked with * where possible.

	number of clones (OTUs) in libraries from depth [m] below ground surface of:									
Phylogenetic affiliation	**5.7**	**6.3**	**6.7**	**7.2**	**7.4**	**8.1**	**9.1**	**9.8**	**10.2**	**11.7**
Amoebozoa										
-Lobosea	5 (2)									
-unidentified	2 (1)		2 (1)		1	1		6 (1)		
Alveolata										
-Oligotrichia	11 (1)*				2 (1)*					
-Heterotrichida			10 (1)*		3 (1)*					
- Trichostomatia		1								
- Peniculida		1*	2 (1)*							
- Scuticociliatia	3 (1)									
- Stichotrichia		1								
- Hymenostomatia			1							
- Peritrichia		37 (1)*							1*	
- Colpodea									1	
Cercozoa										
- Reticulosida	2 (1)									
- Cercomonadida	19 (7)*	1*	13 (1)*						1*	
Euglenozoa										
- Kinetoplastida		1			11 (1)*	28 (1)*		43 (1)*	1*	
Jakobida		1								
Malawimonidae-related		1								
Choanozoa -related			4 (1)							
Basidiomycota										
-Pucciniomycotina	2 (2)		2 (1)	31 (2)	17 (2)	14 (1)			6 (2)	26 (1)
-Agaricomycotina			1	6 (1)	1				1	3 (2)
-unidentified	1		3 (1)	4 (1)	1				1	
Ascomycota										
-Pezizomycotina					4 (1)		1	1	16 (3)	19 (3)
unidentified fungi	1									
Chlorophyta										
- Trebouxiophhyceae			1*	10 (1)*			8 (1)*		3 (1)*	
- Chlorophyceae									1	
Streptophyta	1		8 (2)	7 (1)		2 (1)	2 (1)		20 (6)	2 (1)
Metazoa	11 (1)	5 (2)	6 (3)		10 (2)	6 (1)	40 (1)			
clones (OTUs) in total	58 (19)	49 (10)	53 (15)	54 (5)	50 (11)	51 (5)	51 (4)	50 (3)	52 (19)	50 (7)

Rarefaction curves of the single clone libraries are displayed in Fig. 8. Some libraries (from 7.2, 8.1, 9.1 and 9.8 m) showed a very low diversity (≤ 5 expected $OTU_{97\%}$) with few dominant lineages and a low amount of singleton clones detected (Table 8). Here, rarefaction curves were indicative of diversity saturation, as shown also by the estimated $Chao1_{97\%}$ (Fig. 7B). In contrast, other libraries, such as the one from the capillary fringe (6.3 m) were far from reaching saturation due to one dominating OTU and 70 % of singletons. Apparent highest eukaryote diversity (19 OTUs) was detected in the libraries of the unsaturated sample (Chao = 28) and at 10.2 m depth (Chao = 30) followed by the plume core with 15 OTUs (Chao = 16). Obviously, these differences in diversity were also reflected in the Shannon *H'* indices for clone OTUs as defined by 97 % sequence similarity (Fig. 7B). Compared with *H'* calculated for T-RF diversity, both approaches resulted in similar trends below the contamination plume (e.g. the highest diversity was at 10.2 m depth). However, in the first three samples T-RFLP analysis underestimated clone OTU diversity (5.7, 6.7 m), or overestimated it (6.3 m).

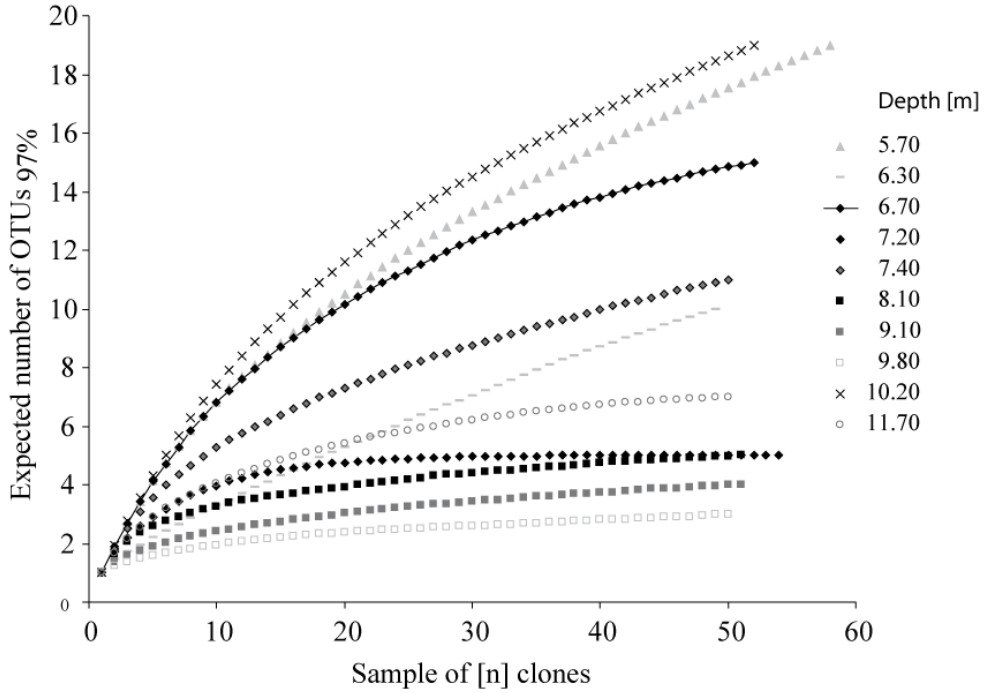

Fig. 8. Rarefaction analysis of the 10 eukaryotic 18S rRNA gene clone libraries generated from Flingern sediments. Library names indicate sampling depth below ground surface. Calculations were performed at a level of 97 % sequence similarity cut-off for distinct OTUs. Values are given in Table A.5.

In total, 59 different OTUs (97 % sequence similarity) were detected through the depth profile belonging to various eukaryotic phyla (Table 8). I detected protists of the *Alveolata, Amoebozoa, Cercozoa, Chlorophyta, Choanozoa, Euglenozoa, Jakobida,* but also yeast-like fungi of the *Basidiomycota* and *Ascomycota*, as well as sequences of non-targeted higher plants and animals. The phylogenetic placement of selected representative clones is given in Fig. 9. Further information and results of NCBI Blast matches for each OTU are summarised in Table 9.

Table 9 Affiliation and T-RFs (predicted and measured) of $OTU_{97\%}$ detected at the Düsseldorf- Flingern site.

Σ clones	found in depths [m]	T-RF measured	T-RF predicted	BLAST match (Accession No.); sequence similarity (%)	Taxonomy
5	5.7	423	423	Thaumatomonadida environmental sample (EF024039); 96	Cercozoa
1	5.7	257	258	Cercomonadida environmental sample (EF023604); 95	Cercozoa
24	5.7, 6.3, 6.7, 10.2	420	420	uncultured eukaryote (EU091848); 99	Cercozoa
1	5.7		429	uncultured cercozoan clone (AY620294); 93	Cercozoa
1	5.7	257	258	Cercomonadidae environmental sample (EF023604); 92	Cercozoa
1	5.7	176	177	uncultured cercozoan (AM114800); 95	Cercozoa
1	5.7	423	423	Eocercomonas sp. strain 13-3.5 (AY884316); 96	Cercozoa
2	5.7	176	177	Uncult. eukaryote clone:18S-KM-B-33 (AB238190); 98	Cercozoa
1	10.2	253	253	Pseudoplatyophrya nana (AF060452); 98	Colpodea
83	7.4, 8.1, 9.8, 10.2	188	191	Bodo saltans (AY490224); 99	Euglenozoa
1	6.3		191	Neobodo designis (DQ207585); 98	Euglenozoa
13	6.7, 7.4	204	207	Spirostomum teres (AM398199); 99	Heterotrichida
1	6.7		542	Tetrahymena farleyi (AF184665); 99	Hymenostomatia
1	6.3		402	Andalucia godoyi isolate AND28 (AY965870); 93	Jakobida
1	6.3	336	337	Plagiophyla frontata (Z29440); 97	Litostomatea
3	5.7	420	421	Hartmannellidae environmental sample (EF023499); 93	Lobosea
2	5.7		435	Korotnevella hemistylolepis strain ATCC 50804 (AY121850); 93	Lobosea
1	6.3	231	233	Malawimonas jakobiformis (AY117420) ;89	Malawimonadidae
13	5.7, 7.4	267	268	Halteria grandinella (AF194410); 98	Oligotrichia
3	6.3, 6.7		235	Paramecium tetraurelia (AB252009); 99	Peniculida
38	6.3, 10.2	526	523	Ophistonecta minima (EF417834); 99	Peritrichia
3	5.7		408	Cohnilembus verminus (Z22878); 96	Scuticociliatia
1	6.3		269	Oxytrichidae environmental sample(EF024702); 96	Stichotrichia
2	6.7		414	uncultured eukaryote clone 18S-KM-B-2 (AB238161); 80	unidentified eukaryote
2	5.7	420	421	uncultured eukaryote (EF025032); 87	unidentified eukaryote
8	7.4, 8.1, 9.8	412	412	uncultured eukaryote clone 18S-KM-B-23 (AB238180); 87	unidentified eukaryote
1	5.7		220	uncultured marine eukaryote clone MA1_2H3P (EF527201); 91	unidentified eukaryote
8	6.7, 7.2, 11.7		213	Asterophora lycoperdoides (AJ496254); 99	Agaricomycotina
4	7.4, 10.2, 11.7		419	Cryptococcus vishniacii (AB032657); 99	Agaricomycotina
1	10.2	407	406	Uncultured fungus clone clone WIM108 (AM114819); 96	Basidiomycota
9	5.7, 6.7, 7.2, 7.4		405	Uncultured basidiomycete clone BAQA52 (AF372708); 97	Basidiomycota
12	10.2, 11.7		415	Cladosporium cladosporioides strain MD-2 (EU375523); 99	Pezizomycotina
6	10.2		415	Pleosporales sp. RMF2 (EF532931); 99	Pezizomycotina
16	7.4, 9.8, 11.7	59	66	Trichoderma viride (AF525230); 99	Pezizomycotina
7	9.1, 10.2, 11.7		413	Glyphium elatum (AF346419); 99	Pezizomycotina
90	5.7, 6.7, 7.2, 7.4, 10.2, 11.7	420	419	Sporobolomyces foliicola (AB021671); 99	Pucciniomycotina
2	5.7, 7.4	416	417	Rhodotorula sp. W500 (DQ781315); 99	Pucciniomycotina
2	10.2		410	Sporobolomyces lophatheri (AB126046); 93	Pucciniomycotina
1	10.2	224	227	Bracteacoccus sp. BC2-1 (AF516676); 99	Chlorophyceae
4	6.7		426	Uncultured eukaryotic picoplankton clone P1.39 (AY642707); 84	Choanozoa related
21	6.7, 7.2, 9.1, 10.2	224	225	Trebouxia jamesii (Z68700); 99	Trebouxiophyceae
1	10.2	224	225	Stichococcus sp. NB2VFF10 (AF513370); 100	Trebouxiophyceae
55	7.4, 8.1, 9.1	66	74	Anisochrysa carnea (X89482); 98	Metazoa
11	5.7	68	74	Xenillus tegeocranus (AF022042); 93	Metazoa
1	7.4		187	Mus musculus (NR_003278); 98	Metazoa
5	6.3, 6.7		191	Olavius algarvensis (AF411870); 98	Metazoa
3	6.3	68	74	Stenostomum leucops aquariorum (AJ012519); 94	Metazoa
1	6.7		74	Toxoptera citricida (AY216697); 98	Metazoa
2	6.7		74	uncultured eukaryote clone 18S-KM-B-23 (AB238180); 87	Metazoa
3	5.7, 10.2		211	Fibraurea tinctoria (EU240573); 98	Streptophyta
11	6.7, 10.2, 11.7	76	70	Pinus wallichiana (X75080); 99	Streptophyta

Σ clones	found in depths [m]	T-RF measured	T-RF predicted	BLAST match (Accession No.); sequence similarity (%)	Taxonomy
10	7.2, 9.1, 10.2		211	Caryophyllaceae envir. sample clone Amb_18S_453 (EF023163); 98	Streptophyta
7	8.1, 10.2		211	Caprifoliaceae envir. sample clone Amb_18S_1479 (EF024006); 98	Streptophyta
3	10.2	211	212	Uncult. eukaryote 18S rRNAclone EXP.2-Band_S(0%) (AB273952); 99	Streptophyta
2	10.2		211	Restionaceae envir. sample clone Elev_18S_1510 (EF024965); 99	Streptophyta
1	10.2		211	Hedyosmum arborescens (AF206925); 97	Streptophyta
1	10.2		423	Pinus wallichiana (X75080); 96	Streptophyta
3	6.7		553	Pinus wallichiana (X75080); 96	Streptophyta
1	11.7		423	Pinus wallichiana (X75080); 96	Streptophyta

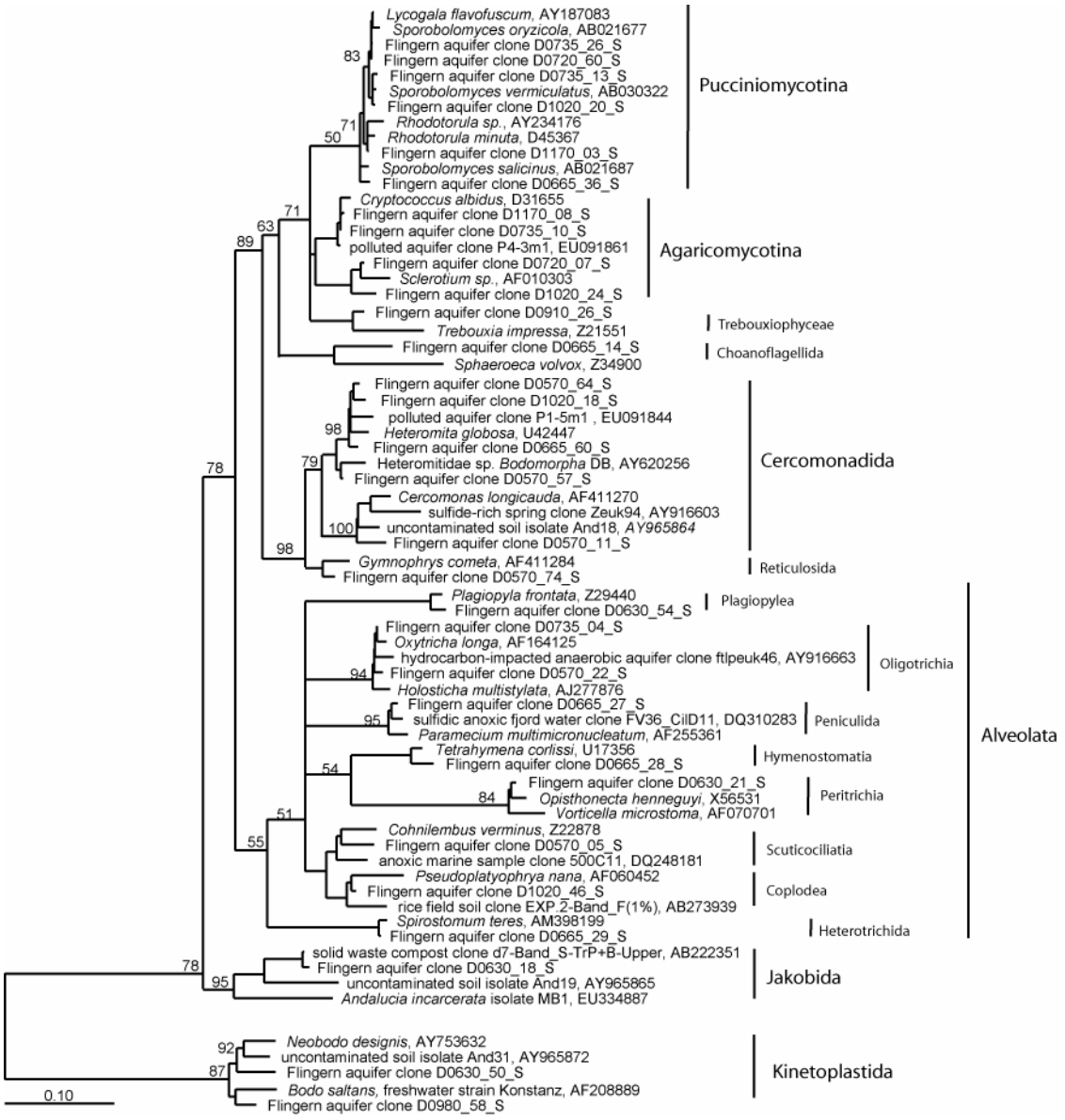

Fig. 9. Phylogenetic tree showing the affiliation of selected clone sequences recovered from depth-resolved sediment samples to other environmental clone sequences and pure culture organisms from public databases. Clone library names indicate sampling depth below ground surface (D + depth [cm]). A core phylogenetic tree was reconstructed by quartet puzzling, shorter clone sequences were subsequently added with the parsimony interactive sequence addition tool of ARB. Puzzling values at nodes indicate the reliability of furcations and are based on 10'000 intermediate puzzling steps. Bar represents 10% sequence divergence. NCBI (GenBank) accession numbers of the sequences are indicated.

Clear shifts in eukaryote communities of the different zones of the aquifer were apparent also in the clone libraries (Table 8). Most ciliates (*Alveolata*) were detected in the samples above 8.1 m depth, also revealing pronounced differences in recovered alveolate communities over depth (members of 9 subclasses were detected). For example, clones related to peritrich *Ophistonecta* spp. (represented by the unique 526 bp T-RF in Fig.7A) clearly dominated (> 75%) the library from the capillary fringe, but were not detected in other zones except one single clone at 10.2 m. Interestingly, in T-RFLP analysis of 6.3 m depth (Fig. 7A), the 526 bp T-RF attributed only 20% to total peak heights. *Spirostomum*-affiliated sequences (*Heterotrichida*) were only found inside the contamination plume and the gradient zone at 7.4 m depth. Like these alveolates, also cercozoan community members were more frequent above the sulphidogenic gradient zone, showing a diverse population (7 OTUs, represented by the 423 and 703 bp T-RFs) in the unsaturated zone sample, with clones related to *Heteromita*, *Bodomorpha* and *Cercomonas* spp., as well as reticulosid *Gymnophrys* spp. contributing over 40 % of the recovered clones. Inside the plume core, only the *Heteromita*-related cercozoans prevailed, represented by the 420 bp T-RF. Another prominent protozoan lineage detected was a *Bodo*-related kinetoplastid, appearing in samples below the contaminant plume. In contrast to cercozoan and ciliate clones, they dominated libraries in the PAH-zone, as reflected also by the abundant 188 bp T-RF in these depths. So far unidentified protists distantly related to *Amoebozoa* were also detected at 9.8 m, and were represented within the 412 bp T-RF found at this depth. However, along with other clones related to *Lobosea* (*Hartmannellidae*) detected at 5.7 m, these putative *Amoebozoa* were not added to the phylogenetic tree (Fig. 9) due to inconsistent branching. Of further interest was one minor protistan OTU (4 clones) related to the *Choanozoa* detected inside the plume core.

However, not only protozoan lineages were detected in the clone libraries. Also other microeukaryotes, mainly yeast-like fungi of the phylum *Basidiomycota* were recovered from most depths (except for the 6.3, 9.1 and 9.8 m samples). These fungi were mostly represented by one OTU (90 clones in total) related to *Sporobolomyces* and *Rhodotorula* strains. The respective T-RFs appear as a number of peaks between 406 and 420 bp length (Table 9). Contrarily, yeast-like *Ascomycota* related to *Trichoderma*- and *Cladosporium* spp. were abundant only in the two deepest layers (Table 8, not shown in Fig. 9). Surprisingly, the 224 bp peak detected at 7.2 and 9.1 m was assignable to unicellular algae related to *Trebouxia* sp..

Furthermore, also clones related to non-targeted (non-protistan) metazoa and higher plants were detected in single samples throughout the sediment core, sometimes in high abundances. They even accounted for 82 % of clones in the sample from 9.1 m, were no protozoan lineages were recovered at all (Table 8). The metazoan clones of the unsaturated zone were identified as members of *Acari* (68 bp T-RF), whereas at the capillary fringe they were affiliated to *Plathelminthes* and *Oligochaetes* (*Olavius* sp. related), the latter also detected inside the contamination plume. In deeper layers, the most abundant metazoan OTU (55 clones in total) was surprisingly related to neopteran insects, dominating the clone library in 9.1 m depth, but also detected in the two layers above. These clones were represented in the 66 bp T-RF. Furthermore, the high ratio of clones related to higher plants sequenced in the sample from 10.2 m was also unexpected.

Of the 59 OTUs detected throughout the sediment core, 36 % were represented by single clones (Table 8), and 27 % of the more abundant OTUs were only found in a single depth. In order to statistically compare the clone libraries and to identify similar communities or community subsets, ∫–LIBSHUFF analysis was conducted. Because of the low diversity of some libraries, OTU overlap allows to predict which library is a subset of another, even if this cannot be derived by ∫–LIBSHUFF alone (Schloss, 2008). Additionally, ∫–LIBSHUFF is designed to work with undersampled libraries and results of comparisons between highly sampled libraries are inaccurate. For those libraries, fractions of shared OTUs between

communities and classical Jaccard indices (J_{clas}) were considered. Thus the community at 7.2 m was, with 4 out of its 5 OTUs shared (J_{clas} = 0.25), clearly a subset of the community in 6.7 m depth. The same holds true for the libraries of 9.8 and 11.7 m, which consisted mainly of OTUs found at 7.4 m, which was similar to 8.1 m, also shown by ∫–LIBSHUFF p-values > 0.18 (all obtained values are given in Table A.6). Contrarily, the eukaryotic population of the unsaturated zone was significantly different to all other investigated samples, as shown also by distinct T-RFLP patterns. A more detailed overview about the presence of specific OTUs in different depths is given in Table 9. Finally, ∫–LIBSHUFF analysis showed no significant difference between libraries of 6.3 and 10.2 m depth, but a low value of J_{clas} 0.074 disproved this finding.

3.3.3 Analysis of eukaryotic communities applying primer set "M"

As described in chapter 2, the eukaryote-specific primer set Euk20f/Euk1179r had been shown to preferentially amplify fungal templates and therefore gave insights into fungal diversity in 7.4, 8.1 and 10.2 m depth. T-RFLP analysis was conducted as described in chapter 2 and corresponding electropherograms are displayed in Fig. 10. A 416 bp T-RF was detected in all 3 samples showing declining relative abundances from 49 % of total peak heights in 7.4 m to 17 % in 10.2 m depth. Contrarily, a 414 bp T-RF became dominant with increasing depth. Other abundant T-RFs were only found in single samples ranging from 59 to 68 bp and 210 to 212 bp. A 419 bp T-RF became only abundant in 8.1 m depth. Calculated Shannon *H'* indices are given in Table 10.

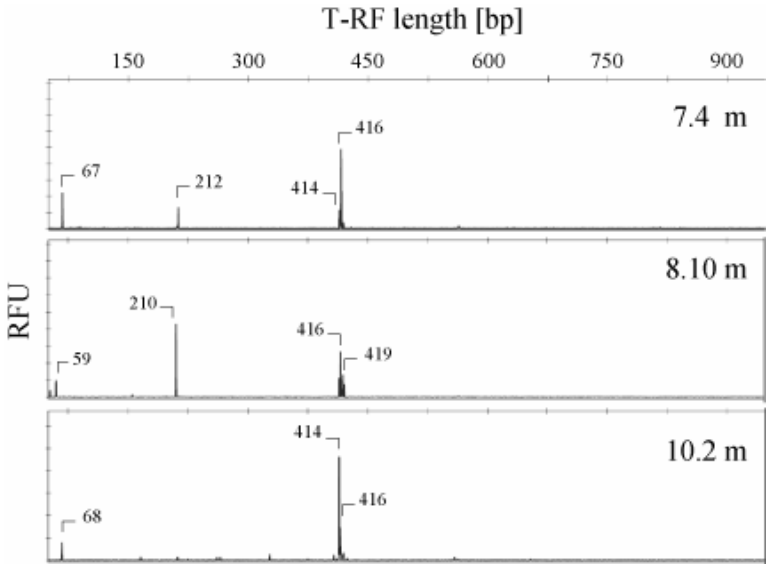

Fig. 10. Electropherograms of samples from the sulphidogenic zone (7.4 m) and the PAH dominated zones below. RFU: relative fluorescence unit.

