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PhyloTrap - Separate Capture of Bacterial and Fungal Small Subunit Ribosomal RNAs by Magnetic Beads

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Abstract

Linking microbial diversity to function is still a challenging task for ecologists, although molecular methods have been vastly improved over the last two decades. Soil environments are generally defined by a high microbial diversity and serve as habitats for globally important communities, driving biogeochemical processes. Especially for nutrient acquisition pathways we are in need of improved tools to investigate how these complex systems function and how we might be able to alter and in uence them by changing abiotic factors.

In this study we aimed to develop a method to specifically separate bacterial and fungal small subunit ribosomal RNA out of a RNA sample by DNA/RNA hybridization combined with a magnetic particle capture approach. Therefore DNA-probes of specific sequences (Bac338 and Euk1379) were tested on capture e ciency (fraction of regained rRNA) and specificity (capturing the target molecule) using RNA from pure-cultures and soil.

Altering the length of Euk1379 in combination with a T-linker sequence increased the capture e ciency 6-fold from 6.6 to 41.5%. Length variation showed no in uence on capture e ciency of Bac338 (20% for all tested probes). Both probes showed specificity in tests with pooled RNA samples from pure-cultures. Bac338 successfully captured SSU rRNA of expected size out of the soil RNA sample, whereas results with Euk1379 were not clear and need further investigation.

We consider the results of this study as very promising in regard of the applicability of the aimed method, since an increase in e ciency for the fungal RNA pool will allow us to obtain su cient material for further downstream analysis steps. This method in combination with stable isotope probing will lead to new insights in nitrogen and carbon assimilation pathways of soil fungal and bacterial communities.

Zusammenfassung

Immer noch stellt das Erforschen des Zusammenhangs zwischen der Diversität von mikrobiellen Gemeinschaften und deren Funktion und Auswirkungen auf unsere Umwelt eine Herausforderung für Ökologen dar, trotzdem sich die molekularbiologischen Methoden in den letzten zwei Dekaden wesentlich verbessert haben. Böden weisen generell eine hohe Diversität von Mikroben auf, welche globale Substratkreisläufe antreiben, sind aber auch wegen ihrer Komplexität ein schwierig zu untersuchendes Habitat. Speziell für nährsto assimilierende Prozesse brauchen wir verbesserte Methoden um die Funktionsweise dieser Systeme zu verstehen, und sie, wenn nötig, gezielt zu beein ussen.

Diese Arbeit zielt darauf ab eine Methode zu entwickeln welche spezifisch bakterielle und pilzliche ribosomale RNS der kleinen Untereinheit des Ribosoms getrennt voneinander aus einer komplexen RNS Probe isoliert. Zu diesem Zweck wurde eine DNS/RNS Hybridisierung mit einer Isolierungsmethode, welche magnetische Nanopartikel nutzt, kombiniert. DNS-Sonden mit spezifischen Sequenzen (Bac338 und Euk1379) wurden auf E zienz (Fraktion der wiedergewonnen rRNS) und Spezifizität (Isolierung des Zielmoleküls) getestet, wobei RNS von Reinkulturen sowie von Bodenproben verwendet wurden.

Eine Verlängerung von Euk1379 führte zu einer 6-fachen Steigerung der E zienz von 6.6 auf 41.5%. Eine Änderung der Länge hatte keinen Ein uss auf die E zienz von Bac338 (20% für alle getesteten Sonden). Mit beiden Sonden konnte spezifisch rRNS aus einer Mischung von RNS aus Reinkulturen gewonnen werden. Mit Bac338 konnte erfolgreich bakterielle rRNS aus einer Bodenprobe isoliert werden. Die Ergebnisse mit Euk1379 in Bodenproben sind nicht eindeutig und bedürfen weiterer Tests.

Die hier präsentierten Ergebnisse sind als vielversprechend einzustufen im Hinblick auf die Anwendbarkeit der angestrebten Methode. Eine Steigerung bei der Isolierung von

pilzlicher rRNS stellt einen entscheidenden Schritt dar, da die Bereitstellung genügend RNS-Materials für die weiteren Analysenmethoden entscheidend für den Erfolg ist. Die angestrebte Methode in Kombination mit stable-isotope-probing (SIP) wird uns helfen wissenschaftliche Fragestellungen zu beantworten, die die Sticksto -, Kohlensto - und Phosphatassimilationswege von pilzlichen und bakteriellen Gemeinschaften in Böden umfassen.

Contents

1 Introduction

Microbial life claims a big part of the whole biomass on earth [Kallmeyer et al., 2012] [Whitman et al., 1998]. These communities drive ecosystem functioning, carry out key processes in global nutrient cycles and in last consequence define the world as we know it [Baldrian et al., 2013]. Diversity of these communities, living in almost every possible habitat imaginable, extends diversity levels of higher life in orders of magnitude, but only one percent can be cultured and described in laboratories with conventional microbiological methods [Dorofeev et al., 2014].

Non-culture based methods allowed a exploration of microbial community structures on a high resolution level. [Foster et al., 2012] [Amann and Ludwig, 2000] [Torsvik et al., 1990]. Soil environments were found to inhabit an incredible high number of microorganisms (up to 10^9 per gram of soil) and show the highest diversities of microbial communities compared to other environments [Delmont et al., 2011]. These methods and the co-evolution of sequencing technologies also changed the view on microbial taxonomy. Was the classification of microorganisms in the pre-molecular era based on morphological descriptions and characteristics of the organisms, we are looking nowadays on sequence similarities of well conserved so called "phylogenetic marker genes". Without a doubt the small subunit (SSU) ribosomal RNA coding gene, also called 16S gene, is the most used phylogenetic marker for prokaryotic organisms with the most advanced databases available [Quast et al., 2013] [DeSantis et al., 2006] [Breitbart et al., 2007]. For fungal communities the internal transcribed spacer region (ITS region), localized between the highly conserved coding regions for ribosomal RNAs, are used as phylogenetic markers beside 18S sequences (SSU rRNA genes of eukaryotic organisms), because a higher resolution can be achieved comparing sequence similarity between these regions [Kõljalg et al., 2013] [Ihrmark et al., 2012]. To classify other eukaryotic organisms phylogenetically, the main marker used today is the 18S rRNA gene.

Studying these phylogenetic markers gives us information about changes in community structures and diversities triggered by changes of environmental factors. Linking this information with ecosystem function helps us to understand complex systems and define key players and factors which control these [Song et al., 2014]. This knowledge allows us to develop prediction models e.g for global warming scenarios or optimize nutrient application in agricultural practice to lower environmental impacts [Blagodatsky and Smith, 2012].

1.0.1 Phylogenetic Marker Genes

1.0.1.1 Prokaryotic Marker Sequences

In order to investigate microbial communities in divers environments using non-culture based approaches, we are in the need to have sequences present in the studied organisms which allow us to interpret phylogenetic distance and evolutionary relationships between them. Ribosomes, complex and abundant structures translating the genetic code of cells to new proteins, represent one of the most essential parts of all living cells we know. The subunits of these complexes consist beside of peptide chains also of RNA molecules. Due to their critical role in the production of peptide chains, these RNA molecules are present in high numbers in active cells. Additionally many sections on these RNA molecules possess highly conserved sequences [Wang et al., 2014] [Janssen, 2006]. These properties combined made them to the most used phylogenetic markers nowadays, and led to the development of taxon-specific primers and probes (Figure 1.1). Although these rRNA sequences are present in the small subunit (SSU) as well as in the large subunit (LSU) of ribosomes, SSU rRNA sequences gained acceptance over the LSU once, probably due to the higher sequencing costs of a longer sequence compared to the smaller one. Nevertheless, it's claimed that databases of SSU rRNA sequences for bacteria reach completeness at the end of this decade [Yarza et al., 2014], mirroring that 16S investigations became a standard method in microbial ecology studies a long time ago and also the advance of next generation sequencing technologies. Not surprisingly also for archeal communities the 16S gene is the most used phylogenetic marker.