Clone libraries were created using extracted DNA of 7.4, 8.1 and 10.2 m depth and 52, 54 and 46 clones were sequenced respectively. Rarefaction curves of the libraries are displayed in Fig. 11.

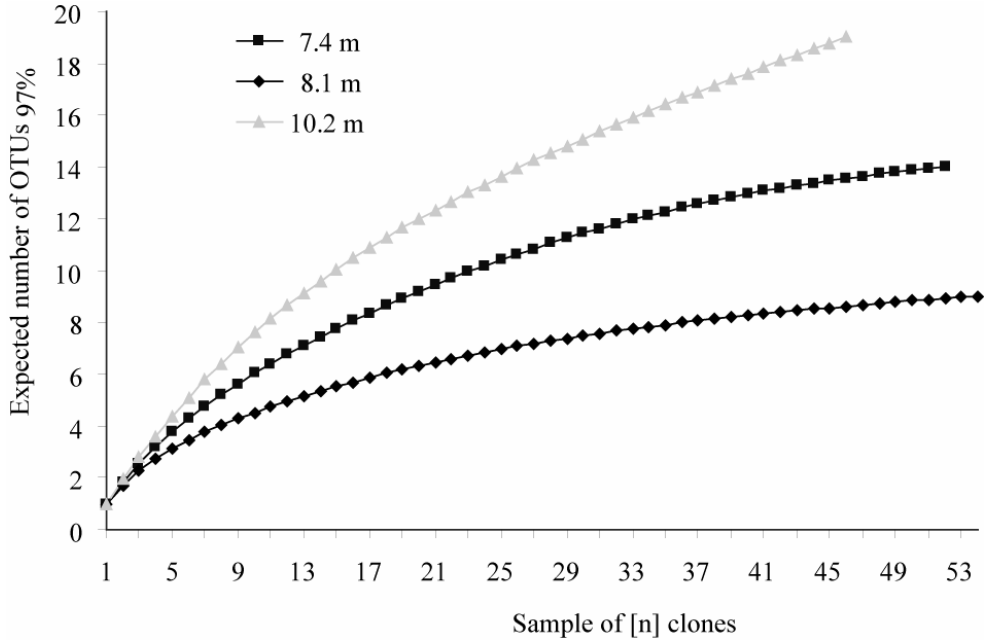

Fig. 11. Rarefaction analysis of the 18S rRNA gene clone libraries generated from Flingern sediments with the "M" primer set. Calculations were performed at a level of 97% sequence similarity cut-off for distinct OTUs. Values are given in Table A.7.

The diversity in 7.4 and 8.1 m depth was covered almost to saturation with 14 ($Chao_{97\%}$ = 14.4) and 9 ($Chao_{97\%}$ = 9.3) $OTUs_{97\%}$, whereas in 10.2 m depth only 19 of estimated 30.2 OTUs were discovered. Comparison of *H'* calculated for $OTU_{97\%}$s and T-RF abundances showed different results of estimated diversity in the samples (Table 10). The sample from 10.2 m depth was found to harbour the most diverse community, whereas by T-RFLP analysis this was from 8.1 m depth. ∫–LIBSHUFF analysis resulted in p-values < 0.0085 (for an experiment-wise error rate of 0.05) for all three comparisons.

In total, 31 OTUs were detected in the three clone libraries of which 74 % were only detected in one depth. Contrarily, 47 % of all clones had been sequenced in all 3 samples. Only a single protozoan clone in 10.2 m depth was detected and identified as cercomonade *Bodomorpha sp.*, two other OTUs were protistan algae of the *Trebouxiophyceae*. Non-targeted metazoan clones comprised 6.6 % of all analysed clones and were identified as oligochaetes (10.2 m) and insects (7.4 m). Additionally, 14.5 % of clones were plants (4 OTUs) and only detected in 8.1 and 10.2 m depth. 77 % of all clones were affiliated to fungi of which 74 % were protists due to yeast or yeast- like morphology. The composition of the libraries is summarised in Table 10.

Table 10 Clone library compositions of eukaryotic 18S rDNA amplified with primer set Euk20f/Euk1179r ("M") of three different depths of the contaminated aquifer.

Phylogenetic affiliation	**number of clones (OTUs) from depth [m] of:**		
	7.4	8.1	10.2
Cercozoa			
- Cercomonadida			1
Basidiomycota			
-Pucciniomycotina	19 (1)	26 (1)	12 (4)
-Agaricomycotina	17 (9)	10 (4)	4 (1)
Ascomycota			
-Pezizomycotina	6 (2)	5 (2)	10 (2)
-Taphrinomycotina			1
- mitosporic	2 (1)		4 (2)
Chytridiomycota			1
Chlorophyta			
- Trebouxiophhyceae			2 (2)
Streptophyta		13 (2)	9 (4)
Metazoa	8 (1)		2 (1)
clones (OTUs) in total	**52 (14)**	**54 (9)**	**46 (19)**
richness estimate Chao1 $_{97\%}$ [b]	14 (14,19)	9 (9,14)	30 (22,67)
diversity estimate H'			
97% OTU	2.15	1.60	2.66
T-RFLP	1.39	1.86	1.73

[b] mean value with 95% confidence intervals given in parentheses.

An overview of the identity of detected OTUs is given in Table 11. Most clones were identified as basidiomycetes and 31 % of clones were related to the yeast *Rhodotorula* sp. (*Pucciniomycotina*) found in all three depths. 17 % were affiliated to *Agaricomycotina*. Only 18 % of the clones were *Ascomycota*, where the majority belonged to *Pezizomycotina* and only 6 clones to fungi *incertae sedis* (mitosporic *Ascomycota*). Compared with results of the "S" libraries, 87 % of the fungal clones (belonging to 12 OTUs) amplified with Euk20f/Euk1179r were detected with both primer combinations.

Table 11 Identified $OTU_{97\%}$s and respective measured T-RFs (predicted in brackets) of libraries amplified with primer set Euk20f/Euk1179r. Protistan lineages are marked in bold.

Depth [m]	Σ clones	T-RF (predicted)	BLAST match (Accession number), sequence similarity	Taxonomy
7.4, 8.1, 10.2	47	416	**Rhodotorula sp. W500 (DQ781315), 99%**	Pucciniomycotina
7.4, 8.1, 10.2	13	415 (416)	Cladosporium cladosporioides strain MD-2 (EU375523), 99%	Pezizomycotina (mold)
7.4, 8.1, 10.2	11	419	**Filobasidium** globisporum (AB075546), 99%	Agaricomycotina
10.2	8	420	**Rhodotorula laryngis (AB126649), 99%**	Pucciniomycotina
7.4, 10.2	7	415	Leptosphaeria maculans Leroy (U04233), 99%	Pezizomycotina (filamentous)
7.4, 8.1	5	212 (213)	Athelia bombacina (M55638), 98%	Agaricomycotina
10.2	3	413	**Coniosporium sp. CBS 665.80 (Y11712), 99%**	mitosporic Ascomycota
7.4, 8.1	3	417 (416)	**Trichosporon dermatis (AB035585), 99%**	Agaricomycotina
7.4	2	212 (213)	Ceriporiopsis pannocincta (AB084590), 96%	Agaricomycotina
8.1	2	419	**Dioszegia hungarica (AB032638)**	Agaricomycotina
7.4	2	405 (406)	**Malassezia pachydermatis AFTOL-ID 856 (DQ457640), 96%**	Basidiomycota
7.4	2	212 (213)	Ceriporiopsis subvermispora (AF334906), 99%	Agaricomycotina
7.4	2	415	Tricladium chaetocladium strain 83-225 (AY357283), 96%	mitosporic Ascomycota (Deuteromycotina)
7.4	2	419	**Cryptococcus laurentii (AB032640), 99%**	Agaricomycotina
7.4	1	212 (213)	**Hyphodontia nespori isolate 3221° (DQ873621), 99%**	Agaricomycotina
8.1	1	59 (66)	Trichoderma viride (AF525230), 99%	Pezizomycotina (filamentous)
10.2	1	416	**Hyphozyma variabilis var. Odora (AJ496240), 99%**	mitosporic Ascomycota (Deuteromycotina)
10.2	1	411	**Rhodotorula hordea isolate AFTOL-ID 674 (AY657013), 93%**	Pucciniomycotina
7.4	1	268	Tulasnella sp. FCUG 2668 isolate AFTOL-ID 622 (AY662665), 96%	Agaricomycotina
10.2	1	417	**Rhodosporidium babjevae (AB073270), 94%**	Pucciniomycotina
10.2	1	419	**Triparticalcar arcticum isolate AFTOL-ID 696 (DQ536480), 99%**	Chytridiomycota
10.2	1	(415)	**Taphrina alni (AJ495834), 99%**	Taphrinomycotina (Ascomycota)
10.2	1	(418)	**Myrmecia bisecta (Z47209), 94%**	Chlorophyta (Trebouxiophyceae)
10.2	1	224 (225)	**Trebouxia asymmetrica (Z21553), 99%**	Chlorophyta (Trebouxiophyceae)
10.2	1	420	Bodomorpha sp. HFCC57 (DQ211596), 99%	Cercozoa
7.4	8	67 (74)	Dorypteryx domestica r KY97 (AY630454), 99%	Insecta
10.2	2	(74)	Rheomorpha neiswestonovae (AY527049), 98%	Oligochaeta
8.1, 10.2	12	210	Luffa quinquefida (AF008957), 99%	Streptophyta
10.2	5	(213)	Pinus wallichiana (X75080), 98%	Streptophyta
8.1, 10.2	4	(417)	Uncultured eukaryote clone Amb_18S_6891 (EF024091), 99%	Streptophyta
10.2	1	(211)	Aesculus pavia (AF206838), 96%	Streptophyta

3.4 Discussion

3.4.1 Depth- related eukaryotic community variation

Both T-RFLP fingerprinting and sequence analysis using primer set Euk20f/Euk516r detected pronounced, but consistent distinctions in protist and eukaryote communities of different redox-zones of the tar-oil contaminated Flingern aquifer. These shifts concerning the distribution and relative abundance of detected lineages (Fig.7A, Table 8) may, to a certain extent, indicate a similar coupling of microeukaryote populations to the local biogeochemical regime as previously found for prokaryotes (Roling et al., 2001, Winderl et al., 2008). This will have to be confirmed further with qPCR using specific primer sets to assess 18S rDNA copy numbers of distinct groups.

The unsaturated sediment sample harboured a diverse (micro-) eukaryotic community clearly distinguishable from saturated samples by characteristic T-RFs and ∫-LIBSHUFF comparison. This hinted towards an extant characteristic vadose zone microeukaryote community, however the analysis of more comparable unsaturated zone samples would have been necessary to further substantiate this hypothesis. Surprisingly, the clone libraries of the capillary fringe (6.3 m) and of 10.2 m depth were not significantly different of each other, although they only shared 2 OTUs. One reason may be the undersampling of diversity at the groundwater table caused by the dominance of one alveolate OTU also found in the deeper zone. Interestingly, the library in 7.2 m was a full subset of the community inside the plume core, but was at the same time devoid of protozoan lineages. Putatively, these were hidden in the sequencing approach due to a dominating abundance of fungi and algae. Consistently, the 188 bp T-RF representative of kinetoplastids was detectable in the respective electropherogram (Fig.7A). The libraries of the PAH dominated samples (down to 9.8 m) were all subsets of the community detected at the lower end of the sulphidogenic zone (7.4 m), with some specific taxa becoming more dominant in the deeper strata.

Community shift were also detected for the fungi-dominated “M” libraries. Here, the H’ values calculated for OTUs confirmed higher eukaryotic diversities in 7.4 and 10.2 m depth found with the Euk20f/Euk516r primer set (Fig. 7B) also for fungi. The T-RFLP assay underestimated detected diversity, most probably due to similar T-RF patterns for different fungal linages (Table 10).

Even though these clear depth-resolved shifts in detectable (micro-) eukaryotic community structure were evident, the relevance or rather the implications of these findings for the importance of the detected protists in on-site processes are much harder to access. Results of PCR-based screenings of community structure have to be discussed carefully, as they may be biased due to various reasons. Complications may be caused by undersampling of a community due to low DNA extraction yields, or by the number of analysed clones, by preferential amplification of specific templates or by differing rRNA gene copy numbers in genomes, all leading to erratic relative abundance interpretations (v. Wintzingerode et al., 1997, Weider et al., 2005). For example, comparing T-RF (20 %) and clone abundances (75 %) for the oligotrich ciliate identified in 6.3 m depth, preferential ligation of ciliate template into plasmids seemed to have taken place obscuring the abundance of cercomonads detected via T-RFLP in respective clone libraries. Especially ciliates are known to harbour multiple 18S rRNA gene copies and therefore, their abundance may be overestimated by PCR approaches. But then, half of the detected subclasses were represented by single clones only, pinpointing problematic conclusions on real cell abundances. Furthermore, the actual activity of an organism detected via DNA is not directly evident, as a template might also stem from

dead cells, resting stages, or otherwise inactive microbes. Unfortunately, generally low protistan abundances in groundwater systems and difficulties in sediment sampling and nucleic acid extraction render surveys of rRNA expression (as a measure of cellular activity) a very challenging task (Weiss and Cozzarelli, 2008). Even though for other habitats such as marine waters these approaches have been proven extremely valuable (Stoeck et al., 2007), I have not been able to perform rRNA-based microeukaryote screenings in this thesis for reasons of yield and quality of extractable rRNA. Keeping these limitations in mind, I will nevertheless discuss aspects of putative activities and ecological functions deducible for the different microeukaryotic lineages detected in the Flingern aquifer.

3.4.2 Potential role of eukaryotic lineages detected in different zones

The unsaturated soil sample harboured a distinct and diverse eukaryotic community, mostly characterised by diverse cercozoan flagellate populations. *Cercozoa* are known as speciose and abundant zooflagellates in soil and freshwater environments (Bass and Cavalier-Smith, 2004). *Heteromita globosa*- related clones have recently been found in PAH-contaminated soil (Lara et al., 2007a) and anaerobic aquifer sediments polluted by landfill leachate, from where an anaerobic culture was successfully isolated (Brad et al., 2008). *Heteromita globosa* soil isolates have been shown to enhance aerobic biodegradation of alkylbenzenes and toluene in batch cultures and to reduce bacterial clogging in sand-filled aquifer columns (Mattison and Harayama, 2001, Mattison et al., 2002, Mattison et al., 2005). Similar functions are conceivable also for the *Cercozoa* detected in the (oxic) vadose zone, as well as the anoxic plume core by influencing microbial processes. Furthermore, a group of clones distantly related to choanozoan sequences was detected inside the plume only. Choanoflagellate clones have been described before from other anoxic sediments (Dawson and Pace, 2002, Slapeta et al., 2005) and oil-polluted seawater (Dalby et al., 2008), and there are hints that they can reproduce under anoxic conditions (Fenchel and Finlay, 1995). A more frequent group of flagellates detected was related to the kinetoplastid *Bodo saltans*, which was only detected at the lower end of the sulphidogenic gradient zone and below, and even dominant in most samples of the PAH zone. Serving as an indicator for low water quality (Jiang and Shen, 2003) and considered to cope with low oxygen concentrations or even anoxia (Stoeck et al., 2005, Bernard, 2000), this ubiquitous nanoflagellate may well be hypothesised to play an active part in the microbial food web underneath the plume. The more or less specific allocation of this lineage to defined depths may indicate that it is more competitive than other protozoa under the constantly reduced conditions under the plume, but at the same time not capable of migrating to the above zones of higher bacterial abundance (Winderl et al., 2008). Higher sulphide concentrations may pose a problem as kinetoplastids were also not detected in other sulphide-rich habitats (Luo et al., 2005).

Furthermore, a surprisingly diverse ciliate community was detected, preferentially in the upper zones of the investigated aquifer, which have been shown to contain highest bacterial 16S rRNA gene copy numbers (Winderl et al., 2008). Ciliate sequences have previously been detected in PAH contaminated soil (Lara et al., 2007b), oxygen-depleted sediment (Slapeta et al., 2005) and a sulphide-rich spring (Luo et al., 2005), but not in an anaerobic aquifer polluted with landfill leachate (Brad et al., 2008). Some closely-related genera have been observed in oxygen-depleted habitats and serve as bioindicator for eutrophic environments, such as heterotrich *Spirostomum teres* or peritrichs related to *Ophistonecta* sp. (Jiang, 2006, Madoni, 2005). All detected ciliate lineages are known to be bacterivores, some of them also preying upon nanoflagellates and the colpodid *Pseudoplatyophrya nana* (one related clone detected at 10.2 m) even on fungi (Petz et al., 1986).

Although the true abundances and activities of protists cannot be unravelled by the applied PCR-based methods, the dominance of ciliate and flagellate amplicons in the upper

strata of the aquifer, where also the highest numbers of bacterial 16S rRNA genes were detected (Winderl et al., 2008), may point towards an active microbial food web. If true, then the detected protozoan community members may well influence microbial contaminant degradation processes in the investigated aquifer.

Very likely, this might also be the case for the detected fungal community, which dominated the clone libraries with increasing depth. Even though they can be found in drinking water and wells (Kanzler et al., 2008), to my knowledge their role in groundwater ecosystems has not been appropriately addressed yet, even though it is known for decades that fungi are capable of aerobically degradation of aromatic hydrocarbons (Middelhoven, 1993). A similar diversity of yeast-like *Basidiomycota* and *Ascomycota* has recently been detected in an anaerobic landfill leachate polluted aquifer (Brad et al., 2008). Comparison of fungal diversity obtained with both primer sets revealed additional OTUs detected in "M" libraries, but the majority of those were singletons or doubletons. Therefore, most abundant fungal lineages seemed to be well assessed by the favoured "S" primer combination covering overall protistan diversity. Clones related to *Rhodotorula* and *Sporobolomyces* spp. formed the most abundant microeukaryote lineage detected in this study (18% of all clones). Psychrophilic members of this group and also of the detected *Cryptococcus* lineage (*Agaricomycotina*) are known for the aerobic degradation of BTEX compounds and PAHs under low temperature (Bergauer et al., 2005). This may be of relevance in generally cold groundwater environments (Ekendahl et al., 2003). Similarly, also the detected ascomycetes (not shown in Fig. 9), the majority related to *Trichoderma* and *Cladosporium* spp., are potential aromatic hydrocarbon degraders and were also found in oxygen-depleted habitats before (Leahy and Colwell, 1990, Potin et al., 2004, Salvo et al., 2005). Even though to my knowledge no data exists proving anaerobic aromatic hydrocarbon degradation pathways in fungi, the repeated detection of these organisms in oxygen-depleted environments may not be at random. Considering their ability to grow by fermentation and combining this with their occurrence at highly contaminated sites and their tolerance to aromatic hydrocarbons, this may indicate a direct or indirect involvement of yeast-like fungi in contaminant breakdown *in situ* (Abd El-Latif et al., 2006, Ekendahl et al., 2003, Hughes et al., 2007).

Chlorophyta were found in changing abundances throughout the aquifer. The majority of clones was closely related to *Trebouxia sp.*, also detected recently in an anoxic aquifer (Brad et al., 2008). Subsurface transport of algal cells introduced by recharge or infiltration is known to take place (Kloep and Röske, 2004). Whether algae can be metabolically active in the subsurface has to my knowledge not been assessed yet. Some algal strains are highly resistant to black oil pollution and non-photosynthetic members of the *Trebouxiophyceae* are capable of petroleum degradation (Safonova et al., 1999, Walker et al., 1975), therefore also here, a potential heterotrophic activity of these organisms *in situ* cannot be excluded. Additionally, osmotrophy of protozoan species may also take place, but knowledge about the importance of this nutritional mode is still scarce (Ekelund and Ronn, 1994). But it is assumed to be more relevant in eutrophic systems, where eukaryotes do not have to compete with prokaryotes. This would be the case in contaminated groundwater.

Finally, in this study, I detected not only complex depth-resolved microeukaryotic communities, but also 18S rRNA genes of multi-cellular organisms, sometimes at high ratios. Interestingly, some metazoan clones detected at the capillary-fringe and inside the contaminant plume were related to *Olavius algarvensis*, a gutless oligochaete with endosymbiotic bacteria forming a syntrophic sulphur-cycle (sulphate- reducers and sulphide-oxidisers) that is only known from marine sediments so far (Dubilier et al., 2001). Also the "M" library of 10.2 m depth, yielded oligochaete clones related to groundwater-dwelling *Aelosoma sp.* suggesting the presence of a higher trophic level in the Flingern sediments. The clone library at 9.1 m consisted of > 75% metazoan clones, and 38 % of the 10.2 m library

were related to higher plants. At the same time these libraries contained no or hardly any protozoa. Clones related to the neopteran *Anisochrysa carnea*, the common green lacewing, were dominant at 9.1 m, but also recovered in the two overlying sediments. Therefore, it is conceivable that contamination may have caused these erratic multi-cellular inputs into selected sediment samples. A possible source could have been an infiltration ditch used for stimulation of *in situ* degradation by nitrate injection at the former gas works site (Eckert and Appelo, 2002). This infiltration ditch is located ~100 m upstream of the sampling well. The fact that these erratic sequences were frequent only in certain sediment depths seems to delineate the possibility of longitudinal transport of neopteran dead biomass, eggs, or even live larvae submerged at the infiltration ditch along specific flow paths in the aquifer. As my samples were derived from only ~3 g of wet sediment, contained multi-cellular non-target material might easily cover the abundance of rare protistan organisms. Similarly, large proportions of metazoa and higher plants in clone libraries have been also detected in a gas condensate-contaminated aquifer (Luo et al., 2005).

3.5 Conclusions

In conclusion, a diverse microeukaryotic community with major shifts in composition through different zones of the aquifer was detected, highlighting the superior ability of my PCR assay to assess over-all protistan diversity in the investigated aquifer sediment samples. Results were similarly supported by the developed T-RFLP assay. Nearly 50 % of all OTUs were related to protozoan lineages hinting at a complex microbial food web in this system, maybe also coupled to abundant fungi via fungivore protozoa. Detected protozoan communities changed with depth. Ciliates and cercomonads seemed to be more abundant in upper parts of the aquifer and were probably coupled to high prokaryotic prey abundances. The hypothesised importance of cercozoans in highly contaminated, anaerobic habitats was further confirmed. In PAH contaminated samples, kinetoplastid *Bodo* sp. dominated the detectable protozoan community. The high numbers of fungal, but also potentially osmotrophic algal clones may indicate a direct involvement of those organisms in biodegradation processes. Interestingly, they comprised the only detected protists found directly beneath the contamination plume, where the hot spot of prokaryotic contaminant degradation is located (Anneser, 2008, Winderl et al., 2008). Over 30 % of singleton OTUs suggests that the real diversity of microeukaryotes was by far greater than observed in some depths. Nevertheless, a similar diverse and structured community of protists in a contaminated aquifer had not been described before. Being now aware of potential key-players in such an environment, I hypothesise that the use of group-specific and also quantitative PCR assays will further advance the knowledge of the role of different microeukaryote lineages for contaminated groundwater ecosystem functioning. Being aware of drawbacks concerning DNA- based methods, concomitant research (e.g. in batch cultures) will be necessary to further prove factual activities *in situ*. Ultimately, specific food web compartments and turnover rates will have to be elucidated to truly understand the influence of food webs on natural attenuation in groundwater.

3.6 References

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4 Unravelling microbial food chains by DNA-stable isotope probing of protistan predators after feeding with ^{13}C-labelled prey– preliminary experiments and a first application

4.1 Introduction

The study of 16S or 18S rRNA genes to assess natural microbial communities overcame major limitations of culture based methods and led to the awareness of cryptic diversities in various environments (Amann et al., 1995, Caron et al., 1999, Epstein and López-García, 2008, Slapeta et al., 2005). Nevertheless, a major drawback of rRNA gene analysis in environmental samples is that the detection of genes alone provides no proof for the activity of identified organisms *in situ*. Amplified DNA may also stem from metabolically inactive (e.g. spores) or dead cells. Therefore, when evaluating the potential role of microbes in the environment, assessing their presence by standard cloning and sequencing alone gives only a hint at species that might be of importance for ecosystem functioning. One promising approach is the direct analysis of rRNA by reverse transcription prior to standard PCR (Stoeck et al., 2007), but then this is limited to environmental samples either very high in biomass or easy to enrich, e.g. by filtration of sea water. Even more powerful, stable isotope probing (SIP) of nucleic acids constitutes a valuable tool allowing the specific identification of organisms actively involved in defined carbon transformation processes (Dumont and Murrell, 2005, Lueders et al., 2004b, Radajewski et al., 2000). In short, this method is based on the incorporation of heavy isotopes into biomass and nucleic acids of organisms growing on a ^{13}C labelled carbon source. Via density gradient centrifugation, labelled DNA or RNA can be resolved from the total community nucleic acids, and with subsequent cloning and sequencing metabolically active organisms are identified by their ^{13}C uptake (Fig.12). The method allows to follow carbon flux via trophic interactions identifying active predators or saprophytic organisms (Lueders et al., 2006, Murase and Frenzel, 2007).

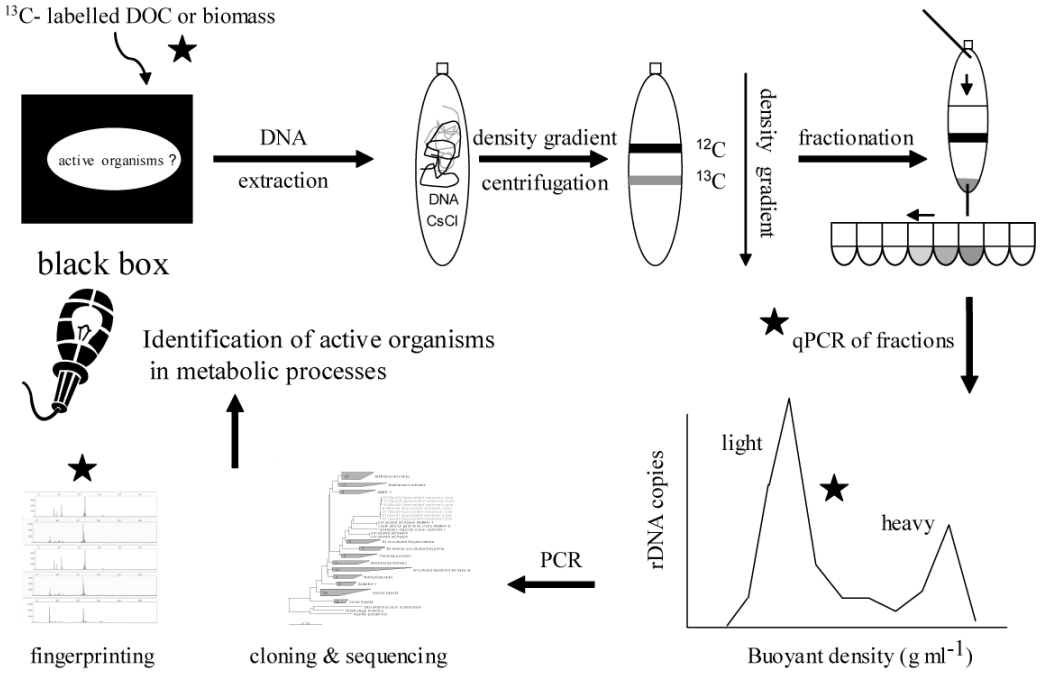

Fig. 12. Overview of workflow using SIP identifying organisms playing an active role in processes of interest. Topics that were assessed by preliminary experiments in this chapter are marked with asterisks.

On this basis, the application of DNA SIP may also be of major use to shed more light on the role of protists in groundwater, e.g. identifying key- players by proving their activity also in anoxic habitats. However, some preconditions must be met before SIP can be applied in more complex experiments. These were addressed in preliminary experiments in this thesis. First, I had to establish a eukaryote 18S rDNA qPCR assay to be able to quantify 18S rDNA in light and heavy gradient fractions (Chapter 4.2). Furthermore, preliminary experiments should answer the following questions:

- How much protistan biomass is produced feeding upon bacteria equivalent to a known amount of substrate and how much is sufficient for subsequent molecular analyses?
- How strong is the maximum label that can be detected in protistan predators feeding solely on ^{13}C labelled biomass?

To answer these questions, a first feeding experiment was conducted by growing different protistan predators on bacteria being equivalent to a known amount of glucose (Chapter 4.3). The cultures were harvested, DNA extracted and via qPCR the protistan 18S rDNA copies were quantified in order to know how much ^{13}C labelled glucose can be used in a subsequent SIP experiment. The latter was done to see how well light and heavy fractions could be resolved under optimal conditions, i.e. with maximal label of DNA produced by feeding predators exclusively with labelled biomass (Chapter 4.4). Finally, the molecular methods established in this thesis were applied on fractions derived from an SIP experiment on anaerobic toluene degradation (Winderl, 2007)(Chapter 4.5).

4.2 Establishment of an 18S rDNA qPCR assay

In short, quantitative real-time PCR is a simple method allowing the quantification of specific genes in mixed (e.g. environmental) template DNA based on the use of fluorescent dyes binding on double stranded DNA (for review see (Zhang and Fang, 2006)). The increase in fluorescence, which is measured after every elongation step, is correlated to the increase in target DNA amplified during PCR. With the help of a dilution series of standard DNA of which the total copy number is known, a standard curve is calculated and unknown DNA templates can be quantified by the evaluation of their fluorescence threshold cycles. Additionally, after PCR the analysis of a melting curve, resulting from reduction in fluorescence during dissociation of double stranded DNA in a temperature gradient, gives information about the specificity of the amplified PCR products.

Therefore, a DNA standard was created by amplifying a fragment of plasmid DNA harbouring a sequenced partial 18S rRNA gene of *Bodo* sp. as insert with binding sites for the eukaryote specific Euk20f and Euk516r primers (clone D0980_41_S identified in chapter 2). The amplicon was quantified with a PicoGreen (Invitrogen) assay testing two dilutions and 4 replicate measurements and yielded 160.8 ± 10.9 ng μl^{-1} DNA concentration. Due to the knowledge of all nucleotides inside one copy (851 base pairs in total), its molecular weight was calculated (517639 Daltons) using the BioEdit software (Hall, 1999), converted into $8.60*10^{-10}$ ng per amplicon and therefore this standard DNA was known to yield $1.87*10^{11}$ copies per µl. The sensitivity of the assay and specificity of the amplified products can be seen in Fig. 13, where a dilution series ranging from 10^7 to 10^0 copies was amplified in qPCR (for detailed protocol see Table A.16).

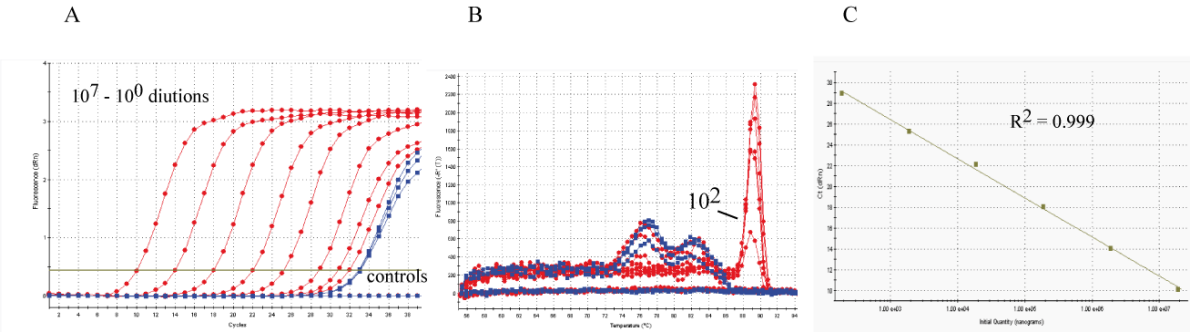

Fig. 13. Amplification plot for dilution series of eukaryotic standard DNA with 3 control samples (A). The dissociation curve (B) reveals unspecific primer-dimer production in water samples and highly diluted standard DNA concentrations of 10^1 and 10^0 18S rDNA cp μl^{-1}. A linear regression was fitted between 10^7 and 10^2 cp μl^{-1} (C).

Due to the formation of primer-dimers in samples with low template content, a detection limit of 10^1 to 10^2 copies μl^{-1} was observed. Thus, further improvements of these limits will be necessary, especially when analysing environmental DNA, where the copy numbers of protistan 18S rDNA may be present only in low concentrations. I already tested different salt concentrations and the use of a Power SybrGreen Master Mix (Applied Biosystems), which provided no further amelioration. Nevertheless, the developed eukaryote qPCR assay will give good approximations of 18S rDNA copy numbers of DNA extracts yielding more than 100 copies per µl, which is demonstrated by the standard curve.

4.3 Preliminary feeding experiment (without labelled glucose)

A feeding experiment was conducted to get an idea of how much labelled substrate used by bacteria would be needed to follow the carbon flow from bacterial consumers to protistan predators. This should prevent later microcosm and culture experiments with cell numbers that were below the detection limits of labelled 18S rDNA in the workflow. It has to be considered, that prey numbers needed to support reproduction of predator populations may vary widely between different lineages. Some examples are summarised in a review by (Ekelund and Ronn, 1994), stating experiments where the number of bacteria consumed per cell division of a predator ranged from 10^2 for microflagellates to 10^5 for *Acanthamoeba* spp., while the yield of carbon (e.g. the protozoan carbon produced per bacterial carbon ingested) may range from 0.2 to 0.6. Obviously a non negligible portion of consumed carbon is lost due to respiration and excretion of DOC. An even more diluted carbon flow can be expected for anaerobic systems.

4.3.1 Experimental setup

E. coli strain *K12* was grown in 50 ml of minimal medium (composition is given in Table A.8) with 28 mM D-glucose (Merk) as single carbon source. 2 ml of the grown culture were harvested, centrifuged at 5000 rpm for one minute and washed with Pratt medium (2 times) to remove any remaining DOC. By OD_{578nm} measurements on a photometer (Varian, Palo Alto, USA) the cell number was estimated to be in a range of 2-5*10^8 cells per ml. One half was supplied into a 15 ml Nunclon culture flask filled with 9.5 ml of Pratt medium, which resulted in a final bacterial prey concentration of about 10^7 cells ml^{-1} used in literature (Sherr et al., 1987) and was equivalent to 5.5 mg glucose spent to grow the bacterial prey.

In short, each culture flask containing 10 ml of suspended bacteria (4 in total) was inoculated with 2 µl of cultures of either *Bodo* sp. (isolated from aquifer sediment, see chapter 5), *Paraphysomonas* sp. JBM10 (obtained from Dr. Chatzinotas, UFZ Leipzig), a ciliate

(related to *Pseudocyrtolophosis* sp., see chapter 5) or a mixed culture with amoebae (containing *Hartmannella* sp. and other yet unidentified species), which were also both isolated from aquifer sediments. The flasks were kept at 16°C in the dark for 17 days. The sparse inoculum of predators was chosen to minimize the input of additional bacteria as potential prey organisms. Growing protistan populations were checked under an inverted microscope (Nikon Diaphot, 400x) twice a week. Observing increased numbers of inactive cells of *Paraphysomonas* sp. and amoebae, additional prey was fed summing up to a glucose equivalent of 6.93 mg glucose per flask or a bacterial number of ~ $4*10^8$ (SD $2.67*10^8$). Finally, the cultures were centrifuged at 4000 rpm for 10 minutes in 15 ml Falcon tubes and cell pellets were stored frozen at -20°C. DNA was extracted after standard protocol, eluted in 30 µl buffer and 1:10 and 1:100 dilutions of DNA were quantified by qPCR in duplicates.

4.3.2 Results

Only weak growth was detected in the *Bodo* sp. culture, suggesting a negative food preference for *E. coli* (see chapter 5.2.2 for more detailed information). In the ciliate culture, cyst formation hinted at unfavourable conditions, e.g. a starving population, but which could not be counteracted by rising food supply. Therefore, both cultures were excluded from further analysis. Results of qPCR showed $4.83*10^6$ (SD $9.01*10^5$) 18S rRNA gene copies μl^{-1} of extract for amoebae and $2.62*10^5$ (SD $1.05*10^5$) for the *Paraphysomonas* sp. culture. Extrapolating on total DNA obtained from harvested cultures, $2.09*10^7$ and $1.14*10^6$ 18S rRNA gene copies of amoeba and *Paraphysomonas* sp. respectively, were generated per mg glucose that was used to produce bacterial prey biomass. These are presumably underestimating values, as not all bacteria had been consumed during the experiment and maybe not all of glucose was used up by the bacteria.

4.3.3 Conclusion

The idea of this feeding experiment was to assess the amount of carbon source that would be needed to detect carbon flow from heterotrophic primary consumers to a predator population large enough to obtain sufficient DNA to be analysed with molecular tools in a SIP gradient. It could be shown that ~ $4*10^8$ bacterial cells (equivalent to ~7 mg glucose) were converted into 10^6 - 10^7 18S rRNA gene copies depending on different predators tested. This does of course not allow any inference on the protistan cells that were produced as eukaryotes are known to harbour multiple gene copies (McGrath and Katz, 2004). For the microflagellate *Paraphysomons* sp., population growth potential was in the range mentioned before (Ekelund and Ronn, 1994)). The higher yield of 18S rRNA genes of the mixed amoebae culture might be caused by various reasons. They may possess more gene copies than *Paraphysomonas sp.*, making a direct comparison of gene copy numbers inappropriate. Another possibility would be a better efficiency of converting bacterial carbon into biomass, maybe caused by lower energy losses due to different feeding strategies, i.e. fast swimming and capturing of motile prey cells by flagellates and contrarily grazing on a biofilm with slower movement by amoebae. Additionally, respiration rates are also dependent on the biovolume of a cell (Fenchel and Finlay, 1983). Furthermore, maybe osmotrophic uptake of egested DOC took place in the mixed culture of amoebae, leading to an enhanced production of biomass. However, independent on the two cultures tested, the general finding was that the amount of 7 mg glucose was enough to follow its fate from conversion into bacterial and later protistan biomass.

4.4 Stable isotope probing of a flagellate pure culture grown on ^{13}C labelled prey

The aim of the second feeding experiment was to assess the density shift between light and heavy eukaryote DNA that can be expected under optimal conditions, i.e. comparing only fully ^{13}C- labelled and unlabelled DNA. Another reason was to get a first idea of how much bulk DNA should be used in density gradient centrifugation to have sufficient amount left in the resulting fractions to exceed the detection limit of subsequent PCR methods. A difficulty here is the relatively small proportion of eukaryote DNA in mixed templates compared to prokaryotic background, making the amount of DNA applied in density centrifugation a crucial topic.

4.4.1 Experimental setup

In this experiment, I used the obtained *Bodo saltans* pure culture (chapter 5.2.5) as predator organism as it had shown the most stable growth behaviour during routine cultivation. As mentioned before, *Bodo saltans* showed an inhibited growing behaviour feeding on *E.coli K12*. Due to that, *Pseudomonas putida* strain *F1* was chosen as prey and grown in 5 ml M9 minimal medium with 28 mM D-glucose. To account for volume losses during sterile filtration through a 0.22 µm filter (MILLEX GP, Millipore) 30,267 mg ^{12}C-glucose (Merk) or 31,267 mg ^{13}C- glucose (Sigma) were dissolved in 6 ml minimal medium. 5ml were filtrated in sterile glass test tubes, inoculated with 500 µl of a grown culture, closed and incubated on a shaker at 30 °C. After 3 days, 100 µl of each culture, having an OD_{578nm} of 0.26 ^{13}C and 0.25 ^{12}C in a 1:10 dilution, was transferred a second time into fresh medium with ^{13}C- or ^{12}C substrate to allow for maximum labelling. After another 3 days, 4.5 ml were harvested, washed 2 times to rid off free glucose or other organic compounds and solved in Pratt medium with a resulting OD_{578nm} of 0.1 in a 1:10 dilution. The rest was used as inoculum for fresh medium. For further analysis of the ^{13}C carbon content in bacterial biomass, 200 µl samples of both bacterial cultures were centrifuged and pellets stored frozen at -20°C. Triplicates were analysed on an elemental analyser (EuroVector). In short, the method is based on burning of biomass to CO_2, NO_x (further reduced to N_2) and H_20 (removed by absorption) with helium as carrier gas. After removal of N_2 by a separating column, CO_2 was transported on-line into a mass spectrometer (Thermo Finnigan), where it was measured in continuous flow against a reference gas.

Duplicates of Nunclon flasks with 9.8 ml Pratt-medium were inoculated with 5 µl of *Bodo saltans* culture and fed with 200 µl of labelled or unlabelled bacteria suspension (equivalent to $4\text{-}10*10^7$ cells estimated by OD_{578nm} measurements). After 7 days of growth, 500 µl of protistan culture were transferred into new flasks containing the same amount of prey. The remaining 9.5 ml were harvested and frozen as described before. This was repeated another two times (after growth for 11 and a third time for 7 days). Finally, DNA was extracted (in total 12 samples) and eluated in 40 µl EB buffer. The amount of bulk DNA was estimated by UV quantification (ND-1000 Spectrophotometer, NanoDrop Technologies) measurement to be between 38 - 54 ng μl^{-1} (Table A.9). Only the last samples (transferred 2 times, after 25 d) were used for SIP centrifugation. Duplicates were pooled and contained approximately 3 µg of DNA, which were used in subsequent density gradient centrifugation (~ 1.72 g ml^{-1} CsCl average density before centrifugation) after a protocol of (Lueders et al., 2004a). In short, after 36h of ultracentrifugation at 44500 rpm, fractions of both gradients (named LR and LS) were collected (11 for the ^{12}C and 13 fractions for the ^{13}C treatment). Refractory indices of the fractions were measured and converted into densities of CsCl (g ml^{-1}) using a pre-determined standard curve ($y = -11.293\ x^2 + 42.6513\ x - 35.9133$). DNA was precipitated, eluated in 25µl EB buffer and stored frozen at -20°C until further analysis.

Finally, to quantify the amount of 18S rRNA gene copies in different fractions, qPCR was performed as described before.

4.4.2 Results

The elemental analyser measurements of carbon isotope ratios in bacterial biomass showed 1.1 AT % (SD = 0.01) (atom percents of ^{13}C of total carbon) for the ^{12}C- glucose fed *Pseudomonas putida* and 94.22 % (SD = 0.56) for the ^{13}C-glucose fed treatment.

The quantification of 18S rRNA genes in qPCR was not possible for all of the investigated fractions of both treatments. Apparently DNA was below detection limits in some fractions, and dissociation curves proved no amplification of specific products. For a better comparison of both gradients independently of absolute gene quantities, ratios of 18S rDNA copies to the maximal value of a treatment were calculated and are displayed in Fig. 14. In total numbers, the ^{12}C gradient fractions reached a peak with $9.15*10^5$ 18S DNA copy numbers at a buoyant density of 1.684 g ml^{-1} CsCl and the ^{13}C- treatment with $1.85*10^4$ copies at a density of 1.711 g ml^{-1} CsCl.

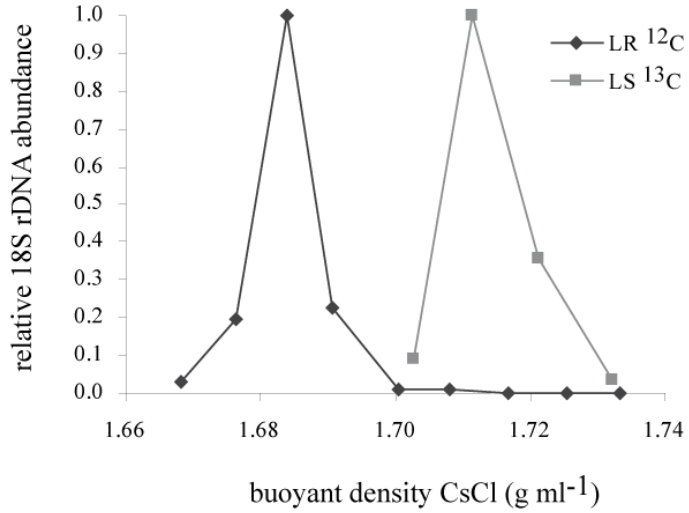

Fig. 14. Comparison of 18S rDNA gene copy distribution in SIP gradient fractions of unlabelled and ^{13}C labelled treatment. Values are given in Table A.10.

4.4.3 Conclusions

Elemental analysis of bacterial biomass proved a nearly complete ^{13}C labelling (94 %) of prey cells after one only transfer (1:50) of a culture that had been grown on labelled substrate. This information may be of interest for further experiments using labelled prey organisms. For >95% labelling, a third transfer may be preferable.

The first systematic SIP assessment of the buoyant density (BD) of fully labelled and unlabelled protozoan DNA showed a good resolution of density fractions containing ^{12}C or ^{13}C 18S rRNA genes. The difference in BD was close to 0.04 g ml^{-1} CsCl, a density shift previously observed also for fully labelled vs. unlabelled prokaryotic genomes (Lueders et al., 2004a). On the other hand, it was also found that the initial ~ 3 µg of DNA loaded into the gradients resulted in numerous fractions below the eukaryotic qPCR detection limit, although the relevant fractions contained enough DNA to be quantified. But then, this experiment was carried out under optimal conditions, i.e. predators were only feeding on labelled or unlabelled prey and DNA was extracted from liquid pure cultures and not from sediment samples. In the case of different preys with unequal labelling and different carbon sources, a

range of intermediate labelling can be expected leading to a wide BD distribution of protistan genomes due to partially labelled DNA. Therefore, PCR detection limits may be a major problem using SIP in more complex samples, where there also might be more non- target (prokaryotic) background in bulk DNA and additionally, potential ^{13}C labelled DNA of interest may constitute only a small proportion of total eukaryotic DNA.

4.5 First application of developed molecular tools for detection of eukaryotic activity during a batch experiment on anaerobic toluene degradation

In this section, I describe the results of the application of the qPCR and T-RFLP fingerprinting assay (Chapter 2) developed in this thesis to a SIP experiment of anaerobic toluene degradation in groundwater sediments conducted by Dr. Christian Winderl (Winderl, 2007). In this experiment, Winderl successfully showed the incorporation of ^{13}C derived from fully labelled toluene into degrader biomass and was able to identify Gram-positive key-players in anaerobic toluene degradation by SIP. In short, ~8 g aquifer sediment samples, obtained from the tar-oil contaminated Testfeld Süd site, were transferred to serum flasks and mixed with 50 ml medium adding ~1 mM ^{13}C-labelled or unlabelled toluene to study biodegradation processes over a period of 100 days. The degradation of ^{13}C of the contaminant was followed via $^{13}CO_2$ and increasing sulphide concentrations (up to 3mM), which substantiated degradation under sulphate-reduction. In the following section, gradients containing 5 µg aliquots of DNA extracted from ~2 g incubated sediment samples of two time points (after 8 and 86 days) of both ^{12}C and ^{13}C toluene treatments were analysed for eukaryotic 18S rDNA.