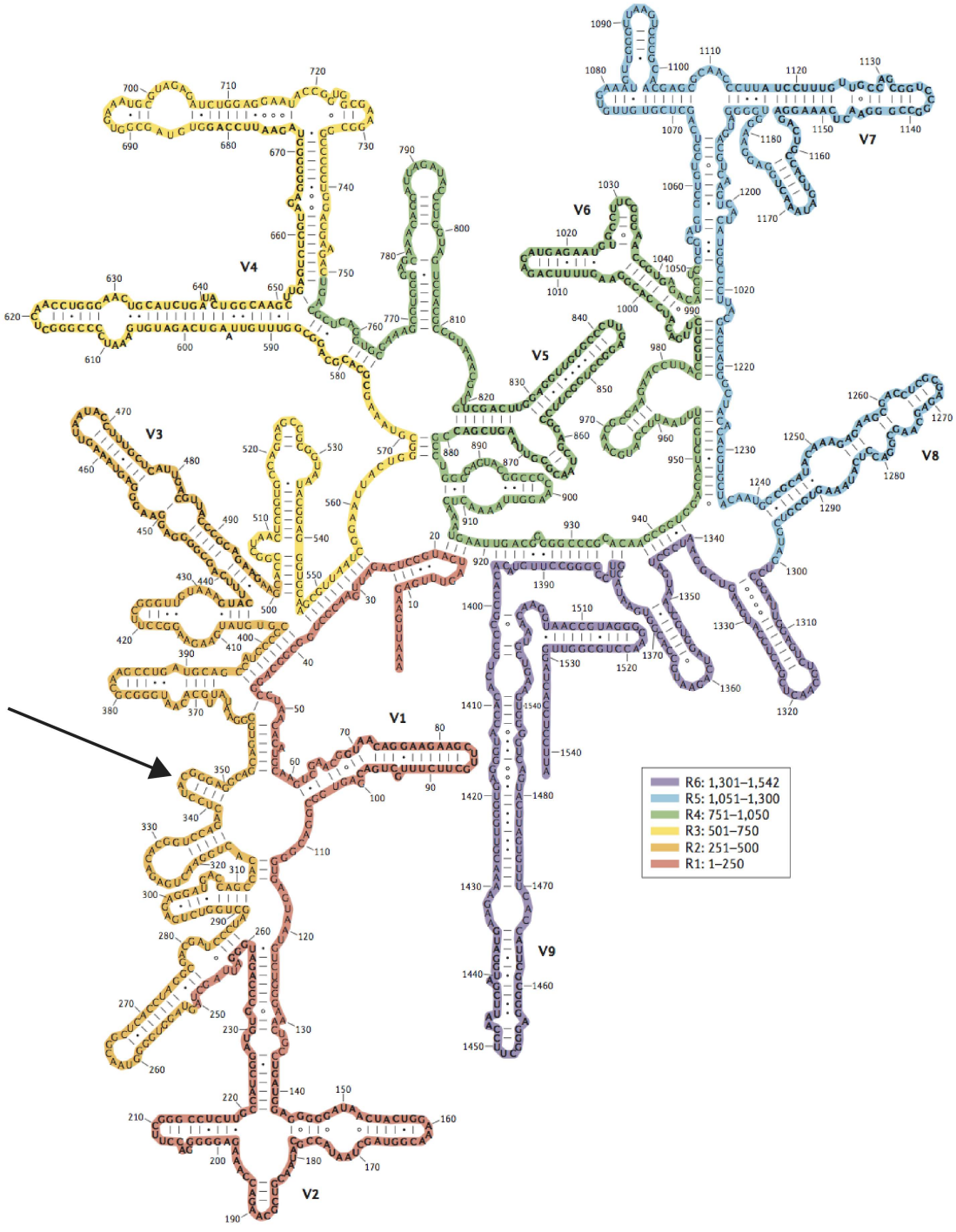

Figure 1.1 Secondary structure of SSU rRNA of *Escherichia coli* [Yarza et al., 2014]. Arrow indicates position of target sequences from the probe used in this study.

1.0.1.2 Eukaryotic Marker Sequences

The analogous SSU rRNA sequence in eukaryotic organisms (18S rRNA) has also evolved to a widely used phylogenetic marker. For fungal communities also the ITS region (Figure 1.2) is used to determine phylogenetic relationships, providing higher resolution down to species level [Hibbett et al., 2011] [Bellemain et al., 2010]. Currently the UNITE database harbors 442 706 fungal ITS sequences and distinguishes between 53 891 different fungal species based on a 98.5% sequence similarity threshold ([Kõljalg et al., 2013]; https://unite.ut.ee). A major drawback of using ITS sequences is the limited use in studies targeting active microbial communities, since these regions are cut-out during transcription from genomic DNA to get functional ribosomal RNA. Although it was shown that active fungal communities can be targeted by quantifying pre-curser rRNA molecules which still contain ITS regions [Anderson and Parkin, 2007], 18S sequences are still the most investigated markers when it comes to identify community members which participate in processes of interest. Additionally, information derived from fungal 18S rRNA sequences can be used to calculate phylogenetic relationships between the organisms in ones sample, which can not be achieved with ITS sequences [Lie et al., 2014].

1.0.2 DNA-Hybridization

DNA-based hybridization techniques are all based on the complementary coded structure of the molecule, favoring Adenin-Thymin and Guanin-Cytosin bonds. Thus a specific se-

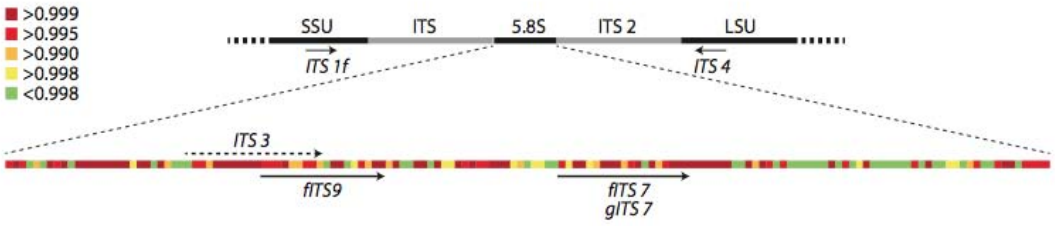

Figure 1.2 Fungal ITS regions. Arrows indicate position of primers on conserved sites on the ribosomal rRNA genes anking the two ITS regions, thus amplifying ITS1, ITS2 or both regions combined for subsequent phylogenetic classification of organisms [Ihrmark et al., 2012].

quence on a single DNA strand (probe) can be used to search it's complementary sequence out of a pool of DNA and bind to it comparable to a key-lock principle. This highly specific property of DNA molecules was used for many methods established in molecular biology, from plotting DNA on membranes and stain them to primer annealing in all kinds of PCR reactions to the design of micro-array chips just to name a few [Amann et al., 1990] [He et al., 2007] [Amann et al., 2001]. For many of these hybridization techniques also RNAs can serve as the targeted molecule. In this study a DNA/RNA hybridization was performed using DNA oligonucleotide probes to target ribosomal RNAs. This was crucial since a translation of the RNAs to cDNA would not be compatible with our goal to use the method in stable isotope probing approaches. Apart from ordering

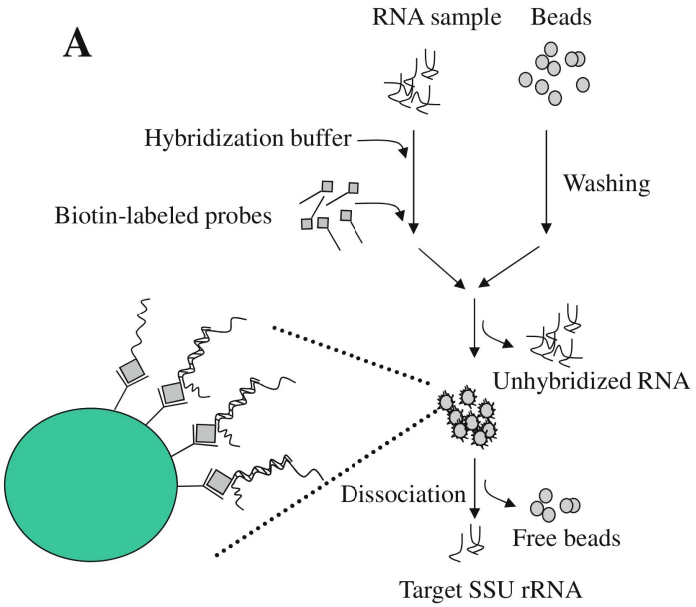

Figure 1.3 Schematic overview on the magnetic bead capture approach used in this study (adapted from [Abed, 2012]). Biotin-labelled probes and RNA are hybridized before adding the steptavidin-coated magentic particles. After separating the captured molecules by applying a magnetic force to retain the particles in the assay, the target molecules are released again by heating the sample.

oligonucleotides which consist of the sequences of interest at companies, many kinds of special modifications on the 3' and 5' end of the probe are available nowadays to add special features for the experiment performed. In this study a biotin molecule was linked at the 5' end of our probes to make use of the highly specific, non-covalently bond between

biotin and streptavidin [Dundas et al., 2013]. By adding streptavidin coated magnetic nano-particles to our hybridization reaction it was possible to retain added probes and bound (hybridized) RNA molecules by applying a magnetic force to the reaction tubes. Similar approaches were used in many studies due to the fast and easy handling to specifically isolate DNA or RNA molecules of interest (Figure 1.3).