First, the applicability of my assays, mostly concerning the detection limits, was tested. In the analysed gradients, ~ 5 µg of bulk DNA was applied and it was of interest if this amount would be sufficient to detect eukaryotic 18S rDNA genes in subsequent gradient fractions. In contrast to the preliminary SIP analysis described before, the extracted DNA was expected to consist of mixed prokaryotic and eukaryotic templates, of which only minor proportions might be labelled. Furthermore, the maximum labelling of key anaerobic toluene degraders detected in this experiment was only ~50 % (Winderl, 2007). In case of non-selective feeding of predators, incorporation of label would depend on the abundance of labelled prey available, leading to intermediate labelled eukaryotes, which may be harder to resolve into different fractions.

Nevertheless, these analyses were an unparalleled opportunity to obtain information about eukaryotic and protistan activity under anoxic, contaminant-degrading conditions. A detection of ^{13}C label in eukaryotic biomass would hint at a role of microeukaryotes in biodegradation, e.g. by feeding on degraders (concerning protozoa) or by participation in biodegradation pathways which may be true for fungi. A first hint on eukaryotic activities during this experiment was achieved by the ability to enrich small amoebae (< 5 µm) after incubation of only 25 µl of medium obtained when the experiment was stopped after 100 days (see Chapter 5.2.5 for more details).

4.5.1 Experimental setup

As mentioned before, I could directly use fractions of DNA of four of the gradients originating from the experiment carried out by Dr. Christian Winderl in 2007 (Winderl, 2007). The DNA had been kept stored frozen at -30 °C in the meantime. Real-time qPCR was conducted and in case of specific amplification products detected by dissociation curves, T-RFLP fingerprinting was done subsequently after the protocol described in chapter 2.

4.5.2 Results

In all four gradients analysed, specific amplification of 18S rDNA was detected by qPCR, although the number of fractions per gradient with a valid signal differed between both samples. The absolute quantities of eukaryotic 18S rRNA gene copies in fractions are displayed in Fig. 15. The day 8 gradients turned out to contain insufficient eukaryotic DNA for meaningful results. Maximal numbers of gene copies quantified in single fractions ranged from $7.6*10^3$ (^{13}C) and $2.2*10^4$ (^{12}C) after 8 days to $4.9*10^4$ and $6.1*10^4$ cp μl^{-1} after 86 d, respectively. Unfortunately, most fractions supposed to harbour 'heavy' DNA were not evaluable due to DNA contents below detection limits.

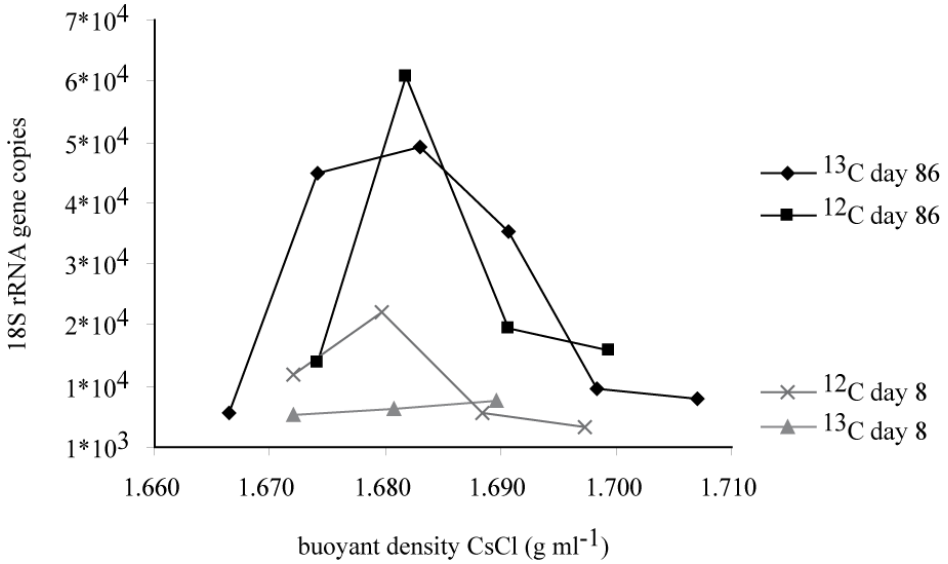

Fig. 15. Comparison of 18S rDNA copy number distribution in SIP gradient fractions of unlabelled and ^{13}C labelled toluene fed treatments after 8 and 86 days. Values are given in Table A.11.

Although information about 18S rDNA copy numbers in intermediate and heavy fractions was scarce, T-RFLP analysis was conducted for fractions showing specific PCR products during amplification of qPCR. Due to enhanced primer-dimer formation in samples with low template concentrations, not all fractions showing a specific amplification during qPCR were evaluable via T-RFLP as dimers accounted for the majority of fluorescence signal measured. This was true especially for fractions derived after 8 days and therefore only results of the 86 day gradients are presented, for which electropherograms are displayed in Fig. 16.

In all fractions of both treatments a T-RF of 189 bp length was detected. Other T-RFs differed considerably in abundances between both gradients. Most of all a 420 bp T-RF dominant in ^{12}C fractions appeared only in very low abundances in ^{13}C electropherograms (< 5% of total peak heights). Contrarily, different peaks in the range of 406 to 417 bp were more prominent in the ^{13}C-toluene treatment. Additionally, a 213 bp T-RF was only detected in one single fraction of the unlabelled gradient. To access density-dependent shifts of exemplary T-RFs found in all gradients, their abundances in both gradients after 86 days are displayed in Fig. 17.

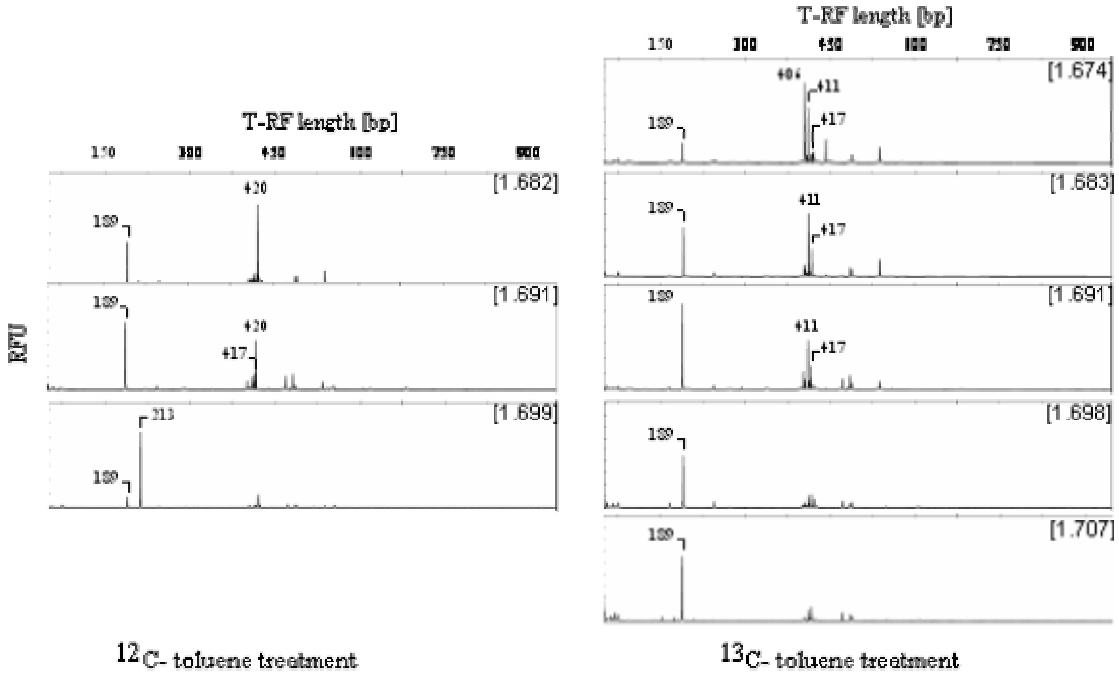

Fig. 16. Electropherograms of different fractions of gradients after 86 days. Respective buoyant densities are given in brackets. Dominant T-RFs are named by length (bp). RFU: relative fluorescence unit.

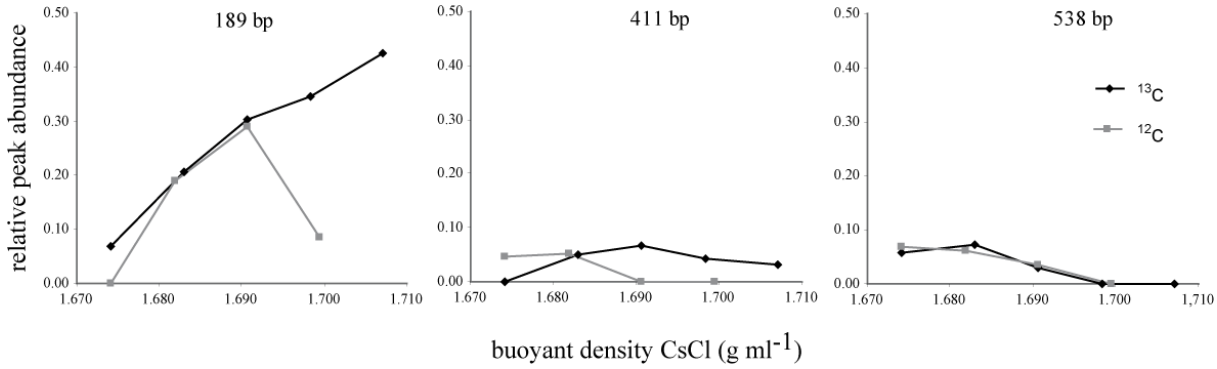

Fig. 17. Comparison of exemplary T-RFs and their density-dependent abundance shifts in both gradients after 86 days. Values are given in Table A.12.

Even though it was not possible to directly compare fractions of high BD, the 189 bp T-RF reached its abundance maximum at a higher CsCl concentration compared to the unlabelled treatment and was clearly more abundant in even 'heavier' fractions. Other T-RFs did not display such pronounced (411 bp T-RF) or any (538 bp T-RF) density-dependent abundance shifts between labelled and unlabelled treatments.

4.5.3 Conclusions

The investigated SIP gradients had been loaded with ~ 5µg DNA extracted from harvested laboratory microcosms, compared to ~3 µg used in my preliminary pure culture SIP experiment. Thus, although more DNA was used, the inability to amplify 18S rDNA in numerous samples of the second SIP experiment, most of all in fractions with higher densities, pinpoints the relevance of the detection limits and a sufficient amount of DNA to be used in gradients to assess eukaryotic genes. In general, eukaryote DNA will constitute a minor proportion in mixed templates compared to prokaryotes and therefore, more DNA should be used for future gradients.

Nevertheless, the comparison of 18S rRNA gene copy numbers of fractions after 8 and 86 days (Fig. 15) indicated relative eukaryotic population growth during the experiment, where anaerobic toluene degradation took place (Winderl, 2007). By reaching its maximum in intermediate densities and being still detectable in analysable heavy fractions, the dominant 189 bp T-RF putatively represents microeukaryotes that incorporated labelled carbon originating from added toluene. The single 213 bp T-RF detected in the ^{12}C fraction with a density of 1.70 g ml^{-1} CsCl was probably an unfortunate contamination inside the DNA, as it was not found in any adjacent fractions. A contamination during PCR for T-RFLP analysis can be excluded, as multiple controls without template proved no amplification of products. Contrarily, a 420 bp T-RF was detectable in all gradients, but was only dominant in the ^{12}C toluene fed one after 86 days. One possible explanation may be population shifts, e.g. displaying predator-prey oscillations (Hausmann et al., 2003), leading to different abundances to be observed at specific time-points.

Nevertheless, true isotopic labelling can be hypothesized for the organisms behind the 189 bp T-RF based on these results. This was not to be expected, as degrading bacteria were only labelled to an extent of about 50% (Winderl, 2007). Without sequencing any conclusion about the identity will be highly hypothetic. But a detectable label is hinting either on an effective predator characterized by frequent cell divisions to allow incorporation of considerable amounts of ^{13}C in nucleic acids by feeding on weakly labelled prey. This might be true for the enriched amoebae as their population can be estimated to have been very large and their small body size may favour label uptake in DNA by short generation times. But then this would also be true for nanoflagellates and the 189 bp T-RF could as well stem from kinetoplastid *Bodo sp*, for which a T-RF of 188 bp was measured at the contaminated Düsseldorf site. Last but not least, the label may be uncoupled to predation of prokaryotic degraders and be derived from assimilation of labelled toluene directly or from degradation products (also by osmotrophy).

To summarize, this first application of SIP analysis and associated molecular methods highlighted some important topics to be considered for further SIP experiments to reveal the potential roles of eukaryotes during biodegradation. First, due to expected lower DNA ratios in mixed templates, much more DNA has to be applied for density gradient centrifugation than would be necessary for analyses of prokaryotic nucleic acids. Additionally, full labelling cannot be expected, intensifying problems concerning the detection limits of eukaryote SIP, especially in anoxic groundwater habitats. Hinting at an active eukaryote community coping with anoxia and consisting of members differently related to biodegradation processes (indicated by differential behaviour of T-RFs), SIP analysis may be a useful tool shedding light on degradation processes and permit a functional characterization of microeukaryotes involved. However, the experimental set-up for SIP analysis to assess heterotrophic processes and the potential impact of labelled eukaryotes on degradation will remain a challenge.

4.6 General considerations using SIP to identify eukaryotic key-players

The major problem that became obvious during this first application of SIP analysis targeted on microeukaryotes was the low number of 18S rDNA, most of all in heavy fractions. As already mentioned above, this was not only due to the amount of bulk DNA used for the creation of gradients, but also of the label strength to be expected at all when considering the experimental setup. A very important point was that the toluene added constituted not the single carbon source, because the batch cultures were inoculated with environmental samples of a contaminated site, most probably enriched with DOC and microbial biomass creating a non negligible ^{12}C pool that probably was cycled additionally and constituted a considerable problem in the ^{13}C treatments and potential conclusions of the experiment. Furthermore, additional carbon input was potentially provided by the bicarbonate buffer of the applied freshwater medium. Being interested in the fate of a labelled contaminant added, this causes no major problem because degraders will incorporate specifically the heavy carbon supplied as substrate and can be identified, although additional ^{12}C carbon is present. Other bacteria may reach only a weak labelling with time, e.g. due to assimilation of labelled intermediates excreted by degraders or autotrophic fixation of $^{13}CO_2$. But then, only 50 % of label was detected in bacteria after more than 50 days, when also $^{13}CO_2$ and sulphide concentrations increased considerably. Unfortunately, the experimental setup did not allow for the monitoring of toluene decrease quantitatively. Therefore, without the identification of all microorganisms involved, conclusions are hypothetical as the presence of eukaryotic degraders, potentially competing with prokaryotes at early time points, cannot be excluded.

Contrarily, a ^{12}C background will obscure implications correlating label strength with the involvement in processes of eukaryotes. Under this condition, a clear label in protists, which by the way is dependent on time or rather on cell divisions, can only be expected under the following assumptions:

- A predator is feeding selectively on labelled prey due to preferential feeding.
- A predator is not selectively feeding, but labelled prey is highly abundant (which was not the case for identified degraders in this experiment (Winderl, 2007).
- The labelled substrate itself is degraded by the microeukaryote (e.g. possible for algae or fungi).
- Labelled degradation products are assimilated, e.g. by osmotrophy.

In all other cases, a range of labelling efficiency can be expected, complicating the clear detectability of labelled DNA. Especially, weak labelling can be expected at earlier time points of the experiment or may be a result of:

- non-selective feeding of predators and labelled prey is no dominant part of a prey community.
- selective feeding of predator causing population oscillations and meanwhile switching of food preferences to alternative prey. A label would then fluctuate over time in a population.
- A carnivore predator is feeding on labelled or unlabelled bacterivores. This effect would become more pronounced over time.

Contrarily, exhibiting no label at all during a SIP experiment can be a result of:

- metabolic inactivity of organisms.

- selective feeding on unlabelled prey which may be dependent on prey frequencies.
- anti-predatory defences of labelled prey, deflecting predation pressure on other prey organisms.

Therefore, it is clear, that adequate ^{12}C backgrounds in SIP incubations batch cultures will complicate the interpretation of eukaryote SIP results. It will be difficult to correlate label strength to relevance for the degradation process, i.e. the role of protozoa is harder to delineate as that of primary consuming prokaryotes or also directly degrading eukaryotes. For example, the detection of a strong label in protozoa can hint at selectively feeding on the degrader population and effect biodegradation negatively, but then this may be dependent on nutrient supply. In nutrient limited systems, predators may still enhance degradation processes due to recycling of essential resources. Contrarily, no label does not necessarily imply that a species is not involved in biodegradation, because it can very well influence processes indirectly, e.g. by consuming potential competitors of degraders (comparable to the impact of predators on competing prokaryotes by 'killing the winner' (Pernthaler, 2005)), which also may be of importance under limited conditions.

To conclude, the detection of labelled eukaryotes in this experiment is only proving their metabolic activity, without providing any conclusion about their qualitative impact on biodegradation itself. Contrarily, it cannot be clearly deduced from the absence of incorporation of ^{13}C to inactivity and therefore the experimental setup to explore eukaryotic significance in biodegradation processes will be very challenging.

4.7 Outlook

The cultivation of small amoebae out of very low amounts of liquid samples of the experiment investigating anaerobic biodegradation of toluene hinted at a high abundance of protistan predators and made an involvement of eukaryotic microorganisms at least likely. As pinpointed, the experimental set-up did not allow for a conclusion about the quality of an impact on the processes. For this, SIP experiments under controlled and initially more simple conditions will have to be conducted. No generalisations about the role or impact of protists may be possible, as this group is comprised by a variety of organisms with differing ecological features. First, nutritional modes like osmotrophy, bacterivory or carnivory may influence prokaryotic communities and species therein in different ways and therefore a prediction on the influence on processes will be hard. Second, possible impacts will most likely, be dependent on the trophic status of an environment, e.g. under nutrient limitations foraging of predators may constitute a positive effect even preying upon degraders. Third, the application of stable isotope probing may be a valuable tool to access the importance of eukaryotes and the microbial loop on the natural attenuation potential of groundwater, but experimental-setups have to be elaborate. Most of all, this is due to a lack of knowledge concerning basic ecological coherences on the microbial level. Nevertheless, application of molecular tools may help shedding light on the ecological processes on the micro-scale.

4.8 References

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5 Cultivation experiments

In this chapter, experiments aiming at the cultivation of protists from aquifer sediment samples are summarised. The first part is dealing with the comparison of oxic and anoxic quantification and qualification of protistan community members from aquifer sediments of two depths of the Düsseldorf-Flingern site. Afterwards, findings on general aspects of protistan cultivation from groundwater are summarised, such as choice of medium and prey supplied, or troubleshooting such as avoidance of intense fungal growth in cultures. Finally, some obtained pure and mixed cultures of protists are described. Concluding remarks summarise the results of these cultivation experiments.

5.1 Aerobic and anaerobic qualification and quantification of protists from contaminated aquifer sediments

5.1.1 Introduction

Molecular tools are promising to study microeukaryote communities of groundwater samples, allowing for respective identification (cloning and sequencing) and quantification (qPCR). However, major drawbacks of these methods are e.g. the multiple 18S rRNA gene copies extant in different eukaryotes, obscuring actual cell numbers and abundances of different lineages or amplification of templates stemming from inactive organisms. Additionally, as discussed in chapter 2 and 4, PCR biases concerning selective amplification of templates or effective detection limits when analysing samples with low biomass, and therefore low target DNA concentrations, have to be considered.

The latter is also true for direct cell counts and microscopic identification of protists, especially investigating aquifer sediment samples, as these approaches are also limited due to low abundances and small body sizes of protists. Therefore, cultivation and enumeration of indigenous eukaryotes may add further information on protistan activities and communities in groundwater. But then, most studies to date have enriched or cultivated protists under oxic conditions, running the risk of proliferation of inactive organisms or cysts, which may obscure the real importance of detected species *in situ*. An interest in active microorganisms originating from oxygen-depleted habitats like contaminated aquifers makes anaerobic cultivation absolutely necessary. First, reproduction under anaerobic conditions would be an important indicator of activity *in situ*. And second, cultivation with atmospheric oxygen may be selective, as oxygen is toxic to many anaerobes, and important key-players may be outcompeted by organisms less abundant or even inactive under natural conditions.

For a comparison of diversity and structure of the microeukaryotic community obtained for the Flingern site by molecular tools (Chapter 3) quantitative (MPN and LAM) and qualitative (enrichment) cultivations were set up. Comparative cultivation was done under oxic and anoxic conditions. In short, the MPN (most probable number) method is based on the recalculation of original cell numbers by observation of growth in replicated dilution series, whereas in LAM (liquid aliquot method), protistan abundances are estimated by the frequency of their occurrence in replicated aliquots (Butler and Rogerson, 1995). The qualitative study of protists is based on enrichment, where sediment samples are covered with water or medium and subsamples are inspected occasionally to assess successional community changes and identify abundant community members. The methods access total numbers of protists (active + encysted) and as for all cultivation based methods, the real diversity may be underestimated due to a selection of organisms favoured under the applied cultivation conditions (Ekelund and Ronn, 1994).

Sediment samples used for the aerobic and anaerobic cultivation experiments were derived from a second drilling campaign in March 2006, next to the multilevel well installed in 2005. Two depths were sampled, from 6.4 m at the capillary fringe (> 500 µM BTEX) and at 7.6 m underneath the contamination plume. Prior to transportation, sediments had been covered with anoxic water.

5.1.2 MPN and LAM quantification

Triplicate LAM plates and duplicate MPN plates were prepared for each sediment sample, either in the lab or in an anoxic glovebox with a nitrogen and hydrogen atmosphere. LAM was conducted in 24 well plates (Nunclon). Each well contained a sterilised wheat grain with 1 ml of Pratt-medium (see Table A.8) and was inoculated with 25 µl sediment sampled by using a tip-cut 100 µl pipette. One well was not inoculated as control. 96 well plates were used for MPN counts. The first well was inoculated with 50µl sediment (7 replicates) and further diluted (1:4) in the following 11 wells of a row. The last row of a plate served as control. A quinoa grain was supplied as carbon source for bacterial growth in each well. Plates were sealed with Petri dish seal tape (Roth) to avoid desiccation. All cultures were stored in the dark at room temperature as temperature control was not possible inside the anoxic tent. The plates were examined once a week under an inverted microscope (Nikon Diaphot). Only a 20x objective was available initially, later a 40x objective was used.

A preliminary test was conducted to assess time-dependent oxygen-saturation of plates when carried from the anoxic tent to the microscope and back. The aim was to know for which time span oxygen-depletion was maintained. Pieces of oxygen-sensitive sensor strips were attached at one well bottom of a 24 and 96 well plate and oxygen concentrations were monitored via a non-invasive optode-array measuring system (Microx 1/FIBOX, PreSens, Regensburg, Germany).

5.1.2.1 Results

The development of oxygen concentrations inside the two plate types after being removed from the anoxic tent is displayed in Fig. 18. Suboxic conditions (< 0.3 mg l^{-1} dissolved oxygen) were guaranteed for half an hour when handling the 96 well plate outside the anoxic tent, whereas the 24 well plate was already hypoxic (~ 2mg l^{-1} dissolved oxygen) by then.