1.0.3 Stable Isotope Probing (SIP) in Microbial Ecology

Stable isotopes of elements have the same number of protons but di er in the number of neutrons. Consequently these atoms have the same physico-chemical properties but we can distinguish them by their mass. Naturally occurring ratios of these isotopes are used to get information of the origin of ones sample, since specific ratios can be linked to specific processes or environments [Snider et al., 2015] [Yin et al., 2010]. Stable Isotope Probing or SIP stands in contrast to that as in this approach stable heavier isotopes (e.g. C^{13} or N^{15}) are added artificially within an experimental setup. By subsequent determination of isotope ratios one can track the pathway of these atoms through the complex degradation and transformation network of microbial communities [Radajewski et al., 2000].

The ratio of the heavier isotope over the normal occurring isotope (isotope ratio) in ones sample is determined by mass spectrometric analysis technologies mainly di ering in precision [Abraham, 2014]. Information about this isotopic ratio combined with the identification of the compound allows us then to follow a substrate through their fate in the metabolism of organisms [Chokkathukalam et al., 2014].

More advanced approaches using stable isotopes became very important in microbial ecology recently. Microbes which utilize the above mentioned substrates as e.g. a carbon or nitrogen source will incorporate these stable heavier isotopes also in their biomass. Subsequent isolating and analysis of phylogenetic biomarkers casts light on which members are active in the community and participate on the process of interest. Several methods to identify target communities after addition of an isotope labeled substrate have been developed and are summarized in figure 1.4 [Evershed et al., 2006] [Friedrich, 2005] [Musat et al., 2008] [Pawelczyk et al., 2011].

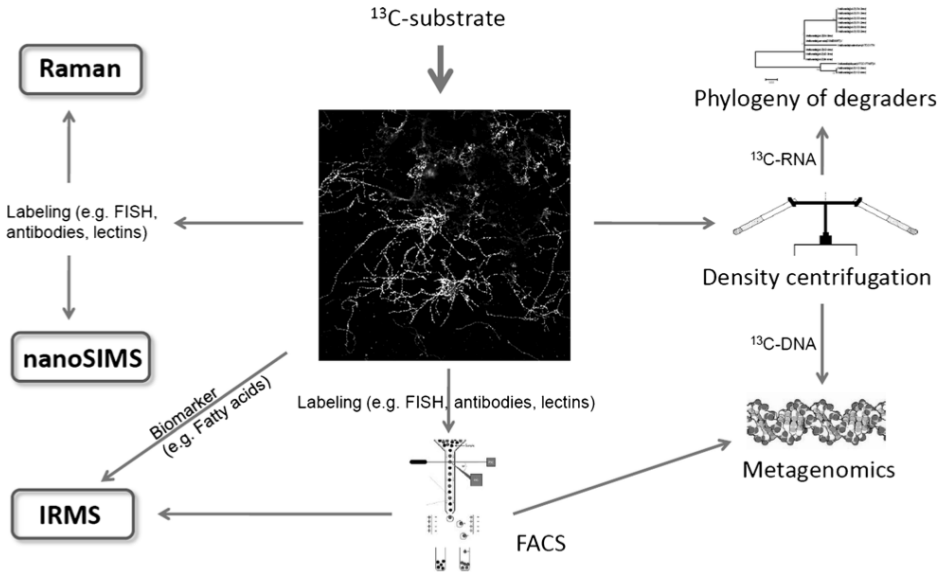

Figure 1.4 Overview of SIP-based methods in microbial ecology (adapted from Abraham [Abraham, 2014])

This study approaches the analysis of ribosomal rRNA after a domain specific capturing similar to a study of MacGregor et al. [MacGregor et al., 2002]. Density gradient centrifugation methods were developed to separate the light and heavy fraction of RNA or DNA molecules. This method can be realized by using C^{13} isotopes due to high incorporation rates of carbon in nucleic acids. In contrast to that our method would allow us to track other nutrient elements which are incorporated in rRNA like nitrogen or phosphor. By first separating the RNA pool via hybridization and subsequent isotope ratio determination we will be able to link nutrient uptake and phylogenetic information. Nevertheless it should be mentioned, that the phylogenetic resolution might be better by applying a density gradient approach under certain circumstances. Figure 1.5 gives an overview on the working principle and di erent steps of the PhyloTrap.

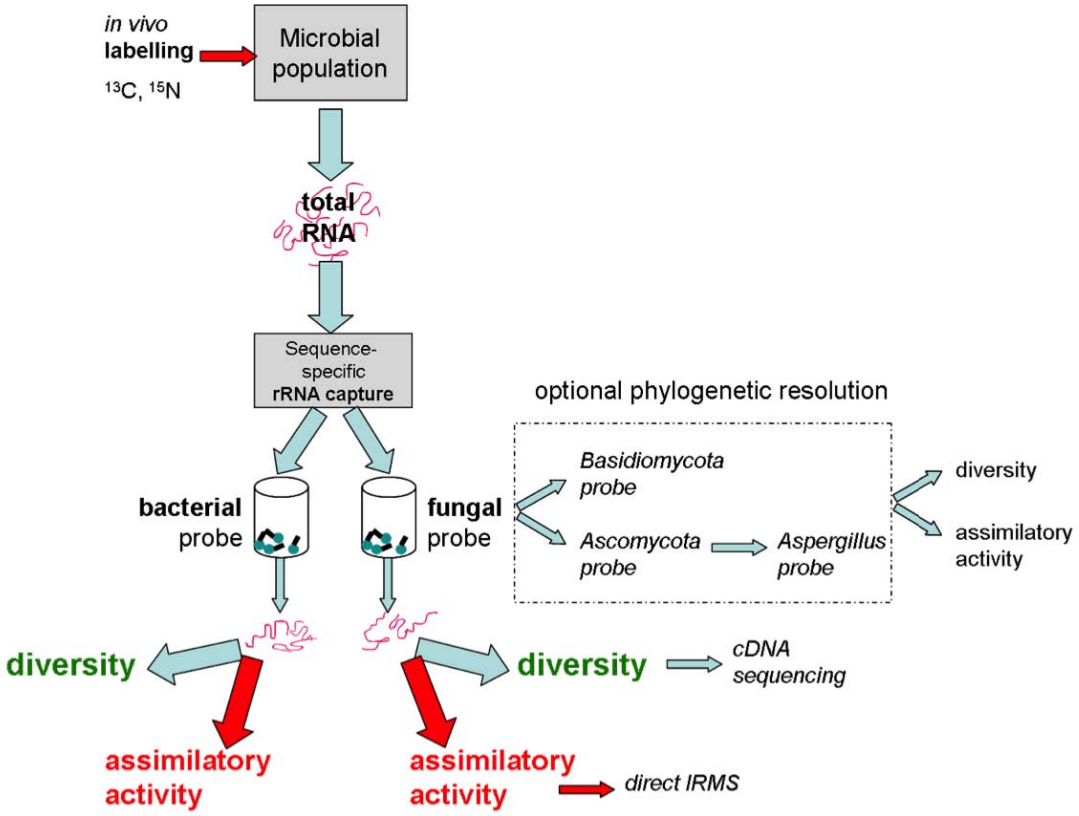

Figure 1.5 Overview of single steps in the PhyloTrap approach. Instead of a density gradient centrifugation step this approach separates the RNA sample by hybridization with phylogenetic specific probes. Subsequently information on diversity (cDNA sequencing) and activity in assimilation (direct IRMS) can be achieved. Figure created by Sandra Moll.

2 Aim of Work

In this study we aimed to develop a method to specifically capture small-subunit ribosomal RNA molecules from RNA mixtures according to their bacterial and fungal origin using DNA/RNA hybridization combined with a magnetic particle capturing approach. First steps towards this goal included testing the probes on performance in pure-culture RNA and the optimization of hybridization conditions in the assay.

Dividing specifically ones pool of rRNA on domain level has the potential to be a standard method in microbial ecology studies in the future. Especially in combination with a stable isotopic probing approach we would be able to answer questions of high importance regarding community function in complex environments. While we are able nowadays to separate C^{13}-labelled nuclear acids by density gradient centrifugation and analyse communities subsequently by a sequencing approach, similar experiments with stable isotopes from di erent elements are still di cult to realize.

By first isolating the fungal and bacterial pool of environmental RNA sample and direct measurement of the isotopic ratio in these pools we could target e.g. processes in nitrogen- or phosphor-cycling communities. In combination with the sequencing of these samples we could add one dimension of information to these studies. Finally, by the further development of this approach to specifically target organism groups of lower phylogenetic levels we could track the fate of elements through microbial communities, which would represent a big step in the development of microbial ecology based research.

3 Material & Methods

All worksteps were carried out in regard to avoid RNA degradation. This included working on ice or cool temperatures whenever possible and the use of DEPC treated H_2O (DEPC-H_2O) in all solutions and reactions. To avoid contamination with RNases from autoclaved pipette tips only tips from a fresh opened bag were used and put in DEPC treated tip boxes using unused disposable gloves. For hybridization, capture reactions and storage of RNA, Eppendorf LoBind© reaction tubes were used to avoid loss of RNA due to interactions with the polymer surface of tubes. All measurements of RNA concentrations were performed using the Quant-iT^{TM} RiboGreen© RNA Assay Kit (Invitrogen^{TM}) according to the manual in a microtiter plate reader (Enspire 2300 Multilabel Reader).

3.1 RNA Isolation

3.1.1 Fungal RNA Isolation

Doratomyces spores were harvested from malt extract medium (ME-medium) by washing the fungus with PBS-Tween bu er and counted under the microscope. 100 mL of ME-Broth was inoculated with the spore suspension resulting in a spore concentration of 10^6 per mL culture media. The culture was inoculated over night in an erlenmeyer ask at 30°C on a rotary shaker at 160 rpm. Grown mycelia was filtered through a sterile filter tissue and dried by pressing in-between sterile filter paper. Dried mycelia was wrapped in sterile aluminum foil and put into liquid nitrogen for subsequent RNA isolation and storage at -80°C.

Frozen mycelia was put into a pre-cooled mortar and grinded to a fine frozen powder.

Avoiding thawing of the powder, approximately 200 mg were transferred in a pre-cooled 2.0 mL reaction tube. 1 mL of peqGOLD TriFast^{TM} (peqlab) was added and suspension was vortexed for 10 sec. After incubation at room temperature (RT) for 5 min 0.2 mL of chloroform was added and mix was vortexed. Another incubation at RT for 5 min was followed by a centrifugation at 12 000 g for 10 min at 4°C. RNA in the clear hydrophillic phase on top of the three phase mixture was transferred in a new RNAse-free reaction tube. For RNA precipitation 0.5 mL isopropanol was added subsequently mixed and incubated on ice for 10 min. Centrifugation at 12 000 g for 10 min at 4°C resulted in a RNA pellet which was washed 2 times with 75% ethanol by vortexing and centrifugation at the same parameters as described before. The pellet was dried under sterile conditions for 5 min and finally diluted in DEPC-H_2O.

To remove co-extracted genomic DNA, a DNA digestion step was performed using the DNase I, RNase-free endonuclease (Thermo Scientific) according to the manual (Removal of Genomic DNA from RNA Preparations). To avoid hydrolysation of the RNA the heat inactivation step of the enzyme was replaced by a additional cleaning step using the RNeasy MiniElute Cleanup Kit (QUIAGEN). This cleaning step was performed according to the manual except that elution of RNA from the silica gel matrix was performed in 3 steps to maximize the yield of cleaned RNA (e.g. 3 x 16,6 μL to elute in 50 μL). Still present phenols from the isolation step as well as tRNAs and small mRNA were removed from the RNA in this step.

This RNA isolation procedure and cleaning steps resulted in high quality RNA mainly consisting of rRNA. Quality of the RNA was controlled by gel electrophoreses resulting in two bands at 1.8 and 3.3 kb representing the small and large subunit ribosomal RNA respectively and no smear on the gel. Concentrations of RNA were measured with the Quant-iT^{TM} RiboGreen© RNA Assay Kit (Invitrogen^{TM}) according to the manual in a microtiter plate reader (Enspire 2300 Multilabel Reader) subsequently aliquoted and stored at -20°C for further use.

3.1.2 Bacterial RNA Isolation

Raoultella terrigena was grown as pure culture in TB broth over night at 37°C on a rotary shaker (200 rpm). On the next day 20 mL of TB broth was transferred under sterile conditions in a 50 mL Greiner reaction tube, inoculated with 1 mL of the over night culture and incubated on a rotary shaker at 200 rpm at 37°C. The cultures were grown to a OD_{600} of 0.8, put on ice and subsequently spun down in a pre-cooled centrifuge at 5 500 rpm for 5 min at 4°C. The pellet was resuspended in 2 mL peqGOLD TriFast^{TM} and 2 times 1 mL of the suspension was transferred in two new 2 mL reaction tubes to perform the RNA isolation according to the manual of peqlab for bacterial cultures. Further downstream steps to purify, quantify and quality check of RNA were performed as described in 3.1.1 for fungal RNA.

3.2 Bacterial and Fungal Specific Probe Design

The probes tested in this study were based on already available probe sequences specific to bacterial and eukaryotic SSU rRNA molecules (Bac338 and EUK1379, [Amann et al., 1990] and [Hicks, 1992] respectively). Euk1379 targets eukaryotic organisms in general, but is also specific to fungal sequences. Nevertheless, since in the environments we aimed to study mainly by bacteria and fungi are present, we could make use of this general sequence for our purposes. To avoid confusion, probe Bac338 will be named further "bacterial" probe and Euk1379 "fungal" probe. The core sequences of the two probes were extended by on base pair on both ends to determine the e ect of sequence elongation on capture e ciency. Additionally a T-linker sequence was added to the probes to enhance hybridization performance due to sterical hindrance. A Biotin molecule was linked to the T-linker sequence, so they could bind to the streptavidin coated beads in the assay. Figure 3.1 gives an overview in probe design, all probes were ordered from Thermo Fisher Scientific GmbH.