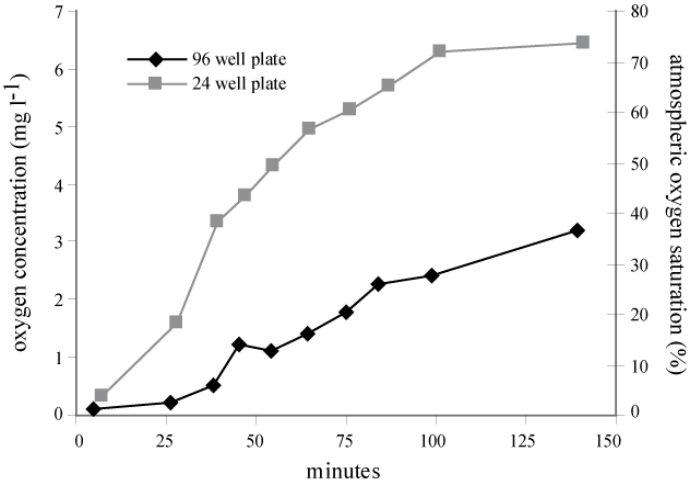

Fig. 18. Development of oxygen concentration and saturation inside sealed microtiter plates during air exposure. Values are given in Table A.13.

A systematic microscopic investigation of the MPN and LAM plates was unfortunately not possible in the subsequent experiment. This was due to various reasons. Prokaryotic biofilms on the bottom of the wells and mineral particles of the sediment dispersing the transmitting light caused severe problems connected to suboptimal optics. It was not possible to focus through the bottom layer and therefore, to observe free-swimming organisms. Only cells attached to the bottom were visible, but then also due to small cell sizes, I was not able to distinguish protozoan cysts from yeast-like fungi or 'pseudo-cysts' of amoebae. Moving amoebae or attached nanoflagellates were also not observable due to biofilm growth and low contrast. Additionally, plates of the 6.4 m sample treated under oxic conditions showed intense growth of fungal hyphae already after one week, connecting wells and leading to cross-contamination which was evident by prokaryotic and eukaryotic growth also in control wells of 96 and 24 well plates. Therefore, no quantitative evaluation was possible. Furthermore, fungal growth caused fast desiccation of the MPN plates and also worsened optical quality. The latter was especially a problem of the 7.6 m sample treated with atmospheric oxygen concentrations, where mold-like pigmented crusts covered the well floors. Additionally, also plates stored in the anoxic tent showed problematic evaporation of medium due to frequent gas exchange necessary for work in the anoxic tent. Furthermore, sterile conditions could not be guaranteed when working in the glove box and therefore, after additional supply of medium, control wells showed contamination. Due to these unexpected problems during cultivation, the experiment was stopped after 9 weeks. Triplicate culture flasks inoculated with 100 µl sediment at the beginning of the experiment were used for subsequent isolation efforts described in later parts of this chapter.

5.1.2.2 Discussion

Unfortunately, this cultivation-based quantification experiment failed due to various reasons. First, the quinoa grains supplied as unselective carbon source for indigenous prokaryotes provided too much nutrients and lead to the formation of thick prokaryotic biofilms and fungal growth. Intensive bacterial growth often suppresses reproduction of amoebae and other protozoa (Smirnov, 2003). Proliferation of fungi was also promoted by cultivation at room temperature, which was done for identical treatment of anoxic and oxic cultures. All subsequent (oxic) cultivation experiments were conducted at 16 °C, where hyphal growth was no longer experienced as that problematic.

For MPN counts in 96 well plates, quinoa grains evidently yielded too much substrate, and I recommend the use of quinoa grains as substrate and dilution series using 24 well plates for further investigations. However, suboxic conditions in 24 well plates are hard to maintain even for a few minutes outside the anoxic tent (Fig. 18). 96 well plates proved to be problematic due to desiccation and cross-contamination and therefore, the use of sealable culture flasks, which also provide better optical features compared to plates, may serve as alternative. Last but not least, detected protists were in general small (< 20 µm), which was more pronounced in anaerobic cultures where small amoebae (<10 µm) were infrequently observed, rendering any closer identification impossible at the available magnification of the inverted microscope. For application of a 100x objective, microscopy of subsamples on microscope slides would have been necessary, connected to additional opening of plates, leading to even higher contamination potential. Additionally, it has to be considered that oxygen may be toxic to some protozoa, which can encyst within minutes (Ekelund and Ronn, 1994). In summary, an appropriate instrumentation for an optimal workflow in oxic / anoxic MPN counts of protists is presently not established at the Institute of Groundwater Ecology, and respective investments are a prerequisite for related efforts in the near future.

5.1.3 Qualitative enrichment of protists

2 culture flasks (Nunclon) per depth of Flingern sediment (6.4 m and 7.6 m) were filled with sediment samples to a volume of 25 ml and covered with 5 ml Pratt medium described in Table A.8 (no additional carbon source supplied). This was done either in the lab for aerobic treatments or inside a glove box with a nitrogen and hydrogen atmosphere, where the added medium had been autoclaved anoxically prior to use. In order to assure equal temperature conditions, both treatments were stored at room temperature as sample conditioning was not possible inside the anoxic tent.

Subsamples were taken with a 1000 µl pipette with a cut tip at different time points (one day after drilling when the experiment was started and after 6, 12, 16, 22, 30 and 40 days) and stored frozen in 1.5 ml Eppendorf cups at -20°C. Duplicate DNA was extracted after standard protocol (respective sediment amounts are summarised in Table A.14), pooled and eluted in 40 µl EB buffer. T-RFLP analysis was conducted digesting 25 ng of amplification product (NanoDrop quantified) after the protocol described in chapter 2. Additionally, at each subsampling day, one MPN and one LAM plate were prepared as described before. Plates and flasks were stored at room temperature in the dark.

5.1.3.1 Results

The same problems as discussed above were encountered in the quantification of protists by MPN and LAM. Therefore, cultivation-based cell number estimations were not possible. Nevertheless, for the subsequent cultivation and isolation of protists, subsamples from wells were taken and examined further on in separate experiments, described in chapter 5.2.5. Furthermore, a total of 13 T-RFLP analyses per depth were conducted from frozen subsamples. The respective electropherograms are displayed in Fig. 19 and 20.

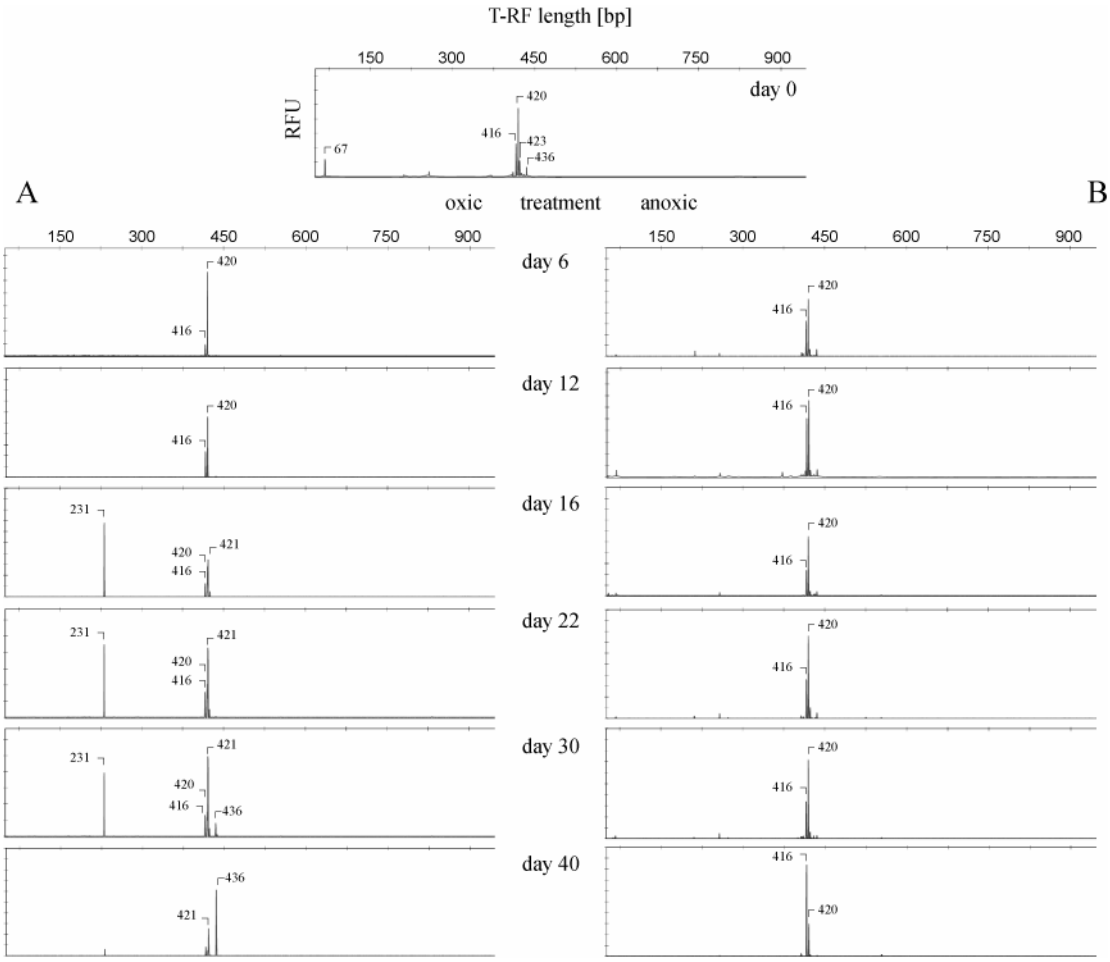

Fig. 19. Time-resolved T-RFLP electropherograms for oxic (A) and anoxic (B) enrichment of sediment samples originating from 6.4 m depth.

The comparisons of oxic and anoxic treatment of samples from 6.4 m depth displayed enrichment of different organisms concerning T-RF abundances and revealed substantial successional shifts over time for the enrichment under atmospheric oxygen. Cultivated without oxygen, only two dominant T-RFs (416 and 420 bp) were present. The same was observed for the oxic treatment until day 16, where peaks of 231 and 421 bp length appeared and became dominant subsequently until day 30. From there on, a 436 bp T-RF arose and became dominant at day 40.

The eukaryotic community at 7.6 m depth was dominated by a T-RF of 416 bp at the beginning of the enrichment experiment (Fig. 20). During both treatments, this T-RF continued to be highly abundant. However, an additional 407 bp peak appeared under oxic

conditions between 12 and 22 days together with a 189 bp T-RF. Both peaks were also observed under anoxic conditions. After 22 days with atmospheric oxygen, a 414 bp peak arose together with a 406 bp T-RF.

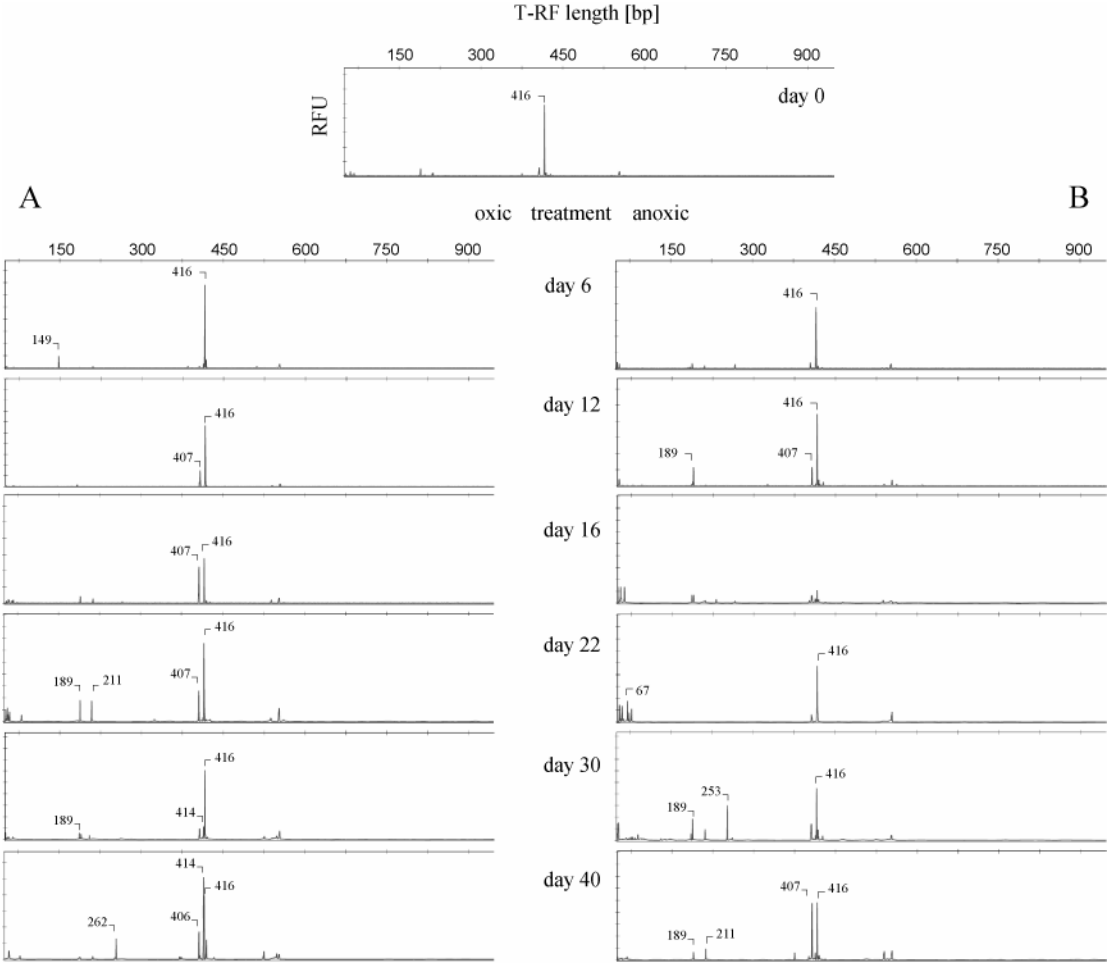

Fig. 20. Time-resolved T-RFLP electropherograms for oxic (A) and anoxic (B) enrichment of sediment sample originating from 7.6 m depth.

To better illustrate the appearance and dynamics of T-RFs over time for both treatments, the relative abundances (proportion of total peak heights) of dominant peaks are displayed in Fig. 21. The oxic enrichment of the community from 6.4 m depth harboured a more diverse community, obviously connected to the appearance of the 231 and 421 bp T-RFs. Furthermore, the abundance of a 416 bp T-RF was increasing under anoxic conditions, but declined with time under atmospheric oxygen. In contrast, the 436 bp T-RF strongly increased towards the end of the oxic incubation only. The 420 bp T-RF, abundant already at the start of incubation, showed a more or less stable relative abundance over 4 weeks without oxygen, before decreasing towards the end. Contrarily, it reached highest abundance (80 %) in the first two weeks with oxygen, but then strongly declined afterwards.

The samples from 7.6 m depth did not show such pronounced discrepancies between treatments. The dominant 416 bp T-RF displayed abundance oscillations in both treatments. Minor differences were observed during the first 20 days, were a 407 bp T-RF arose under oxic conditions, but declined subsequently and was displaced by an appearing 414 bp T-RF. Contrarily, rising of the 407 bp T-RF was delayed in the anoxic sample, and the 414 bp T-RF

remained low. Additionally, despite the 416 bp T-RF no other dominant peak was detected, e.g. after 16 days of anoxic treatment calculated eveness was 0.95 and 0.97 after 30 days, respectively.

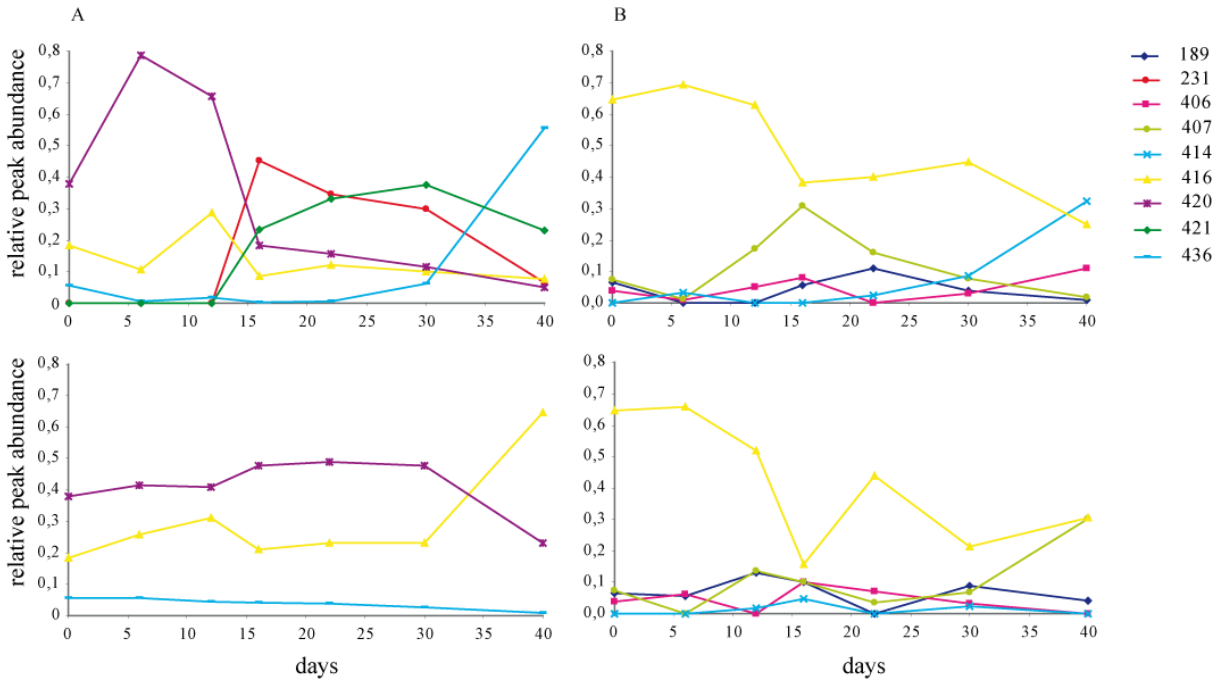

Fig. 21. Overview of T-RFs and abundance shifts during enrichment of sediment samples from 6.4 m depth (A) and 7.6 m depth (B) with (above) and without (below) atmospheric oxygen available. Values are given in Table A.15.

5.1.3.2 Discussion

The comparison of T-RFLP analyses and distinct peak abundances of sediment samples during 6 weeks of incubation with or without atmospheric oxygen showed pronounced successions of different T-RFs. Therefore, these results indicate at the selective cultivation of diverging protistan community members. But then, from T-RF abundance shifts alone, conclusions about microbial growth can be obscured, as any increase in abundance can have two reasons: either a population is reproducing, or it is stable (e.g. as cysts) while the abundance of other community members is declining over time. In the latter case, a decrease of amplification product of PCR would have been expected, which was not observed by UV-quantification of amplicons (Table A.14).

Even though the samples originated from a second drilling campaign in 2006, suggestions about the organisms represented by the observed T-RFs are possible by comparison to the cloning and sequencing results from the same site half a year earlier (chapter 3). Additionally, isolation efforts described in a subsequent part of the chapter allowed some further identification. The 416 bp T-RF abundant in all treatments can be related to the yeast-like *Rhodotorula* sp., which was isolated from 6.4 m depth after 40 days of enrichment culture. An experiment described in chapter 5.2.4 also substantiates its aerobic and anaerobic growth. In Table 12, further dominant peaks for which affiliated lineages can be assumed via sequence information are summarised.

Table 12 Dominant peaks identified by existing clone sequence data.

6.4 m	T-RF	potential lineage	detected in [m] [a]	7.6 m	T-RF	potential lineage	detected in [m] [a]
	231	Malawimonidae	6.3		189	Kinetoplastida	7.4, 8.1, 9.8
	231	Peniculida	6.3, 6.7		211	Streptophyta	7.2
	416	Rhodotorula	5.7, 7.4		253	Colpodea	10.2
	420	Rhodotorula	5.7, 7.4		406	Pucciniomycotina	6.7
	420	Cercomonadida	5.7, 6.7		407	Pucciniomycotina	7.2, 7.4
	420	Lobosea	5.7		414	Lobosea	6.7
	423	Cercomonadida	5.7				
	436	Lobosea	5.7				

[a] Depth [m] of clone libraries described in chapter 3.

The 420 bp peak was not clearly attributable to a specific lineage and may even comprise organisms of the *Basidiomycota*, *Cercozoa* and *Lobosea*, probably to different extents over time. Therefore, no conclusion about anaerobic growth of the latter two is possible based on these results. But as discussed in chapter 3, anaerobic reproduction of e.g. cercomonads is highly probable. The 231 bp T-RF, appearing only under aerobic incubation of the 6.4 m sediments, could be affiliated to *Malawimonidae* or to a ciliate related to *Paramecium* sp.. As the height of the peak indicates a frequent organism, small flagellates (*Malawimonas* sp. < 5 µm) may be more likely, as they would not have been detectable during microscopy. But then members of the genus *Paramecium* are known to harbour repeated genes coding for rRNA in chromosomes (micro- and macronucleus) and even free extrachromosomal rRNA copies (Findly and Gall, 1978). The dominant T-RF in the same treatment after 40 days (436 bp) may belong to an amoebaen species related to *Neoparamoeba* sp. (*Lobosea*). Also the 414 bp T-RF that became frequent in the aerobic treatment of 7.6 m depth, could be connected to amoebae, this time related to *Saccamoeba* sp.. The finding that both peaks only appeared in the oxic treatment does not necessarily exclude a relevance of amoebae also under anoxic conditions.

Both 406 / 407 bp T-RFs detected in the 7.6 m sample may stem from basidiomycetes of the subphylum *Pucciniomycotina*. T-RFLP results indicate growth of the latter in both treatments. Additionally, the 189 bp peak in both treatments is probably assigned to *Bodo* sp., which was frequently found in clone libraries of the sulphidogenic gradient zone and beneath. As discussed in chapter 3, this species may very well grow under both oxic and anoxic conditions. Finally, the 253 bp peak, only abundant at day 30 of the anoxic treatment (7.6 m), may hint at a mycophageous colpodid ciliate related to *Pseudoplatyophrya* sp., of which a single clone was sequenced in 10.2 m depth.

To summarise, even though the quantification experiment failed, it could be shown that dependent on oxygen availability different communities developed in incubated sediment cultures. In total, anoxic cultures may have been dominated by fungi, which may have further obscured potential growth of protozoan communities. No additional carbon source was supplied for the enrichment cultures and as both samples were contaminated (6.4 m with BTEX, 7.6 m with PAH), a fungal role in direct anaerobic degradation, or by metabolising prokaryotic degradation products can be hypothesized (e.g. benzoate consumption). For *Rhodotorula* sp. anaerobic growth on benzoate is demonstrated (Chapter 5.2.4). Contrarily, it can be assumed that under aerobic conditions, those carbon sources were degraded more easily, which would explain the shift from a fungal dominated to a protozoan dominated eukaryotic community, from day 16 and 22 on in both samples. Available dissolved organic

carbon might have been depleted up to then, and cycling of nutrients by predation would have become beneficial for degradation. Certainly, these assumptions require more profound investigation in future experiments.

5.2 Cultivation and isolation experiments

In this part of the thesis, different cultivation efforts of groundwater protists were summarised. In the beginning of the thesis, different media were tested (chapter 5.2.1), and prey organisms and their carbon sources were varied during the work with protistan cultures for reasons described in chapter 5.1.2. With the awareness of problematic fungal proliferation in sediment samples of the Düsseldorf-Flingern site, the application of a fungicide was examined (Chapter 5.2.3). Another experiment dealt with the comparison of carbon sources metabolised by a fungal culture under aerobic and oxygen-depleted conditions (Chapter 5.2.4). And last but not least, efforts were made to isolate protistan pure cultures or defined mixed cultures by dilution or micromanipulation of single cells. A description of isolation methods and information about the obtained cultures is given in chapter 5.2.5.