Eukaryote probes

	Euk1379	nt	%GC	Tm
GCGACGGGCGGTGTG	**TACAAAGGGCAGGGAC**	GTAATC		
	TACAAAGGGCAGGGAC	16	56.2	57.7
G	**TACAAAGGGCAGGGAC**	G 18	61.1	63.6
TG	**TACAAAGGGCAGGGAC**	GT 20	55.0	67.0
GTG	**TACAAAGGGCAGGGAC**	GTA 22	54.6	67.2
TGTG	**TACAAAGGGCAGGGAC**	GTAA 24	50.0	70.3

Name	Probe
Euk1379	Biotin-TACAAAGGGCAGGGAC
Euk1379-TL	Biotin-TTTTTTACAAAGGGCAGGGAC
Euk1379+2-TL	Biotin-TTTTTGTACAAAGGGCAGGGACG
Euk1379+4-TL	Biotin-TTTTTTGTACAAAGGGCAGGGACGT
Euk1379+6-TL	Biotin-TTTTTGTGTACAAAGGGCAGGGACGTA
Euk1379+8-TL	Biotin-TTTTTTGTGTACAAAGGGCAGGGACGTAA

Prokaryote probes

	Bac338			
SCAANATTCCCCACT	**GCTGCCTCCCGTAGGAGT**	CTGGNCCG		
	GCTGCCTCCCGTAGGAGT	18	66.7	64.9
T	**GCTGCCTCCCGTAGGAGT**	C 20	65.0	69.2
CT	**GCTGCCTCCCGTAGGAGT**	CT 22	63.6	70.1
ACT	**GCTGCCTCCCGTAGGAGT**	CTG 24	62.5	72.6
CACT	**GCTGCCTCCCGTAGGAGT**	CTGG 26	65.4	76.8

Name	Probe
Bact338	Biotin-GCTGCCTCCCGTAGGAGT
Bact338-TL	Biotin-TTTTTGCTGCCTCCCGTAGGAGT
Bact338+2-TL	Biotin-TTTTTTGCTGCCTCCCGTAGGAGTC
Bact338+4-TL	Biotin-TTTTTCTGCTGCCTCCCGTAGGAGTCT
Bact338+6-TL	Biotin-TTTTTACTGCTGCCTCCCGTAGGAGTCTG
Bact338+8-TL	Biotin-TTTTTCACTGCTGCCTCCCGTAGGAGTCTGG

Figure 3.1 Overview on probe sequences used in this study.

3.3 SSU rRNA Capture

3.3.1 Hybridization of Bacterial and Fungal Specific Probes with SSU rRNA

For hybridization assay 1 to 2 μg of RNA was used, hybridization bu er and the biotin-labelled probe (12.5 to 25 pmol = 12 to 14 times (bacterial and fungal probe respectively) the molar concentration of the targeted SSU rRNA) was added in a 1.5 mL reaction tube (Eppendorf LoBind©). Endvolume of the reaction was 50 μL with following assay concentrations: 5x SSC; 0.1% N- laurylsarcosine; 0.1% NaCl; 0.02% SDS. To guarantee that SDS is not precipitated, hybridization bu er stock was aliquoted in 1.5 mL reaction tubes and put on a thermal shaker block (Eppendorf Thermomixer© compact) at 70°C for 5 min with no shaking before hybridization assay was set up.
The assay was mixed gently by pipetting and spun down before it was put on the thermal shaker block at 70°C for 10 min at 450 rpm. Thermal shaker was set to RT and shaking was continued at 450 rpm over night for hybridization of the probes and SSU rRNA.

3.3.2 Capture of Hybridized Probes with Magnetic Beads

After hybridization reaction over night the assays were spun down to collect condensed liquid from the reaction tube cap and put on ice. The volume of streptavidin-coated magnetic beads (Dynabeads© M-280 Streptavidin from Invitrogen^{TM}) used per reaction was calculated with the specific binding capacity of the beads (0.2 pmol per μg beads according to the distributor). A 4 times higher binding capacity of the beads than the molar amount of probe used was chosen. Beads were washed by gentle up and down pipetting and in-between collection with a magnetic particle collector using the following steps: 2 times solution A using 2 times the volume of used beads; 1 time with solution B using 2 times the volume of used beads; 2 time 0.5 x SSC bu er using 2 times the volume of used beads. Washed beads were resuspended in 0.1% blocking solution (Roche Applied Science) and put on the thermal shaker block for 1 hour at RT. Shaking speed was adjusted that beads did not settle down in the reaction tube depending on the volume. After blocking reaction the beads were washed with 0.5 x SSC bu er (2 times the volume

of used beads) and finally resuspended in 0.5 x SSC bu er using the original volume of beads calculated.

Washed and blocked beads were added to the hybridization mix and put back on the thermal shaker block at RT for 2 hours. Shaking speed was adjusted that beads did not settle down in the reaction tube (1150 rpm for 75 μL reaction). Reaction tubes were put back on the magnet and unhybridized RNA was transferred to a new reaction tube. For further analysis these unhybridized RNA samples were cleaned from bu er salts and reaction components using a size exclusion chromatography step by performing the protocol of Chroma Spin^{TM} 400 mini spin colums (Clonetech). Too high bu er concentrations prevent analysis with the electrophoresis based Agilent Bioanalyzer system due to increased conductivity of the sample. Unhybridized RNA samples were stored at -20°C for further analysis and use. Beads were washed 3 times with 7.5 x SSC bu er (4 times the volume of used beads) and collected in-between on the magnetic particle collector.

For elution of the captured SSU rRNA 50 μL of ultra-pure water (Promega Nuclease free water) was added to the beads and tubes were put on thermal shaker block for 2 min at 60°C and 70°C for fungal and bacterial probe respectively at 1150 rpm. After proper settling of beads towards the magnet the RNA was transferred with the clear supernatant to a new reaction tube avoiding the take up of beads. Eluted RNA could be directly used for quantification measurements and analysis with Agilent Bioanalyzer.

3.4 Analysis of RNA Samples on Agilent Bioanalyzer System

To ensure the capture of the domain specific SSU rRNAs samples were analyzed using RNA 6000 pico chips (Agilent) and run on the Bioanalyzer system according to the manual. Electropherograms were exported from the 2100 expert software. In this system a capillary electrophoreses approach on the RNA sample provides a separation of RNA molecules according to their length in a very high resolution, allowing the identification of bacterial and fungal SSU rRNA molecules in the same sample. Also peak area in the electropherograms is correlated to the concentration of the RNA molecule of a specific length similar to a chromatographic approach. Whereas absolute quantification of in-

dividual RNA molecules are di cult to realize with this method, a relative change in concentration can be easily observed by comparing peak heights and areas derived from the same chip.

3.5 Buffers, Media & Solutions

10 % N-laurylsarcosine	3 g N-laurylsarcosine fill up with 30 mL DEPC-H_2O
10 % NaCl	10 g NaCl fill up to 100 mL with H_2O add 100 μL DEPC stir for 2 h at RT autoclave (121°C; 20 min)
10 % Sodium-dodecyl-sulfate	3 g SDS fill up to 30 mL with DEPC-H_2O
10 % Blocking solution stock	10 % blocking reagent (w/v) dissolve in maleic acid bu er autoclave (121°C; 20 min)
Hybridization bu er stock	50 mL of 20x SSC 2 mL of 10 % N-laurylsarcosine 2 mL of 10 % NaCl 400 μL of 10 % SDS
Maleic acid bu er	maleic acid (100 mM) NaCl (150 mM) adjust pH with conc. NaOH
RNA loading bu er	50 % Glycerin EDTA 10 mM 0.001 % Bromphenol Blue
Solution A	0.8 g NaOH (0.1 M) 0.584 g NaCl (0.05 M) fill up to 200 mL with H_2O add 200 μL DEPC and stir 2 h at RT autoclave (121°C; 20 min)
Solution B	1.169 g NaCl (0.1 M)

	fill up to 200 mL with H_2O
	add 200 μL DEPC and stir 2 h at RT
	autoclave (121°C; 20 min)
SSC bu er (20x)	35.06 g NaCl (3 M)
	17.64 g Na-citrate (0.3 M)
	fill up to 200 mL with H_2O
	set pH with conc. NaOH
	add 200 μL DEPC and stir 2 h at RT
	autoclave (121°C; 20 min)
TAE bu er (50x)	242 g Tris base
	dissolve in 750 mL H_2O
	add 57.1 mL acetic acid
	add 100 mL of 0.5 M EDTA
	fill up to 1000 mL with H_2O
	adjust pH to 8.5
TAE bu er (1x) for gels	20 mL TAE bu er (50x)
	fill up to 1000 mL with H_2O
Agarose gel 0.7% (GelRed 1:20000)	3.5 g agarose
	fill up to 500 mL with TAE bu er (1x)
	melt in microwave
	let cool down - add 25 μL of GelRed
PBS-Tween-Bu er	
ME-medium	

3.6 Reagents & Equipment

Reagent/Substance	**Distributor**
Agarose	
Blocking reagent	Roche
Bromphenol blue	
DEPC	Carl Roth
Dynabeads© M-280 Streptavidin	Invitrogen^{TM}
EDTA	Carl Roth
Ethanol	
Formamide	Carl Roth
GelRed^{TM}	Biotium
Glycerin	Carl Roth
Isopropanol	
Maleic acid	
Na-Citrate	Carl Roth
NaCl	Carl Roth
Na_2HPO_4	
NaOH	Carl Roth
N-laurylsarcosine	Merck
Oligonucleotides	Thermo Fisher Scientific
Quant-iT^{TM} RiboGreen© RNA Assay Kit	Invitrogen^{TM}
Sodium dodecyl sulfate	
Tris	Carl Roth
Trizol© Reagent	Invitrogen^{TM}
Yeast tRNA	Invitrogen^{TM}

4 Results

4.1 Determination of Optimal Probe-Length

Preliminary experiments showed specificity of the probe sequences, but the capture efficiency was low especially for Euk1379. By testing di erent lengths of probe sequences on the ability to capture the SSU rRNA out of a fungal or bacterial RNA, we determined optimal probe length for further experiments. Capture e ciency was calculated by dividing the amount of RNA determined in the eluted sample by the amount of SSU rRNA added in the hybridization assay. All probes tested had a linker sequence of 5 successive T-bases on the 5'-end of the probe sequence, since preliminary data showed minor performance of probes without a linker sequence.