5.2.1 Choice of medium

Prior to cultivation experiments of groundwater protists, different media (Cereal grass infusion medium (Andren et al., 1999), bottled water (Volvic, Danone Waters Deutschland GmbH) (Twagilimana et al., 1998) and inorganic Pratt medium) were tested upon a cercozoan strain of *Cercomonas longicauda* (CCAP 1910/2, SAMS research services Ltd, Scotland) and an enrichment culture obtained from a kerosene-contaminated aquifer near Berlin (GWM2). Details about medium preparation and respective ingredients are given in Table A.8. Volvic water and Pratt medium had to be supplied with an additional carbon source (autoclaved wheat grains) to support bacterial prey growth. Experiments were conducted in 7 ml medium in Nunclon flasks closed with filter caps. Comparing population growth of *Cercomonas longicauda* and the mixed GWM2 culture under the microscope (Zeiss, Axioskop; 100x objective) no qualitative preferences of media concerning cell numbers and their motility were detectable after 1 and 2 weeks. For reasons of defined medium contents (most important a defined organic carbon source for later experiments) and turbidity of the cereal grass infusion medium, I chose Pratt medium for further use.

5.2.2 Prey organisms and carbon sources

Prey organisms and carbon sources supplied were switched during the duration of this thesis recognising strengths and constraints of different experimental setups. In the beginning, cultures were fed by addition of autoclaved spelt wheat (*Triticum spelta*) or quinoa (*Chenopodium quinoa*) grains, which should support unselective growth of indigenous bacteria matching different feeding preferences of diverse predators. However, applied grains also served as food source for fungi, leading to problematic fungal growth and dominance most of all under room temperature. Therefore, I subsequently offered *E. coli* strain K12 as direct food source without any carbon to be added. In general, bacterial prey was raised in mineral minimal medium (M9, composition is given in Table A.8) with glucose (28 mM) or benzoate (2 mM) as carbon source, which was added by sterile filtration through a 0.22 µm filter (Millipore). Harvested aliquots were centrifuged for 5 minutes at 5000 rpm, the pellet was washed with sterile water, centrifuged again and suspended in the medium to be used in. The final concentration of bacteria fed to the culture resulted in ~10^7 cells ml^{-1} and was estimated by OD_{578nm} measurements.

As already mentioned in chapter 4.3, there were hints that some predators were not able to grow properly on *E. coli* K12. Therefore, the qualitative growth of *Bodo* sp., *Paraphysomonas sp.* and a mixed amoebae culture were tested by offering *E. coli* K12 (~ 3 µm cell length) or *Pseudomonas putida* strain F1 (~ 2 µm) as prey in 1 ml medium on a 24 well plate (4 replicate wells each). The plate was stored at 16°C in the dark and expected after 7 and 14 days under an inverted microscope. The small flagellate *Bodo* sp. preferred *Pseudomonas putida* as prey organism, whereas amoebae did not show a clear preference. Contrarily, *Paraphysomonas* sp. exhibited higher cell abundances in *E. coli* fed wells. A third prey type was introduced for feeding specific small amoebae enriched from the SIP experiment of Winderl (2008) described in chapter 4. The first time detecting these very small cells, it was obvious that those amoebae were not able to phagocytose larger prey supplied. After adding smaller sized (~ 1µm) *Thiobacillus thiophilus D24TN* (isolated from the Düsseldorf aquifer by Claudia Kellermann, in prep.) as food source, large respective amoebal populations could be achieved.

Protozoan preference of different prokaryotic prey types can have various reasons. For the small amoebae, it was obvious that prey choice was dependent on the size of the prey, but protozoan predation may also be influenced by prey behaviour like motility or other characteristics. Additionally, defence mechanisms of potential prey such as the release of toxins will also lead to negative preference of specific prey types (Fig. 22).

Therefore, it has to be considered, that feeding single bacterial strains may lead to highly selective cultivation of protozoan community members, contrarily to addition of universal carbon sources, where proliferation of a diverse prokaryotic prey community may meet the demands of numerous predator species. But specifically feeding of prey organisms is an option to avoid fungal growth, most of all at the beginning of protozoan enrichments.

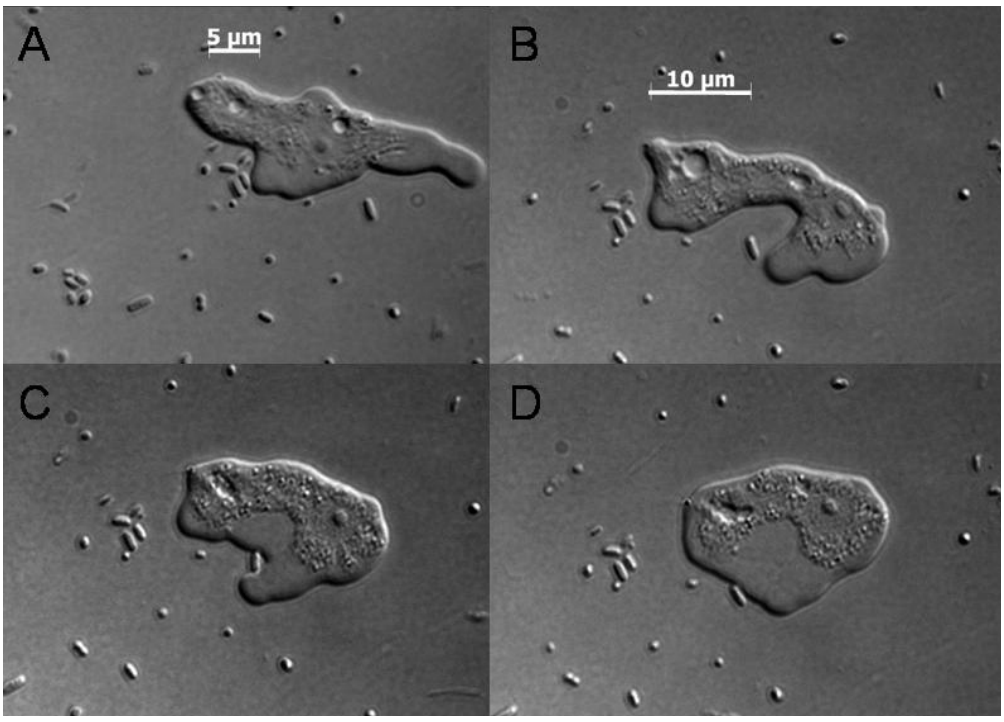

Fig. 22. Example of negative prey selection (A-D) observed in the mixed amoebae culture (GWM2).

5.2.3 Application of fungicide

Facing the failure of quantification experiments due to intense fungal growth, I tested the application of the antimycotic Nystatin. The substance is a polyene antifungal drug to which many molds and yeast infections are sensitive due to interaction with ergostol in the fungal cell membrane. Concentrations of 0.5 – 20 µg ml^{-1} are creating fungi-static conditions, whereas concentrations up to 80 µg ml^{-1} act fungicidal. Its potential efficiency but also possible effects on different protozoan species were assessed in an experiment conducted in a 24 well plate with 1 ml Pratt medium and a quinoa grain. Using a stock solution of Nystatin (50 mg ml^{-1} DMSO, stored frozen at -20°C) different concentrations were prepared (0, 5, 10, 20, 40 and 50 µg ml^{-1}). Rows were inoculated with *Bodo* sp., *Rhodotorula* sp., *Sporidiobolus* sp. D24TN (a basidiomycetous yeast isolated by Claudia Kellermannat my insitute) and a *Hartmannella* sp. dominated mixed amoebal culture (GWM2). The plate was stored at 16°C in the dark. After one week, cell growth was examined under an inverted microscope. Growth of *Sporidiobolus* sp. was only detected in concentrations up to 10 µg ml^{-1}, but *Rhodotorula sp.* was still to be observed at the highest applied concentration of 50 µg ml^{-1} Nystatin. *Hartmannella* sp. was found to cope with the fungicide. Contrarily, growth of *Bodo sp.* was not to be detected in any well containing Nystatin. For further validation, I repeated the experiment with *Bodo* sp. and again no growth was to be observed. Additionally, a ciliate culture (a *Pseudocyrtolophosis*- related colpodid) still mixed with fungi was also treated with 50 µg ml^{-1} Nystatin, but showed resistance of both organisms to the fungicide. Therefore, Nystatin proved not only to affect fungi to different extents, but also to selectively prevent growth of specific protozoan species. As avoidance of fungal spread was not guaranteed, Nystatin was not applied in any further experiments.

5.2.4 Fungal growth experiment

The results of T-RFLP analysis during enrichment (Chapter 5.1.3) hinted at fungal growth under aerobic and anaerobic conditions. To assess their growth potential and the usability of different organic substrates, I tested *Rhodotorula* sp. and *Sporidiobolus* sp. D24TN (both isolated from the contaminated Düsseldorf-Flingern site) offering 3 different carbon sources (1 mM glucose, 1 mM or 5 mM benzoate and 1 mM phenol) in a fungal mineral medium (Santos and Linardi, 2001) described in Table A.8. Experiments were conducted in serum bottles with 50 ml medium (autoclaved anoxically). For the creation of oxygen-depleted conditions, half of the bottles had been closed with butyl stoppers and the atmospheric gas phase was exchanged by sparging with nitrogen gas. 100 µl of fungal cultures were used as inoculum. The bottles were stored at 16°C in the dark and 50 µl subsamples were evaluated qualitatively under the microscope after 1 week. In all 8 treatments growth of *Rhodotorula* sp. was observed, but substantially less with phenol as carbon source. Contrarily, *Sprodiobolus* sp. reproduced only in samples with atmospheric oxygen available and glucose or benzoate as carbon source. Therefore, it can be concluded that *Rhodotorula* sp. can very well grow under both aerobic and anaerobic conditions using glucose, benzoate and to lesser extents also phenol as carbon source. The latter will have to be further substantiated, as the lower population size observed could also hint at growth on prokaryotic phenol degradation products, as axenic cultures were not used.

5.2.5 Establishment of protistan cultures

To obtain groundwater protistan cultures for subsequent lab experiments, subsamples of the oxic incubation of the Düsseldorf-Flingern sediment (6.4 m, day 40) were utilised. Additionally, flasks containing 10 ml Pratt-medium and a wheat grain had been inoculated with 100 µl sediment of 6.4 m throughout the enrichment experiment. Furthermore, cultures from a kerosene-contaminated aquifer (Brand, near Berlin) were obtained via dilutions of 15 ml sediment from a drilling core (GWM2, 18 m depth).

For the documentation of the isolated protozoa, microscopic pictures taken at optimal magnification are of key importance. I used a Zeiss Axioskop in DIC (differential interference contrast) settings at 1000 x magnification. However, due to the fast movement of some cells and especially also to visualise structures like flagellae, different methods described in (Lee and Soldo, 1992) were tested to immobilise or slow down cellular movement. 2.5 % (w/v) $MgCl_2$*6 H_2O, 10% (v/v) Ethanol or a solution containing 1 mM $CaCl_2$, 1 mM KCl, 5 mM TrisHCl and 0.125 mM $NiCl_2$*6H_2O were applied on different protozoan cultures. Best results were achieved with the latter solution and resulted photographs are given Fig. 23.

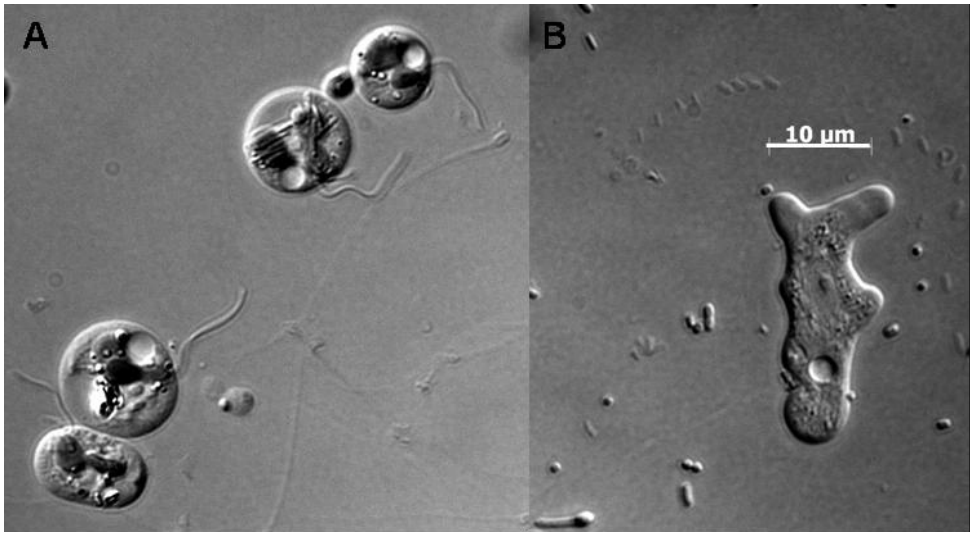

Fig. 23. Pictures of (A) Paraphysomonas sp. culture, with visible flagellae and vacuoles and (B) an unidentified amoeba (GWM2 culture) (magnification 1000x)

Cultures were obtained using different strategies. In sediment samples of GWM2, which had continuously been stored at 16°C after sampling, problematic fungal growth was never encountered. Therefore, 24 well plates were appropriate for conducting dilution series to isolate abundant species. A pure culture of *Bodo* sp. was obtained by transfers of small aliquots of a *Bodo*-dominated community, for which T-RFLP analysis had been conducted (Fig. 24). An obtained culture of amoebae consisted of different species, which was obvious by microscopy where diverse morphotypes were detected (Fig. 25) and T-RFLP analysis (Fig. 24) even though 100% of 16 sequenced clones were related to *Hartmannella vermiformis* (> 99%).

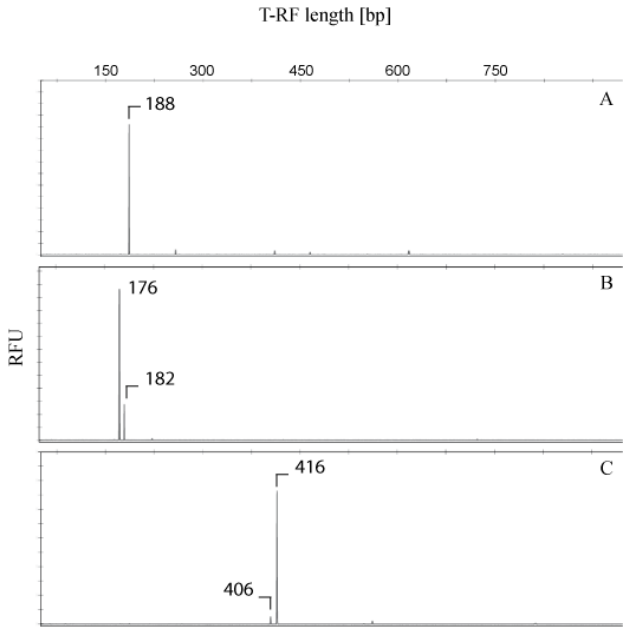

Fig. 24. Electropherograms of protistan cultures: (A) *Bodo* sp. dominated, (B) *Hartmannella* sp. dominated amoebae (C*) Pseudocyrtolophosis* sp. dominated by *Rhodotorula* sp..

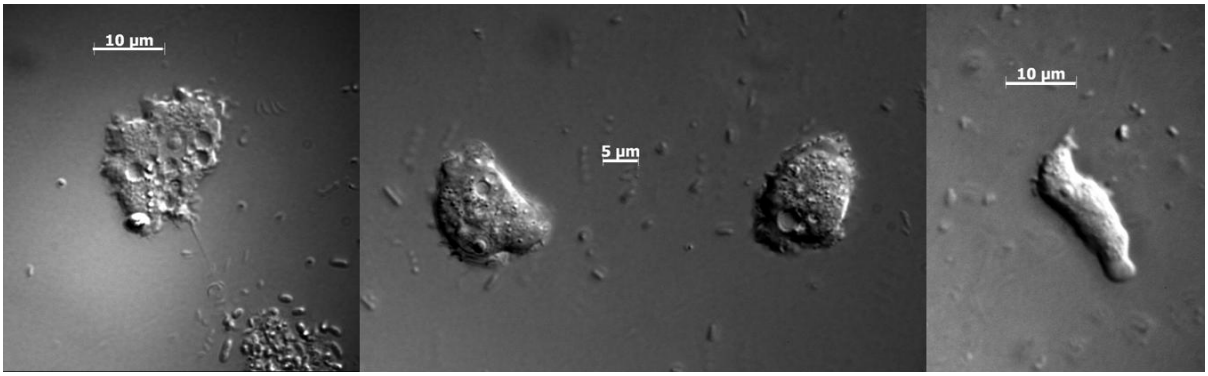

Fig. 25. Pictures of different morphotypes detected in mixed amoebaen culture. On the right the identified *Hartmannella sp*..

During the enrichment experiment at each examined time point, 100 µl of sediment was diluted in 10 ml Pratt medium and supplied with a wheat grain. After observing intense fungal growth inside the plates, but also in the flasks, the latter were placed at 16°C. To avoid further fungal spread, subsamples were transferred in additional flasks without added carbon source, but were fed with *E. coli* K12 instead. After further transfers, a wheat grain was added. Unfortunately, fungal hyphae growth was observed again, although not that intense as it had been under room temperature. To get rid of fungi, I decided to isolate single protozoan cells by micromanipulation.

For this purpose, the tips of Pasteur pipettes (glass) were fixed with a clamp and elongated by heating. The resulting width of each pipette tip was checked under the inverted microscope with a micrometer to be in a range of 20 to 50 µm. For micromanipulation of single cells under the inverted microscope, the pipette was connected with a PVC-hose of 1 m length, which ended in a pipette pumper. The length of the tube allowed very small amounts of medium to be handled. The pipette was fixed in a micromanipulator allowing precise positioning of the pipette in three dimensions. To avoid cross-contamination, a 0.22 µm filter

was inserted between pipette and hose. Samples were treated on microscope slides with a cavity. Once a cell was captured, it was transferred in a well containing 1 ml Pratt medium and a quinoa grain.

This approach was very time consuming. Due to that, it was not possible to wash cells prior to transfer in a well, because they were lost frequently in the droplet due to their small sizes and motility. Additionally, some cells did not reproduce (e.g. cercozoan cells), maybe they were killed due to shearing forces inside the tight pipettes or negative selectivity of cultivation conditions (e.g. *E. coli* as prey). A ciliate culture identified as colpodid related to *Pseudocyrtolophosis* sp. (97 % sequence similarity by Blast analysis) was only obtained in combination with *Rhodotorula* sp. and originated from a subsample after 40 days of oxic enrichment cultivation, even though its 406 bp T-RF was not detected in T-RFLP analysis (Chapter 5.1.3). As other colpodids like *Pseudoplatyophrya* sp. are known to be mycophageous, this may also be true for this species. The micromanipulation experiments were conducted prior to the observation that protozoan species were very selective concerning their prey organisms. Therefore, feeding only *E. coli* K12 led to selective cultivation conditions which prevented e.g. isolation of *Bodo* sp. from Düsseldorf sediment, even though individuals were observed occasionally in cultures. Additionally, for carnivorous protozoa it has to be considered, that they may depend on the presence of sufficient eukaryotic prey. An overview of the cultures obtained in this thesis and some of their features are summarised in Table 13.

Table 13 Overview of cultures obtained from contaminated aquifer sediments.

name	origin	carbon source	culture type	next BLAST hit	T-RF
Bodo sp.	GWM2	*Pseudomonas putida* F1	pure	AY490224	188
Amoebae	GWM2	*E.coli* K12	mixed	M95168	176
Pseudocyrtolophosis sp.	D 6.4 m	*E.coli* K12, Rhodotorula?	mixed	EU264564	406
Rhodotorula sp.	D 6.4 m	Glucose, benzoate	pure	X69853	416
Sporidiobolus D24TN	D 11.2 m	Glucose, benzoate	pure	AB021694	~551
Amoebae_2	Testfeld Sued	*Thiobacillus thiophilus* D24TN	mixed	n.a.	n.a.

5.3 Summary and conclusions of cultivation experiments

Cultivation and isolation of protists from contaminated groundwater habitats is of special interest as controlled lab experiments with obtained cultures may help shedding light on the potential role of eukaryotes on the natural attenuation capacity of groundwater. However, cultivation efforts during my thesis proved to be very challenging and were connected to substantial problems. Contrarily to investigations of pristine groundwater samples (Otto, 2004, Suess, 2001), especially fungal proliferation posed a severe problem in enrichment cultures of contaminated sediment samples which could not be avoided by fungicide application. Selective cultivation efficiency for distinct protozoan species was also observed using different bacterial prey organisms. Therefore, employed enrichment conditions may have been very selective and failed to adequately recover abundant species active in natural samples. This may be especially true for the ciliate culture originating of a sample, where its T-RF was originally not detectable. Nevertheless, it was shown that cultivation under oxic and anoxic conditions led to succession of different successional eukaryotic communities, which were most probably dominated by fungal species during the first 2-3 weeks of enrichment culture. As both samples were contaminated with aromatic hydrocarbons (BTEX and PAH), it can be hypothesised that fungi may effect biodegradation,

either by direct degradation of compounds, which is very likely under oxic conditions or by metabolism of prokaryotic products, which was shown for the *Rhodotorula* sp. culture growing on benzoate. To summarise, the establishment of protistan cultures from groundwater habitats and their use in controlled experiments is urgently needed to further assess the role of microeukaryotes for groundwater ecosystem functioning.

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6 General conclusions and outlook

Research on the natural attenuation potential of aquifers has to date mostly been focused on degradation by aerobic and anaerobic prokaryotes (Andreoni and Gianfreda, 2007). Contrarily, knowledge about eukaryotic communities in such systems and the identity of their members, but also insights in their activities and potential effects on biodegradation are still scarce. Therefore, one aim of this thesis was to assess microeukaryote diversity and community structure in different compartments of a tar-oil contaminated sandy aquifer. For this, appropriate molecular methods first had to be established and refined prior to application.

A comparison of different eukaryote specific primer sets revealed pronounced differences in their capability to assess microeukaryotic diversity in contaminated aquifer sediment samples. Full- length 18S rRNA gene primer combinations applied to study eukaryotic diversity in various environments (Diez et al., 2001b, Medlin et al., 1988) proved to be problematic investigating samples of the Düsseldorf-Flingern site by erroneous preferential amplification of archaeal templates. This was most probably due to a high background of archaeal 16S rRNA genes (> 10^4 gene copies g^{-1} sediment) compared to eukaryotic target DNA. Weaknesses in primer specificity were also validated by database screenings. Two combinations shown to most specifically amplify eukaryotic 18S rRNA genes were further evaluated, but were found to differ widely in their ability to assess diversity of distinct protistan lineages. While the "M" combination (Euk20f/Euk1179r) preferentially amplified fungal templates, the "S" combination (Euk20f/Euk516r) displayed a superior ability to detect apparent overall eukaryotic diversity in samples of the Flingern site by amplification of protozoan, but also dominant fungal templates. The utility of the established assay also in other aquifers will have to be further investigated, but the findings in the first part of the thesis also highlight general constraints of molecular tools that have to be considered. Especially, estimations of abundances of different eukaryotic groups by clone ratios are controversial, which was revealed by the differential amplification of non-target or preferred eukaryotic lineages using different primer sets. The latter had also been found in a multi-primer assay analysing marine samples (Stoeck et al., 2006), but not in another study (Diez et al., 2001a). To assess abundance shifts of distinct community members, qPCR using group-specific primers or probes will be necessary. A T-RFLP fingerprinting assay developed in this thesis (Euringer and Lueders, in press) for the rapid screening of protistan community shifts, was proven to work well in the analysed aquifer samples by comparing diversity estimates calculated by T-RF and clonal OTU abundances. As for the primer sets, the general applicability of the assay will have to be proven in further investigations at other sites.