4.1.1 Fungal Probes

The continuous addition of bases at both sides of the initial used fungal specific probe sequence (Euk1379) increased the capture e ciency 6-fold (Figure 4.1).The initial amount of SSU rRNA added from *Doratomyces* to these assays was 260 to 520 ng ($\sim$ 1 to 2 μg total RNA). Using probe Euk1379-TL (16 bp) only 6,6% of the added SSU rRNA could be recovered. By adding only one base-pair at the 3' and 5' end of the probe sequence, we could increase the capture e ciency 3-fold to 26.4%. Best results could be obtained by adding 3 bp at both ends of the probe, leading to a recovery of 41.5% of the initial used SSU rRNA in the assay. A further elongation of probe length had no e ect on capture e ciency, thus the optimal probe length under these conditions was 22 bp (Euk1379+6-TL).

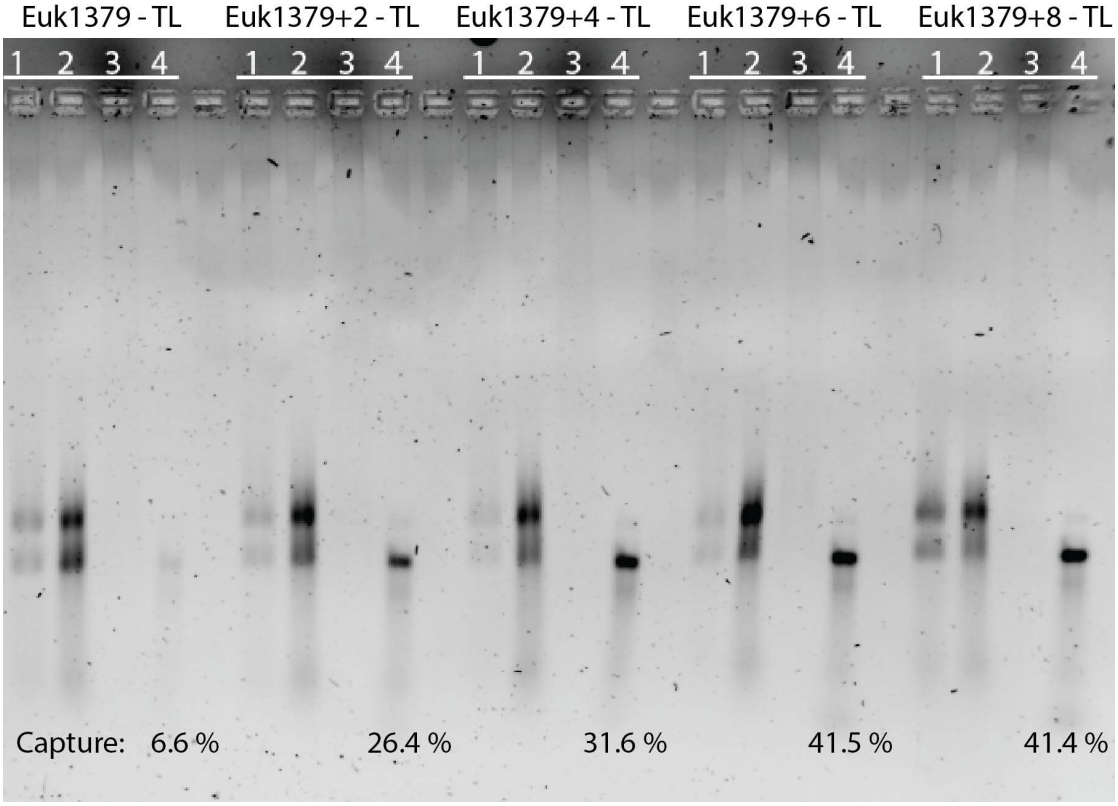

Figure 4.1 Capture of SSU rRNA from pure-culture RNA (*Doratomyces*) using domain speci c probes of di erent length on 0.7% agarose gel stained with GelRed^{TM}. Used probes are indicated in the graph. For each hybridization reaction slots represent as follows: (1) fungal RNA not used in hybridization reaction; (2) unhybridized fungal RNA from hybridization reaction; (3) washing step of hybridization reaction; (4) eluted RNA after bead capture. Percentage of RNA captured was calculated from values of RNA concentration measurements.

4.1.2 Bacterial Probes

For bacterial specific probes an elongation of the probe sequences did not lead to an increase in capture e ciency (Figure 4.2). Using probe Bac338-TL (18 bp) or probe Bac338+8-TL (26 bp) always resulted in a recovery of approximately 20% of the initial added SSU rRNA of *Raoultella terrigena* (initial added SSU rRNA: 280 to 550 ng ($\sim$ 1 to 2 μg of total RNA)). Thus the optimal length of the bacterial probe was considered to be 18 bp for further experiments.

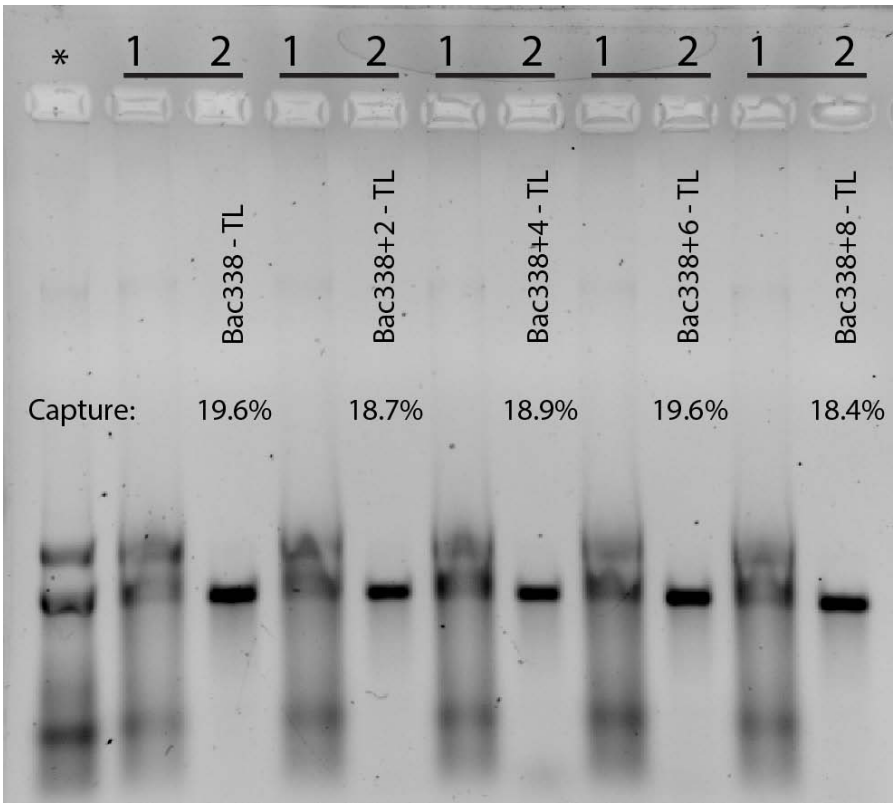

Figure 4.2 Capture of SSU rRNA from pure-culture RNA (*R.terrigena*) using domain speci c probes of di erent length on 0.7% agarose gel stained with GelRed^{TM}. Used probes are indicated in the graph. For each hybridization reaction slots represent as follows: (*) bacterial RNA not used in hybridization reaction; (1) unhybridized bacterial RNA from hybridization reaction; (2) eluted RNA after bead capture. Percentage of RNA captured was calculated from values of RNA concentration measurements.