Only after the establishment of these necessary molecular tools, it was possible to successfully assess protistan communities through distinct redox-zones of the Düsseldorf-Flingern aquifer. By sampling a drilling core during the installation of a multi-level well, depth-resolved eukaryotic communities were characterised, which was to my knowledge never done before for a comparable habitat. Additionally, the site had been very well investigated concerning its biogeochemical and microbial parameters (Anneser, 2008, Winderl, 2007). By cloning and sequencing, a very diverse protistan community was detected inside the contaminated aquifer, which showed depth-related differences in composition also revealed by T-RFLP fingerprinting.

Alveolates of 9 different subclasses were identified, predominantly inside the contamination plume and its upper fringes, where also highest prey numbers were located (Anneser, 2008, Winderl, 2007). As half of the ciliate lineages were represented by single clones only, their true diversity may have not been fully assessed, and the application of

ciliate-specific primer sets may give further details about their true diversity and distribution, as it was previously shown for a PAH contaminated soil, where a comparable diverse community of ciliates has been detected (Lara et al., 2007). Additionally, also cercozoan flagellates were found in the upper part of the aquifer and *Heteromita*- related clones were detected inside the contamination plume. Influences of these protists on bacterial communities and activities at the site are very likely, as near relatives have also been described from other contaminated groundwater habitats and have been isolated under anaerobic conditions (Brad et al., 2008). A positive effect on aerobic biodegradation was already shown in batch cultures, where also the toxicity of different aromatic hydrocarbons was assessed by IC_{50} (half maximal inhibiting concentration) determination, which was 2-3 mM benzene, 0.5 mM toluene and 0.1 mM ethylbenzene for a *Heteromita globosa* strain (Mattison et al., 2005). At the Flingern site, toluene concentrations inside the plume (6.7 m depth) were between 35 and 57 mg l^{-1} (~ 0.4- 0.6 mM) (Anneser, 2008). Therefore, contaminant concentrations inside the plume were in a range that could be coped with by distinct microeukaryotes. Additionally, steep gradients at the plume fringes, clearly identified as a hot spot for bacterial degraders and degradation activities (Anneser, 2008, Winderl, 2007) seem to represent a habitat for distinct microeukaryote organisms. Here, contrarily to ciliates and cercozoans, another protozoan lineage (the kinetoplastid *Bodo* sp.) was identified to be dominant and was only detected beneath the contamination plume. However, without additional activity-based investigations, reasons for this can only be hypothesised. Potentially, BTEX concentrations were toxic for these populations in the upper zones, but also ecological reasons such as superior competitiveness with declining prey abundances are conceivable. Without more detailed knowledge about *in situ* abundances, I cannot exclude that kinetoplastid presence in upper zones was just obscured by high numbers of other protistan lineages. Again, group-specific qPCR approaches will be necessary and of major importance for a more detailed insight in abundances of distinct protozoan lineages in different aquifer compartments. However, in concordance with other studies concerning protozoan diversity in contaminated groundwater (Brad et al., 2008, Luo et al., 2005), the results of this thesis indicate an active microbial food web, which may influence biodegradation *in situ*.

However, due to the large diversity of detected predators and their deducible functional diversity (such as prey preferences, amount of consumed prey, feeding strategies and nutritional modes), a generalisation of effects on biodegradation is not possible. Osmotrophy has been observed for different protozoan groups, but is still insufficiently investigated up today (Boenigk and Arndt, 2002, Ekelund and Ronn, 1994, Finlay and Esteban, 1998). This might also explain different findings in studies concerning qualitative effects of protozoa on biodegradation, where differences of ecological features of organisms or even the discrimination between protozoa and fungi were not considered (Kota et al., 1999, Zarda et al., 1998). The identification of potential key-players by molecular tools may therefore be an essential prerequisite shedding light on the importance of microbial food webs for the natural attenuation of groundwater. But then, actual activities of distinct groups under conditions like those found in different compartments of the Düsseldorf-Flingern aquifer, will have to be further substantiated.

Results of this thesis are also suggesting an involvement of non-protozoan protists for the ecosystem functioning of groundwater. A large fungal diversity of yeasts, molds, yeast-like or filamentous *Basidio*- and *Ascomycota* was detected, similar to other polluted aquifers investigated recently, where either *Pucciniomycotina* or *Pezizomycotina* had been most abundant (Brad et al., 2008, Luo et al., 2005). In the depth-resolved survey of the Flingern aquifer, the latter was only abundant in lower parts of the aquifer. Highest relative abundance of fungi was found in the sulphidogenic gradient zone beneath the plume, where also the hot spot of prokaryotic biodegradation activity is presumed (Winderl, 2007). To my knowledge

the role of fungi in contaminated aquifers has not been addressed yet and pathways of anaerobic degradation of aromatic hydrocarbons in fungi are unknown up today. But an involvement in biodegradation is very likely, at least by metabolism of degradation products such as benzoate (Wright, 1993). Especially basidiomycetous yeasts related to *Rhodotorula* sp. and *Cryptococcus* sp., but also detected ascomycetes like *Cladosporium* sp. are of special interest, as they are known for aerobic degradation of aromatic hydrocarbons (Atlas, 1981, Middelhoven, 1993, Potin et al., 2004). Additionally, the enrichments conducted in this thesis indicated aerobic and anaerobic growth of fungi from the Flingern site and a preliminary experiment with the obtained *Rhodotorula* sp. culture hinted at the use of benzoate as carbon source also under oxygen-depleted conditions.

Therefore, a functional relevance of protists in contaminated aquifers can be hypothesised not only via predation, but also by direct involvement in biodegradation. Here, fundamental research on the utilisation of aromatic hydrocarbons and degradation products for microeukaryotes will be essential, most of all concerning anaerobic pathways. Moreover, although some of the detected algae of the *Trebouxiophyceae* may have been transported passively through the aquifer, this group comprises genera active in aerobic biodegradation (Brad et al., 2008, Kloep and Röske, 2004, Ueno et al., 2002). Osmotrophy of protists is thought to be more important in habitats rich in organic compounds, where microeukaryotes do not have to compete for substrates with prokaryotes (Ekelund and Ronn, 1994).

To summarise, food webs in the Flingern aquifer can be considered to be very simple as the presence of higher trophic levels was only indicated by oligochaetes such as the groundwater dwelling *Aeolosoma* sp. detected in deeper parts (10.2 m depth). However, it has to be considered that DNA was extracted out of 2-4 g sediment only, whereby metazoan populations may have not been adequately sampled. The microbial loop with protists as potential grazers and decomposers of organic matter seems to be of particular importance for biodegradation also in aquifers, but the role and relevance of specific groups and lineages remains to be further assessed.

A promising approach to detect activity and role of eukaryotes is stable isotope probing of nucleic acids (SIP) (Dumont and Murrell, 2005, Lueders et al., 2004). In first experiments, the applicability and also crucible detection limits of DNA-SIP were evaluated for protists, by following ^{13}C from labelled bacterial prey biomass to a flagellate predator in a short food chain under optimal conditions. For that purpose a eukaryotic qPCR assay was developed, with a detection limit of ~10^2 18S rRNA gene copies μl^{-1}. By application of eukaryotic PCR/T-RFLP and qPCR assays developed in this thesis on samples of a SIP experiment dealing with anaerobic toluene degradation (Winderl, 2007), first indications on an active role of eukaryotes in carbon turnover were achieved. Even though bacterial degraders had been found to be labelled to 50 %, a label in eukaryotic DNA was detectable. But also major drawbacks were revealed that have to be considered, most of all the amount of eukaryotic DNA needed to overcome detection limits, but also problematic background of ^{12}C that might obscure conclusions of SIP experiments. Further investigations will be necessary, but are very promising.

Cultivation and isolation of protists is a further essential prerequisite to assess potential roles and impacts of different protistan groups in groundwater ecosystems in dedicated experiments. Unfortunately, enrichment and cultivation experiments turned out to be especially problematic and challenging during my thesis, most of all due to excessive fungal growth and selective cultivation conditions. Nevertheless, comparison of oxic and anoxic treatments of enrichment cultures proved successional development of differing communities, highlighting the need of rigid anaerobic cultivation techniques to be established

also for protists. Finally, relevance of fungi in contaminated aquifers was also indicated by their predominance in cultures.

In summary, the presented data proves a diverse microeukaryotic community to be extant in different zones of the contaminated Flingern aquifer. Variations in composition are indicative for a coupling to biogeochemical and microbial processes, but further respective investigations will be needed. Influence of protists on processes such as natural attenuation is very likely, but the role of different lineages, most of all protozoa involved in food webs, but also fungal decomposers potentially active in direct contaminant turnover will have to be elucidated in future. Prospective application of the established assays and SIP approaches may further promote a better understanding of the influence of microeukaryote populations on the natural attenuation potential of groundwater.

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Appendix

Table A.1 *In silico* dataset of the 243 microeukaryote 18S rDNA sequences used to predict the suitability of different restriction enzymes for protistan T-RFLP fingerprinting in groundwater environments. Given are NCBI accession numbers, names and the affiliation to major eukaryotic lineages.

Accession No.	Full name	Lineage
AF290541	*Cryothecomonas aestivalis*	Cercozoa
AF411265	*Allantion* sp. ATCC 50734	Cercozoa
AF411266	*Cercomonas* sp. LargeSA	Cercozoa
AF411267	*Cercomonas* alexieffi	Cercozoa
AF411268	*Cercomonas plasmodialis*	Cercozoa
AF411270	*Cercomonas longicauda*	Cercozoa
AF411272	*Cercomonas* sp. ATCC 50367	Cercozoa
AF411276	*Bodomorpha minima*	Cercozoa
AF411278	*Metopion*-like sp. (SA)	Cercozoa
AF411279	*Rigidomastix*-like sp. (SA)	Cercozoa
AF411284	*Gymnophrys cometa*	Cercozoa
AF534712	*Cercomonas* sp. SmallSA	Cercozoa
AJ418792	*Trinema enchelys*	Cercozoa
AY642694	uncultured eukaryotic picoplankton	Cercozoa
DQ303924	*Protaspis grandis*	Cercozoa
EF023110	eimeriidae environmental sample	Cercozoa
EU091844	uncultured Banisveld clone	Cercozoa
EU091848	uncultured Banisveld clone	Cercozoa
U42446	*Thaumatomonas* sp.	Cercozoa
U42447	*Heteromita globosa*	Cercozoa
U42448	*Cercomonas* ATCC50316	Cercozoa
U42449	*Cercomonas* ATCC50317	Cercozoa
U42450	*Cercomonas* ATCC50318	Cercozoa
X77692	*Euglypha rotunda*	Cercozoa
X81811	*Paulinella chromatophora*	Cercozoa
X56531	*Opisthonecta henneguyi*	Alveolata
AB252009	*Paramecium tetraurelia*	Alveolata
AF060452	*Pseudoplatyophrya nana*	Alveolata
AF060453	*Bresslaua vorax*	Alveolata
AF060454	*Platyophrya vorax*	Alveolata
AF011461	*Stachyamoeba* sp. ATCC 50324	Heterolobosa
AF011463	*Monopylocystis visvesvarai*	Heterolobosa
AF439350	*Heteramoeba clara*	Heterolobosa
AF439351	*Sawyeria marylandensis*	Heterolobosa
AJ224890	*Paravahlkampfia ustiana*	Heterolobosa
AJ550994	*Paravahlkampfia* sp. li3_03	Heterolobosa
AY394431	Uncultured *Vahlkampfia*	Heterolobosa
AY965862	Soil amoeba AND12	Heterolobosa
DQ979962	*Pleurostomum flabellatum* strain EHF1	Heterolobosa
M18732	*Naegleria gruberi*	Heterolobosa
M98050	*Paratetramitus jugosus*	Heterolobosa
M98051	*Tetramitus rostratus*	Heterolobosa
M98052	*Vahlkampfia lobospinosa*	Heterolobosa
X94430	*Psalteriomonas lanterna*	Heterolobosa
AY121849	*Platyamoeba plurinucleolus*	Lobosa
AY121853	*Vannella aberdonica*	Lobosa
AY183888	*Vannella miroides*	Lobosa
AY929904	*Vannella* sp. strain S2M2/I	Lobosa
AY929906	*Vannella* sp. strain S98M8/I	Lobosa
AY929910	*Vannella* sp. strain 4432/I	Lobosa
AY929915	*Platyamoeba* sp. strain SS8FJ1/I	Lobosa
AY929917	*Platyamoeba* sp. strain SBV1/I	Lobosa
AY929918	*Platyamoeba* sp. strain AFSM6/I	Lobosa
AY929919	*Platyamoeba* sp. strain PMCH/II	Lobosa
AY929920	*Platyamoeba* sp. strain DB282	Lobosa
DQ229953	*Platyamoeba contorta* isolate W51C#4	Lobosa
DQ229954	*Platyamoeba contorta* isolate W51C#5	Lobosa
DQ913101	*Vannella epipetala clone* 9	Lobosa
AF114438	*Acanthamoeba castellanii*	Lobosa
M13435	*Acanthamoeba castellanii*	Lobosa

Accession No.	Full name	Lineage
AF164121	*Sterkiella histriomuscorum*	Alveolata
AF164123	*Stylonychia mytilus*	Alveolata
AF164124	*Stylonychia lemnae*	Alveolata
AF164125	*Oxytricha longa*	Alveolata
AF164126	*Oxytricha* sp.	Alveolata
AF164127	*Paraurostyla weissei*	Alveolata
AF164133	*Gastrostyla steinei*	Alveolata
AF164135	*Cyrtohymena citrina*	Alveolata
AF164683	*Oxytricha* sp.	Alveolata
AF164684	*Oxytricha* sp.	Alveolata
AF217655	*Paramecium caudatum*	Alveolata
AF335514	*Epistylis chrysemydis*	Alveolata
AF335516	*Epistylis urceolata*	Alveolata
AF335518	*Vorticella campanula*	Alveolata
AF357144	*Stentor polymorphus*	Alveolata
AF357145	*Stentor coeruleus*	Alveolata
AF357913	*Stentor roeseli*	Alveolata
AF396973	*Tetmemena pustulata*	Alveolata
AF401522	*Carchesium polypinum*	Alveolata
AF401523	*Zoothamnium arbuscula*	Alveolata
AJ310499	*Stylonychia mytilus*	Alveolata
AY547546	*Anoplophrya marylandensis*	Alveolata
L26447	*Homalozoon vermiculare*	Alveolata
L26448	*Loxophyllum utricullariae*	Alveolata
M97908	*Colpoda inflata*	Alveolata
U27814	*Dasytricha ruminantium*	Alveolata
U27815	*Entodinium simplex*	Alveolata
U47620	*Eufolliculina uhligi*	Alveolata
U51554	*Anophyroides haemophila*	Alveolata
U57763	*Epidinium caudatum*	Alveolata
U57764	*Diplodinium dentatum*	Alveolata
U57765	*Entodinium caudatum*	Alveolata
U57766	*Eudiplodinium maggii*	Alveolata

Accession No.	Full name	Lineage
U07403	*Acanthamoeba castellanii*	Lobosa
AF005995	*Acanthamoeba lugdunensis*	Lobosa
U07407	*Acanthamoeba polyphaga*	Lobosa
U07402	*Acanthamoeba polyphaga*	Lobosa
U07408	*Acanthamoeba* sp.	Lobosa
AF293895	*Echinamoeba exundans*	Lobosa
AF293898	*Leptomyxa reticulata*	Lobosa
AF293899	*Paraflabellula hoguae*	Lobosa
AF293902	*Saccamoeba limax*	Lobosa
AF426157	*Hartmannella vermiformis*	Lobosa
AY121848	*Neoparamoeba aestuarina* strain ATCC 50744	Lobosa
AY121850	*Korotnevella hemistylolepis*	Lobosa
AY121852	*Neoparamoeba aestuarina* strain ATCC 50806	Lobosa
AY183891	*Vexillifera armata*	Lobosa
AY183893	*Korotnevella stella*	Lobosa
AY183894	*Neoparamoeba pemaquidensis*	Lobosa
AY277797	*Paraflabellula hoguae*	Lobosa
AY294147	*Hartmannella cantabrigiensis*	Lobosa
AY502959	*Hartmannella vermiformis* isolate KWR-1	Lobosa
AY502960	*Hartmannella vermiformis* isolate KWR-2	Lobosa
AY502961	*Hartmannella vermiformis* isolate KWR-3	Lobosa
AY680840	*Hartmannella vermiformis*	Lobosa
AY686573	*Korotnevella stella*	Lobosa
AY686576	*Pseudoparamoeba pagei*	Lobosa
AY714352	*Neoparamoeba pemaquidensis* strain GILLNOR1	Lobosa
AY714359	*Neoparamoeba pemaquidensis* strain SEDST1	Lobosa
AY714369	*Filamoeba sinensis* strain CH26	Lobosa
AY929908	*Vannella* sp. strain RSL/I	Lobosa
DQ084363	*Hartmannella vermiformis* clone 4480	Lobosa
DQ084364	*Hartmannella vermiformis* clone 4391	Lobosa
DQ084365	*Hartmannella vermiformis* clone TN102	Lobosa
DQ084366	*Hartmannella vermiformis* clone PFG	Lobosa
DQ190241	*Hartmannella abertawensis* strain Page 1980	Lobosa

Accession No.	Full name	Lineage
U57767	*Polyplastron multivesiculatum*	Alveolata
U57768	*Ophryoscolex purkynjei*	Alveolata
U57769	*Dasytricha ruminantium*	Alveolata
U57770	*Isotricha intestinalis*	Alveolata
U57771	*Didinium nasutum*	Alveolata
U80313	*Enchelyodon* sp.	Alveolata
U82204	*Bursaria truncatella*	Alveolata
U97109	*Coleps hirtus*	Alveolata
U97111	*Prorodon viridis*	Alveolata
X03772	*Paramecium tetraurelia*	Alveolata
X56172	*Tetrahymena capricornis*	Alveolata
X56532	*Colpidium campylum*	Alveolata
X65149	*Obertrumia aurea*	Alveolata
X65150	*Furgasonia blochmanni*	Alveolata
X65151	*Pseudomicrothorax dubius 2*	Alveolata
X65152	*Climacostomum virens*	Alveolata
X71140	*Prorodon teres*	Alveolata
X76646	*Coleps* sp.	Alveolata
AB007814	*Trypanosoma theileri*	Euglenozoa
AF016320	*Phytomonas serpens*	Euglenozoa
AF245382	*Trypanosoma cruzi*	Euglenozoa
AJ223562	*Trypanosoma bennetti*	Euglenozoa
AJ223563	*Trypanosoma congolense*	Euglenozoa
AJ223564	*Trypanosoma equiperdum*	Euglenozoa
AJ223565	*Trypanosoma grayi*	Euglenozoa
AJ223566	*Trypanosoma lewisi*	Euglenozoa
AJ223567	*Trypanosoma mega*	Euglenozoa
AJ223568	*Trypanosoma musculi*	Euglenozoa
AJ223569	*Trypanosoma simiae*	Euglenozoa
AJ223570	*Trypanosoma* sp.	Euglenozoa
AJ223571	*Trypanosoma therezieni*	Euglenozoa
AJ223572	*Trypanosoma varani*	Euglenozoa
AY028451	*Bodo edax*	Euglenozoa
AY425021	*Cryptaulaxoides*-like sp. TCS-2003	Euglenozoa
AY425025	*Rhynchomonas nasuta*	Euglenozoa
AY490230	*Bodo saltans*	Euglenozoa
AY753948	uncultured eukaryote clone	Euglenozoa
D89527	*Trypanosoma evansi*	Euglenozoa
L29264	*Crithidia oncopelti*	Euglenozoa
L29266	*Blastocrithidia culicis*	Euglenozoa

Accession No.	Full name	Lineage
DQ229957	*Neoparamoeba aestuarina* isolate W4-3	Lobosa
DQ229958	*Neoparamoeba aestuarina* S131-2	Lobosa
M95168	*Hartmanella vermiformis*	Lobosa
X75515	*Hartmannella vermiformis*	Lobosa
X75514	*Hartmannella vermiformis*	Lobosa
X75513	*Hartmannella vermiformis*	Lobosa
M95168	*Hartmannella vermiformis*	Lobosa
X75513	*H.vermiformis*	Lobosa
M62995	*Friedmannia israeliensis*	Chlorophyta
Z47208	*Myrmecia astigmatica*	Chlorophyta
Z28971	*Myrmecia biatorellae*	Chlorophyta
Z21553	*Trebouxia asymmetrica*	Chlorophyta
Z21551	*Trebouxia impressa*	Chlorophyta
Z68700	*Trebouxia jamesii*	Chlorophyta
Z21552	*Trebouxia magna*	Chlorophyta
AF070445	*Anurofeca richardsi*	Choanozoa
AF100941	*Salpingoeca infusionum*	Choanozoa
AF192386	*Pseudoperkinsus tapetis*	Choanozoa
AF232303	*Ichthyophonus irregularis*	Choanozoa
AF271999	*Monosiga ovata*	Choanozoa
AY642707	uncultured eukaryotic picoplankton	Choanozoa
DQ059032	*Salpingoeca amphoridium*	Choanozoa
L10824	*Diaphanoeca grandis*	Choanozoa
L29455	uncultured clone	Choanozoa
U21336	*Dermocystidum* sp.	Choanozoa
U21337	*Dermocystidium salmonis*	Choanozoa
U25637	*Ichthyophonus hoferi*	Choanozoa
U33180	*Psorospermium haeckelii*	Choanozoa
Y19155	*Amoebidium parasiticum*	Choanozoa
AB021673	*Sporobolomyces inositophilus*	Fungi
AB021675	*Sporobolomyces lactophilus*	Fungi
AB021688	*Sporobolomyces sasicola*	Fungi
AB039285	*Cryptococcus humicolus*	Fungi
AB039378	*Cryptococcus humicolus*	Fungi
AF201298	*Candida humicola*	Fungi
AF229176	*Sporobolomyces yunnanensis*	Fungi
AY082971	uncultured eukaryote clone	Fungi
AY187083	*Lycogala flavofuscum*	Fungi
EU091845	uncultured Banisveld clone	Fungi
EU091847	uncultured Banisveld clone	Fungi

Accession No.	Full name	Lineage
L35076	*Phytomonas* sp.	Euglenozoa
L35077	*Phytomonas* sp.	Euglenozoa
M12676	*Trypanosoma brucei*	Euglenozoa
M31432	*Trypanosoma cruzi* 2	Euglenozoa
M84225	*Leishmania tarentolae*	Euglenozoa
U22315	*Trypanosoma congolense*	Euglenozoa
U22316	*Trypanosoma vivax*	Euglenozoa
U22317	*Trypanosoma congolense*	Euglenozoa
U22318	*Trypanosoma congolense*	Euglenozoa
U22320	*Trypanosoma simiae*	Euglenozoa
U75507	*Trypanosoma evansi*	Euglenozoa
X03450	*Crithidia fasciculata*	Euglenozoa
X07773	*Leishmania donovani*	Euglenozoa
AF011455	*Learamoeba waccamawenis*	Heterolobosa
AF011456	*Singhamoeba hortricola*	Heterolobosa
AF011458	*Acrasis rosea*	Heterolobosa
AF011459	*Plaesiobystra hypersalinica*	Heterolobosa
AF011460	*Heteramoeba clara*	Heterolobosa

Accession No.	Full name	Lineage
EU091853	uncultured Banisveld clone	Fungi
EU091857	uncultured Banisveld clone	Fungi
EU091861	uncultured Banisveld clone	Fungi
EU091862	uncultured Banisveld clone	Fungi
EU091864	uncultured Banisveld clone	Fungi
EU091866	uncultured Banisveld clone	Fungi
EU091867	uncultured Banisveld clone	Fungi
EU091871	uncultured Banisveld clone	Fungi
EU091873	uncultured Banisveld clone	Fungi
EU091874	uncultured Banisveld clone	Fungi
EU091876	uncultured Banisveld clone	Fungi
EU091877	uncultured Banisveld clone	Fungi
X53499	*Leucosporidium scottii*	Fungi
X60180	*Rhodotorula glutinis*	Fungi
X60181	*Sporobolomyces roseus*	Fungi
X83827	*Rhodotorula graminis*	Fungi
X84326	*Rhodotorula mucilaginosa*	Fungi
Y18698	*Capnobotryella renispora*	Fungi
Y18699	*Comminutispora agavaciensis*	Fungi

Table A.2 Theoretical length (bp) and frequency of OTUs predicted by *in silico* digestion as shown in Fig. 4.

	T-RF	Σ clones	frequency		T-RF	Σ clones	frequency
*Bsh*1236I	66	4	0.03	*Msp*I	59	1	0.01
	74	28	0.18		178	7	0.04
	177	1	0.01		228	6	0.04
	178	2	0.01		230	2	0.01
	187	1	0.01		265	4	0.03
	191	39	0.24		266	2	0.01
	206	3	0.02		269	1	0.01
	211	2	0.01		270	24	0.15
	258	2	0.01		272	2	0.01
	268	13	0.08		288	7	0.04
	405	1	0.01		289	15	0.09
	406	1	0.01		343	3	0.02
	408	3	0.02		355	2	0.01
	412	2	0.01		357	3	0.02
	417	2	0.01		361	2	0.01
	419	1	0.01		364	13	0.08
	420	33	0.21		366	2	0.01
	421	5	0.03		369	2	0.01
	423	7	0.04		370	5	0.03
	429	1	0.01		372	7	0.04
	435	1	0.01		378	1	0.01
	566	1	0.01		384	2	0.01
	700	7	0.04		386	8	0.05
					421	39	0.24

Table A.3 Comparisons of T-RF abundances of T-RFLP analyses (4 replicates) of duplicated DNA extracts as shown in Fig. 6.

	mean values			respective standard deviation		
T-RF	extract 1	extract 2	combined extracts	extract 1	extract 2	combined extracts
59	0.024	0.005	0.014	0.018	0.009	0.017
68	0.007	0.008	0.007	0.014	0.015	0.013
189	0.035	0.086	0.060	0.028	0.023	0.036
210	0.039	0.028	0.033	0.025	0.009	0.018
213	0.131	0.031	0.081	0.044	0.037	0.066
223	0.004	0.015	0.010	0.008	0.030	0.021
406	0.012	0.034	0.021	0.024	0.031	0.027
407	0.228	0.197	0.213	0.073	0.047	0.059
414	0.220	0.263	0.242	0.068	0.054	0.061
416	0.032	0.000	0.016	0.009	0.000	0.018
419	0.115	0.006	0.060	0.023	0.012	0.061
420	0.000	0.013	0.007	0.000	0.026	0.018
421	0.024	0.076	0.050	0.021	0.018	0.033
423	0.010	0.000	0.006	0.012	0.000	0.010
427	0.000	0.016	0.008	0.000	0.011	0.011
539	0.008	0.009	0.008	0.010	0.015	0.011
548	0.021	0.032	0.026	0.015	0.009	0.013
Div	0.014	0.008	0.011	0.010	0.016	0.013

Table A.4 Chao1 richness estimates, detected OTUs and calculated Shannon *H'* diversity indices derived for OTU and T-RF abundances as shown in Fig. 7.

Depth [m]	Chao	$OTU_{97\%}$	H' ($OTU_{97\%}$)	H' (T-RFs)
5.70	30.3	19	2.49	1.51
6.30	20.5	10	1.15	1.62
6.65	16.	15	2.36	1.33
7.20	5.0	5	1.56	1.58
7.35	16.0	11	1.90	2.23
8.10	5.0	5	1.14	1.53
9.10	4.0	4	0.69	1.16
9.80	3.0	3	0.63	1.30
10.20	28.0	19	2.61	2.04
11.70	7.0	7	1.41	1.11

Table A.5 Rarefaction analysis of 18S rRNA gene diversity ($OTU_{97\%}$) detected in 10 clone libraries as shown in Fig. 8.

clones analysed	5.7	6.3	6.7	7.2	7.4	8.1	9.1	9.8	10.2	11.7
1	1	1	1	1	1	1	1	1	1	1
2	1.89	1.43	1.89	1.69	1.81	1.64	1.38	1.24	1.91	1.67
3	2.69	1.71	2.70	2.20	2.49	2.05	1.57	1.37	2.76	2.16
4	3.43	1.95	3.44	2.60	3.09	2.36	1.75	1.48	3.57	2.56
5	4.13	2.20	4.12	2.92	3.57	2.58	1.90	1.58	4.32	2.91
6	4.76	2.43	4.72	3.18	3.98	2.75	2.03	1.65	5.02	3.19
7	5.33	2.64	5.29	3.42	4.36	2.90	2.14	1.74	5.68	3.42
8	5.85	2.87	5.83	3.64	4.67	3.03	2.23	1.82	6.30	3.64
9	6.35	3.08	6.34	3.81	4.97	3.16	2.32	1.90	6.87	3.86
10	6.83	3.28	6.81	3.97	5.26	3.27	2.41	1.96	7.42	4.06
11	7.28	3.48	7.22	4.11	5.52	3.38	2.47	2.01	7.93	4.24
12	7.70	3.70	7.60	4.23	5.74	3.45	2.55	2.07	8.40	4.38
13	8.09	3.91	7.97	4.35	5.97	3.53	2.62	2.12	8.86	4.54
14	8.45	4.10	8.35	4.44	6.17	3.59	2.68	2.16	9.30	4.70
15	8.83	4.30	8.69	4.52	6.38	3.64	2.74	2.19	9.73	4.84
16	9.18	4.50	9.02	4.58	6.57	3.70	2.80	2.23	10.14	4.96
17	9.54	4.71	9.31	4.64	6.76	3.77	2.86	2.28	10.53	5.08
18	9.88	4.92	9.61	4.68	6.97	3.82	2.91	2.31	10.91	5.20
19	10.21	5.08	9.91	4.73	7.14	3.88	2.96	2.34	11.27	5.32
20	10.52	5.27	10.17	4.77	7.30	3.93	3.01	2.37	11.60	5.42
21	10.84	5.45	10.43	4.80	7.47	4.00	3.07	2.40	11.93	5.53
22	11.14	5.62	10.68	4.83	7.61	4.05	3.12	2.42	12.27	5.61
23	11.44	5.80	10.90	4.85	7.77	4.11	3.16	2.45	12.59	5.70
24	11.74	5.99	11.12	4.87	7.94	4.14	3.20	2.47	12.88	5.77
25	12.01	6.19	11.32	4.89	8.09	4.19	3.24	2.49	13.20	5.85
26	12.28	6.38	11.52	4.91	8.23	4.24	3.28	2.52	13.48	5.93
27	12.54	6.55	11.75	4.92	8.38	4.29	3.32	2.53	13.75	6.00
28	12.80	6.73	11.95	4.94	8.50	4.34	3.35	2.55	14.01	6.08
29	13.05	6.88	12.17	4.96	8.64	4.37	3.39	2.57	14.28	6.14
30	13.30	7.05	12.34	4.96	8.76	4.41	3.42	2.59	14.52	6.21
31	13.55	7.22	12.52	4.97	8.90	4.44	3.46	2.61	14.77	6.27
32	13.77	7.40	12.68	4.98	9.03	4.48	3.49	2.63	15.01	6.33
33	14.00	7.58	12.84	4.98	9.14	4.50	3.52	2.65	15.25	6.38
34	14.22	7.75	12.99	4.98	9.26	4.54	3.55	2.68	15.46	6.45

clones analysed	5.7	6.3	6.7	7.2	7.4	8.1	9.1	9.8	10.2	11.7
35	14.45	7.92	13.14	4.99	9.39	4.57	3.59	2.70	15.70	6.50
36	14.69	8.07	13.28	5.00	9.49	4.61	3.62	2.72	15.92	6.57
37	14.92	8.25	13.43	5.00	9.61	4.63	3.65	2.74	16.13	6.61
38	15.14	8.41	13.58	5.00	9.74	4.66	3.67	2.76	16.36	6.66
39	15.36	8.55	13.70	5.00	9.86	4.70	3.70	2.78	16.55	6.69
40	15.58	8.70	13.82	5.00	9.98	4.74	3.72	2.79	16.77	6.73
41	15.79	8.86	13.94	5.00	10.09	4.78	3.76	2.81	16.94	6.77
42	16.00	9.01	14.06	5.00	10.19	4.81	3.78	2.83	17.14	6.80
43	16.22	9.16	14.18	5.00	10.28	4.83	3.81	2.85	17.35	6.83
44	16.40	9.29	14.28	5.00	10.39	4.85	3.85	2.87	17.55	6.86
45	16.59	9.43	14.38	5.00	10.50	4.87	3.87	2.88	17.72	6.89
46	16.78	9.58	14.48	5.00	10.62	4.89	3.89	2.90	17.91	6.91
47	16.97	9.71	14.58	5.00	10.71	4.91	3.91	2.92	18.10	6.93
48	17.17	9.85	14.67	5.00	10.81	4.93	3.94	2.95	18.27	6.95
49	17.36	10.00	14.76	5.00	10.90	4.95	3.95	2.97	18.44	6.98
50	17.54		14.84	5.00	11.00	4.98	3.98	3.00	18.63	7.00
51	17.72		14.92	5.00		5.00	4.00		18.82	
52	17.92		15.00	5.00					19.00	
53	18.09			5.00						
54	18.28			5.00						
55	18.47									
56	18.65									
57	18.82									
58	19.00									

Table A.6 Statistical comparison of 18S rRNA gene clone libraries using Cramer von Mises statistic implemented by ∫-LIBSHUFF.

homologous library (X)	p-value for comparison of heterologous library (Y) with X[a]									
Depth [m]	5.7	6.3	6.7	7.2	7.4	8.1	9.1	9.8	10.2	11.7
5.7		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
6.3	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	*0.1243*	0.0000
6.7	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
7.2	0.0000	0.0000	*0.0220*		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
7.4	0.0000	0.0000	0.0000	0.0000		*0.0009*	0.0000	0.0000	0.0000	0.0000
8.1	0.0000	0.0000	0.0000	0.0000	*0.2335*		0.0000	0.0000	0.0000	0.0000
9.1	0.0000	0.0000	0.0000	0.0000	*0.0011*	0.0000		0.0000	0.0000	0.0000
9.8	0.0000	0.0000	0.0000	0.0000	*0.1798*	*0.3664*	0.0000		*0.0034*	0.0000
10.2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000
11.7	0.0000	0.0000	0.0000	0.0000	*0.0008*	0.0000	0.0000	0.0000	0.0000	

[a] Italic values are non-significant with Bonferroni corrected p-values < 0.0006 for a comparison-wise error rate of 0.05.

Table A.7 Rarefaction analysis of 18S rRNA gene diversity ($OTU_{97\%}$) detected in 3 clone libraries as shown in Fig. 11.

Clones analysed	7.4	8.1	10.2
1	1	1	1
2	1.84	1.72	1.93
3	2.54	2.27	2.79
4	3.19	2.74	3.60
5	3.76	3.11	4.38
6	4.29	3.46	5.10
7	4.77	3.76	5.77
8	5.20	4.03	6.40
9	5.61	4.28	7.01
10	6.03	4.51	7.60
11	6.40	4.74	8.13
12	6.77	4.96	8.66
13	7.12	5.16	9.14
14	7.45	5.34	9.60
15	7.76	5.53	10.04
16	8.07	5.69	10.47
17	8.37	5.87	10.86
18	8.66	6.04	11.27
19	8.92	6.22	11.64
20	9.20	6.35	12.00
21	9.42	6.48	12.33
22	9.68	6.61	12.66
23	9.96	6.73	13.01
24	10.18	6.85	13.32
25	10.42	6.95	13.64
26	10.64	7.08	13.96
27	10.85	7.18	14.26
28	11.06	7.28	14.53
29	11.25	7.39	14.80
30	11.45	7.49	15.08
31	11.63	7.59	15.35
32	11.79	7.67	15.62
33	11.97	7.75	15.88
34	12.12	7.84	16.14
35	12.26	7.91	16.40
36	12.42	7.99	16.65
37	12.55	8.06	16.89
38	12.70	8.14	17.14
39	12.83	8.21	17.37
40	12.95	8.29	17.62
41	13.07	8.35	17.85
42	13.16	8.39	18.08
43	13.27	8.47	18.31
44	13.37	8.52	18.55
45	13.47	8.56	18.78
46	13.56	8.62	19.00
47	13.65	8.67	
48	13.74	8.72	
49	13.82	8.78	
50	13.89	8.83	
51	13.95	8.88	
52	14.00	8.92	
		8.96	
		9.00	

Table A.8 Description of media used during the thesis.

Cerial grass infusion medium 2.5 g of powdered wheatgrass (Sanos) was dissolved in 1 litre demineralised water and boiled for 5 minutes. Evaporated water was refilled and 0.5 g Na_2HPO_4 added. After filtration through an ordinary filter paper (Selecta Faltenfilter; diameter 185 mm; Schleicher & Schuell, Dassel, Germany), the medium was filled in Schott bottles and autoclaved for 15 minutes at 121°C. Bottles were stored at 4°C.
Bottled water Aliquots of bottled water were autoclaved for 15 minutes at 121°C and stored at 4°C.
Pratt medium 10 ml of 100 x stock solutions (1 g KNO_3, 0.1 g $MgSO_4$ * 7 H_2O, 0.087 g K_2HPO_4 and 0.01 g $FeCl_3$ * 6 H_2O; each dissolved in 100 ml of demineralised water) were added to 960 ml of demineralised water and portions were autoclaved for 15 minutes at 121°C and stored at 4°C.
M9 minimal medium 3 g KH_2PO_4, 6 g Na_2HPO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.12 g $MgSO_4$, 0.01 g $CaCl_2$ were dissolved in 1 l of demineralised water. 45 ml were autoclaved in serum bottles closed with butyl stoppers and 5 ml glucose solution (5.5 g per litre) was added by sterile filtration through a 0.22 µm filter (Milipore). Bacteria were grown at 30°C on a shaker and stored at 4°C.
Fungi mineral medium 3.4 g KH_2PO_4 , 4.3 g K_2HPO_4, 0.3 g $CaCl_2$*$2H_2O$, 1 g $(NH_4)_2SO_4$, 0.05 g yeast extract were solved in 995 ml demineralised water and 5 ml of a trace element solution (1 mg l^{-1} MnCl* $4H_2O$, 0.6 mg l^{-1} $FeSO_4$*$7H_2O$, 2.6 mg l^{-1} $CaCl_2$ *H_2O, 6 mg l^{-1} Na_2MoO_4*$2H_2O$) was added.

Table A.9 NanoDrop measurement results of extracted DNA. In bold extracts used for gradients.

Transfer	Treatment	DNA (ng µl^{-1})
0	^{12}C 1	64.97
0	^{12}C 2	52.71
0	^{13}C 1	54.12
0	^{13}C 2	60.62
1	^{13}C 1	35.06
1	^{13}C 2	23.55
2	**^{12}C 1**	**48.09**
2	**^{12}C 2**	**48.66**
2	**^{13}C 1**	**38.37**
2	**^{13}C 2**	**53.56**

Table A.10 18S rRNA gene copy numbers in SIP gradient fractions shown in Fig. 14.

Gradient	CsCl (g ml^{-1})	18S rDNA copy numbers µl^{-1}
LR (^{12}C)	1.73	$7.63*10^2$
	1.73	$8.70*10^2$
	1.71	$7.68*10^3$
	1.70	$9.58*10^3$
	1.69	$2.07*10^5$
	1.68	$9.15*10^5$
	1.68	$1.79*10^5$
	1.67	$2.75*10^4$
LS (^{13}C)	1.73	$6.55*10^2$
	1.72	$6.58*10^3$
	1.71	$1.85*10^4$
	1.70	$1.69*10^3$

Table A.11 18S rRNA copy numbers (µl^{-1}) in SIP gradient fractions shown in Fig. 15.

gradient	CsCl (g ml^{-1})	18S rDNA copy numbers	gradient	CsCl (g ml^{-1})	18S rDNA copy numbers
^{12}C 8 days	1.697	$3.30*10^3$	^{13}C 8 days	1.690	$7.63*10^3$
	1.688	$5.75*10^3$		1.681	$6.39*10^3$
	1.680	$2.20*10^4$		1.672	$5.18*10^3$
	1.672	$1.19*10^4$			
^{12}C 86 days	1.699	$1.58*10^4$	^{13}C 86 days	1.707	$7.80*10^3$
	1.691	$1.95*10^4$		1.698	$9.57*10^3$
	1.682	$6.08*10^4$		1.691	$3.55*10^4$
	1.674	$1.37*10^4$		1.683	$4.92*10^4$
				1.674	$4.51*10^4$
				1.666	$5.72*10^3$

Table A.12 Relative abundances of exemplary T-RFs as shown in Fig. 17.

	relative T-RF abundances					
CsCl (g ml^{-1})	**189 (^{13}C)**	**189 (^{12}C)**	**538(^{13}C)**	**538(^{12}C)**	**411 (^{13}C)**	**411(^{12}C)**
1.71	0.43		0.00		0.08	
1.70	0.35	0.09	0.00	0.00	0.08	0.00
1.69	0.30	0.29	0.03	0.03	0.17	0.00
1.68	0.21	0.19	0.07	0.06	0.26	0.05
1.67	0.07	0.00	0.06	0.07	0.20	0.05

Table A.13 Development of oxygen concentrations in plates as shown in Fig.18.

96 well (min)	**athmospheric oxygen saturation (%)**	**mg l^{-1} oxygen**	**24 well (min)**	**athmospheric oxygen saturation (%)**	**mg l^{-1} oxygen**
5	1.3	0.11	7	3.9	0.34
26	2.5	0.22	28	18.3	1.58
38	6.1	0.52	39	38.4	3.32
45	14.2	1.23	47	43.3	3.74
54	12.7	1.10	55	49.2	4.25
64	16.1	1.39	65	56.7	4.90
75	20.6	1.78	76	60.5	5.23
84	26.1	2.25	87	65.2	5.64
99	27.5	2.37	101	71.7	6.20
139	36.7	3.17	141	73.8	6.37

Table A.14 NanoDrop measurement results of extracted DNA from enrichment cultures.

	sediment (g ww)	**DNA (ng µl^{-1})**	**per g**		**sediment (g ww)**	**DNA (ng µl^{-1})**	**per g**
6.4 m	2.56	31.26	**12.21**				
oxic	1.15	28.02	**24.37**	anoxic	1.44	22.80	**15.83**
	1.77	27.35	**15.45**		1.28	32.09	**25.07**
	1.39	43.22	**31.09**		0.90	12.76	**14.18**
	1.40	45.39	**32.42**		1.62	23.54	**14.53**
	1.70	45.01	**26.48**		1.63	18.05	**11.07**
7.6 m	2.22	10.30	**4.64**				
oxic	1.64	8.30	**5.06**	anoxic	1.04	15.39	**14.80**
	1.56	12.17	**7.80**		1.16	11.64	**10.03**
	1.63	8.95	**5.49**		1.80	12.01	**6.67**
	1.22	8.60	**7.05**		1.14	8.86	**7.77**
	1.18	7.80	**6.61**		1.31	11.97	**9.14**
	1.18	8.73	**7.40**		1.18	10.05	**8.52**

Table A.15 Abundances of T-RFs in enrichment cultures as shown in Fig. 21.

sample (m)	treatment	T-RF	relative T-RF abundances at days						
			0	**6**	**12**	**16**	**22**	**30**	**40**
6.4m	oxic	**231**	0.00	0.00	0.00	0.45	0.35	0.30	0.06
		416	0.18	0.11	0.29	0.09	0.12	0.10	0.08
		420	0.38	0.79	0.66	0.18	0.16	0.11	0.05
		421	0.00	0.00	0.00	0.23	0.33	0.38	0.23
		436	0.06	0.01	0.02	0.00	0.01	0.06	0.56
7.6m	oxic	**189**	0.07	0.06	0.13	0.10	0.00	0.09	0.04
		406	0.04	0.06	0.00	0.10	0.07	0.03	0.00
		407	0.07	0.00	0.13	0.10	0.04	0.07	0.30
		414	0.00	0.00	0.02	0.05	0.00	0.02	0.00
		416	0.64	0.66	0.52	0.16	0.44	0.21	0.31
		421	0.00	0.00	0.01	0.00	0.00	0.02	0.03
6.4m	anoxic	**231**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		416	0.18	0.26	0.31	0.21	0.23	0.23	0.65
		420	0.38	0.42	0.41	0.48	0.49	0.48	0.23
		421	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		436	0.06	0.06	0.05	0.04	0.04	0.03	0.01
7.6m	anoxic	**189**	0.07	0.00	0.00	0.06	0.11	0.04	0.01
		406	0.04	0.01	0.05	0.08	0.00	0.03	0.11
		407	0.07	0.01	0.17	0.31	0.16	0.08	0.02
		414	0.00	0.03	0.00	0.00	0.02	0.09	0.32
		416	0.64	0.69	0.63	0.38	0.40	0.45	0.25
		421	0.00	0.00	0.00	0.02	0.01	0.02	0.00

Table A.16 Protocol for eukaryotic qPCR as used in Fig. 13

Scheme for one 50 µl PCR reaction:		**Thermal profile:**	
Nuclease free H_2O	37.15 µl	Denaturation 94 °C	5 min.
10x PCR-Buffer	5 µl		
25 mM $MgCl_2$	3 µl	45 cycles:	
20 µg/µl BSA	0.5 µl	94 °C	30 sec.
10 mM dNTPs	0.5 µl	55 °C	30 sec.
1/500 SybrGreen	0.25 µl	70 °C	30 sec.
1/500 ROX Dye	0.75 µl		
50 µM f-primer.	0.3 µl	Final denaturation 94°C	1 min
50 µM r-primer	0.3 µl	Reassociation 55°C	30 sec.
5 U/µl MBI Taq	0.25 µl	Dissociations ramp 55-95°C	30 min.

Curriculum vitae

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Euringer, K and T. Lueders. Depth-resolved molecular analysis of protistan communities through the redox zones of a BTEX contaminated aquifer. *FEMS Microbiol Ecol* (in preparation)

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Jousset, A., Euringer, K., Lueders T., Bonkowski, M. and S. Scheu. Stable isotope probing of protists differentially feeding on grazing-susceptible and grazing-resistant bacteria in soil. *FEMS Microbiol Ecol* (in preparation)

Selected contributions to national and international scientific meetings:

Euringer K. and T. Lueders. Beginning a molecular characterization of protozoan communities in aquifers. Munich Environmental Microbiology Meeting 2005, 29.11.2005, Neuherberg (Poster presentation)

Euringer K. and T. Lueders. Depth-resolved molecular profile of protozoan communties through a contaminant plume. DGP- Jahrestagung 08. -11.03.2006, Liebenwalde (Poster presentation)

Euringer K. and T. Lueders. Characterization of protozoan communties in pristine and contaminated aquifers. VAAM Jahrestagung 19.-22.03.2006, Jena (Poster presentation)

Euringer K. and T. Lueders. Comparative depth-resolved assessment of protozoan communities in a BTEX contaminated aquifer. VAAM Jahrestagung 01.-04.04.2007, Osnabrück (Poster presentation)

Euringer K. and T. Lueders. Depth-resolved molecular analysis of protozoan communities through the redox zones of a BTEX contaminated aquifer. 10th Symposium on Aquatic Microbial Ecology, 02.-07.09.2007 in Faro, Portugal (Poster presentation)

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