4.2 Testing of Optimized Probes on Capture Specificity

4.2.1 Tests on Pure-Culture RNA

All experiments were carried out using the optimized probes Bac338-TL and Euk1379+6-TL for specific hybridization to bacterial and fungal SSU rRNA sequences respectively. Both probes were tested on RNAs of *Raoultella terrigena* and *Doratomyces* pure cultures serving as positive and negative control assays (Figure 4.3 and 4.4). Eluted RNA from the hybridization assay as well as unhybridized RNA samples were analyzed in the Agilent Bioanalyzer system. Thus the specificity of the probes could be easily tested by identifying the length of the captured SSU rRNA and observation of an increase or decrease in peak-height and -area of peaks in the exported electropherograms. Bac338-

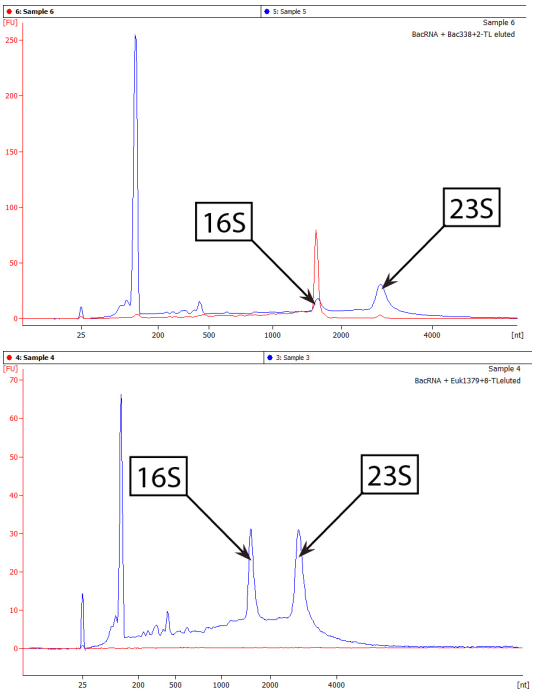

Figure 4.3 Capture of SSU rRNA from pure-culture RNA (*R.terrigena*) using bacterial- and fungal-speci c probes. Samples were run on the Agilent Bioanalyzer system. Blue lines show unhybridized RNA samples, red lines show eluted RNA samples. Bac338-TL and Euk1379+6-TL probes were used in the top and bottom graph respectively.

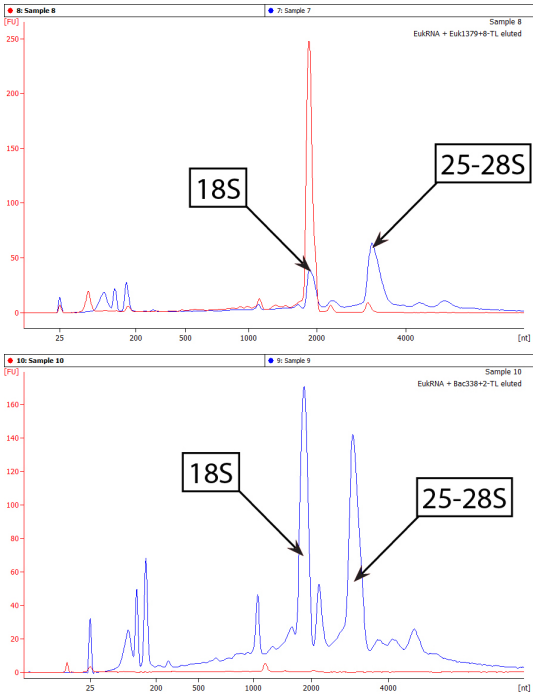

Figure 4.4 Capture of SSU rRNA from pure-culture RNA (*Doratomyces*) using fungal- and bacterial-speci c probes. Samples were run on the Agilent Bioanalyzer system. Blue lines show the unhybridized RNA samples, red lines show the eluted RNA samples. Euk1379+6-TL and Bac338-TL probes were used in the top and bottom graph respectively.

TL could successfully catch SSU rRNA from *R.terrigena* as shown in the top graph of figure 4.3. While the red line shows a clear elution peak on the position of the SSU rRNA molecule, also a decrease in peak area in the unhybridized RNA sample could be observed indicating the specific isolation of the rRNA. The negative control using Euk1379+6-TL and bacterial RNA (Figure 4.3 bottom graph) shows no elution peak. Two similar peaks could be observed in the unhybridized RNA sample on the position of the SSU and LSU rRNA of *R.terrigena*, comparable to the electropherogram from the RNA initially used in the assay (graph not shown).

Likewise Euk1379+6-TL could specifically capture SSU rRNA molecules from *Doratomyces* as shown in the top graph of Figure 4.4. Again a clear elution peak on the position of the fungal SSU rRNA could be observed and a decrease of the correlated peak from the unhybridized sample. No elution peaks were present when Bac338-TL probe and fungal RNA were used in the assay and the unhybridized RNA sample showed a comparable pattern to the RNA initially used in the assay, supporting the result of domain specific capturing since no fungal RNA was hybridized to the bacterial probe.

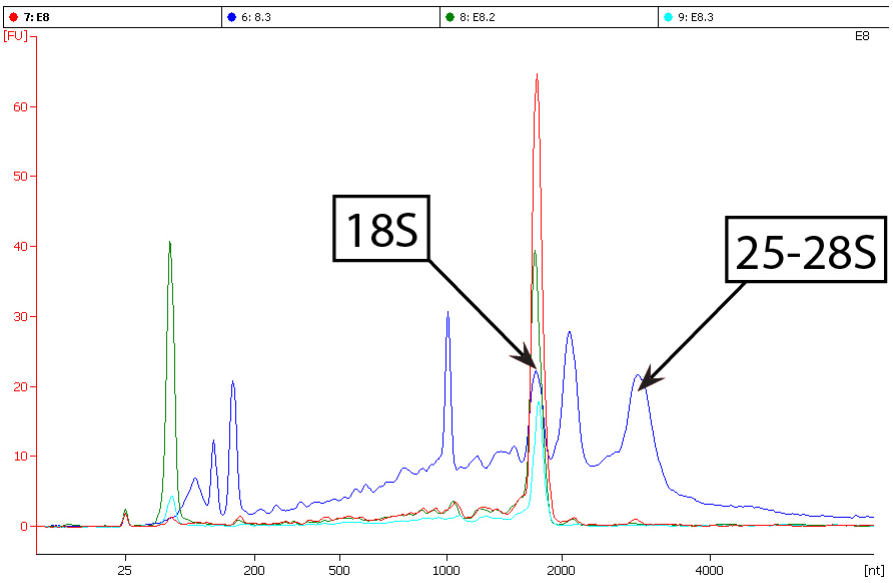

Figure 4.5 Repeated capture of fungal SSU rRNA from pure-culture RNA (*Doratomyces*) using EUK1379+6-TL. Samples were run on the Agilent Bioanalyzer system. Blue line shows the unhybridized RNA sample, red, green and turquoise lines show the eluted RNA samples from the 1^{st}, 2^{nd} and 3^{rd} capture reaction respectively.

An experiment with repeated hybridization reactions to evaluate the possibility of increasing the capture e ciency of SSU rRNA was set-up. For the 2^{nd} and 3^{rd} hybridization reaction the unhybridized sample from the previous reaction was used. Results are shown in figure 4.5. Following this procedure we could increase the capture e ciency from 41.5% to 60%, although this includes a peak of small RNA fragments eluted from the 2^{nd} hybridization reaction with a size of approximately 100 bp. Thus, the actual capture e ciency would be lower than 60%, but an increase of captured SSU rRNA could still be achieved.

4.2.2 Tests on Pooled Pure-Culture RNA

As a next step towards the application of the optimized probes in environmental samples, we tested their performance in a hybridization reaction using one probe and a equimolar pool of bacterial and fungal RNA from pure-cultures (Figure 4.6 and 4.7). For the fungal- as well as the bacterial-specific hybridization reactions we could observe one single peak in the eluted RNA sample.

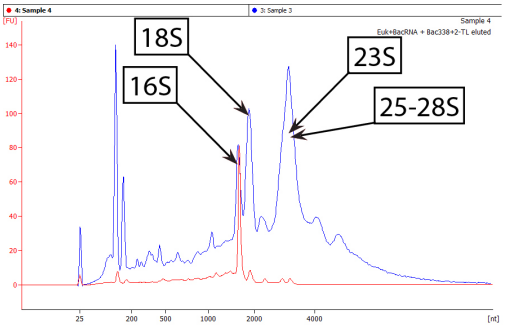

Figure 4.6 Capture of bacterial SSU rRNA from mixed pure-culture RNA (*Doratomyces* and *R.terrigena*) using Bac338-TL. Samples were run on the Agilent Bioanalyzer system. Blue line shows the unhybridized mixed-RNA sample, red line shows the eluted RNA samples.

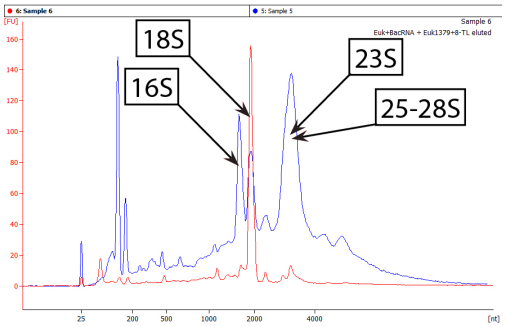

Figure 4.7 Capture of fungal SSU rRNA from mixed pure-culture RNA (*Doratomyces* and *R.terrigena*) using Euk1379+6-TL Samples were run on the Agilent Bioanalyzer system. Blue line shows the unhybridized mixed-RNA sample, red line shows the eluted RNA samples.

At the same time the targeted peak from the unhybridized RNA sample was reduced in height and peak area (16S peak in figure 4.6; 18S peak in figure 4.7). Additionally the capture e ciency of the two hybridization reactions was similar to the values obtained with only bacterial or fungal RNA. 21% of the available bacterial SSU rRNA could be captured whereas 37% of the fungal SSU rRNA in the sample could be isolated. These results show, that the probes performed similar in pooled RNA samples from pure cultures.

4.2.3 Tests on Soil RNA

Finally, probes were tested on a RNA from soil (sample isolated by Sandra Moll) to evaluate applicability of the method on environmental samples. Capture with Euk1379+6-TL was repeated three times, while hybridization and capture with Bac338-TL was done 2 times. Results are shown in figure 4.8. The graph just shows samples of eluted RNA, since the unhybridized RNA sample was completely degraded and showed no peak in the SSU and LSU size range any more.

For the bacterial-specific probe an elution peak of the expected fragment size was observed in both capture reactions. For the fungal-specific probe only in the 2^{nd} hybridization a elution peak showed up, although it comprises RNA fragments of the same size than eluted from the bacterial probe.

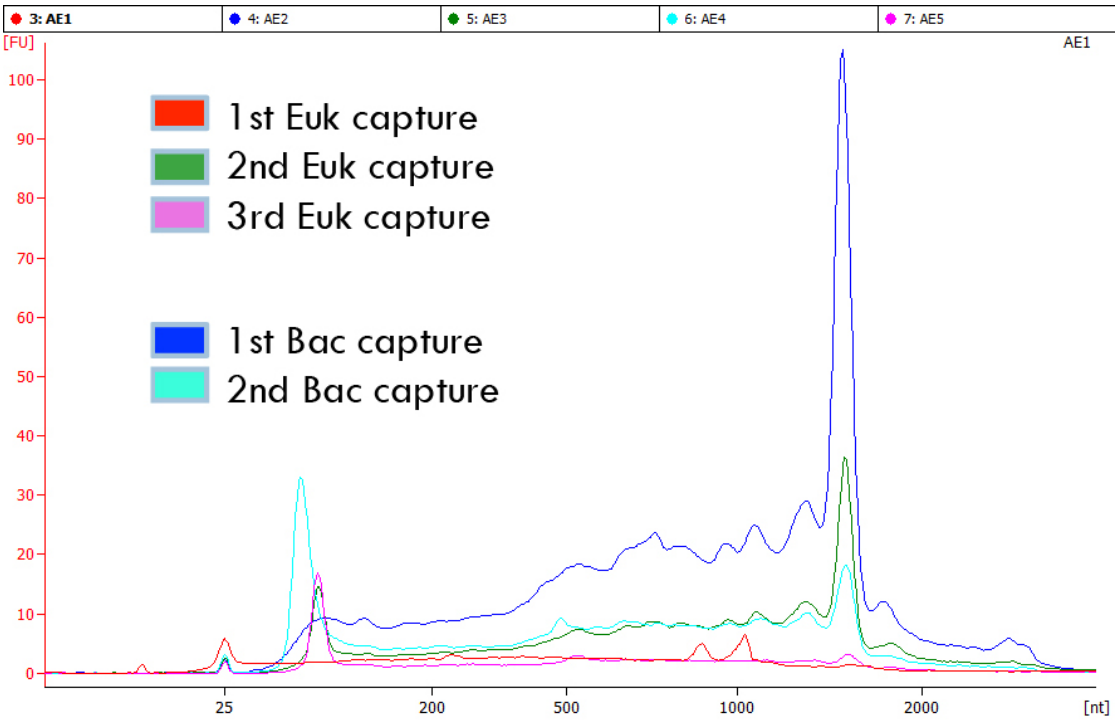

Figure 4.8 Repeated capture of fungal and bacterial SSU rRNA from soil RNA using Bac338-TL and Euk1379+6-TL Samples were run on the Agilent Bioanalyzer system. Blue and turquoise lines show the 1^{st} and 2^{nd} elution from capture reactions using Bac338-TL respectively. Red, green and pink lines show the 1^{st}, 2^{nd} and 3^{rd} elution from capture reactions using Euk1379+6-TL respectively. Unhybridized soil RNA is not shown in the graph.

5 Discussion

In this thesis we aimed to develop a method to separate SSU rRNA from bacteria and fungi from environmental RNA. By separating bacterial and fungal RNA from a complex community, e.g. from a soil environment, we would be able to add an additional tool to currently used methods in microbial ecology. This would translate to more focused experimental designs by a previous separation of information and subsequent application of community profiling and function approaches.
Probes Bac338 and Euk1379 were tested on capture e ciency and specificity in assays containing RNA from pure cultures, mixed pure-culture RNA and RNA from soil.

Capture of Fungal and Bacterial SSU rRNA from Pure-Culture RNA Changing the length of the probes showed clear e ects for the fungal capture e ciency, whereas bacterial probes performance was not e ected. The improvement in regaining of fungal SSU rRNA from 6.6 to 41% from the initial added amount is a very promising result regarding the applicability of the method with environmental samples. In RNA from soil the fraction of fungal RNA is usually very low, thus a high capture e ciency is needed to regain a su cient amount of material for a subsequent mass spectrometric or sequencing approach. Numbers achieved with the bacterial specific probe (∼20%) should be su cient since bacterial rRNA is usually present in excess in soil samples. Additionally it should be mentioned here, that the minimal amount of nuclear acid material needed for possible downstream analyses is constantly decreasing by improvements in detection limits of mass spectrometry or the amount needed to start a sequencing run.
Results using the optimized probes Bac338-TL and Euk1379+6-TL on RNA from pure cultures show the specificity of the two probes in the assays. In all positive controls one elution peak at the correct position in the electropherograms could be observed, whereas

no peaks were present in negative controls. This not just confirms the not binding of the unspecific probe, but also no carry over of RNA due to unspecific bindings to the magnetic particles or other mechanisms throughout the hybridization protocol.

The di erences in shape of the unhybridized samples in the graphs can be explained by many manipulating steps carried out during the protocol. Heating RNAs to 70°C leads inevitably to the degradation of these molecules resulting in many RNAs of smaller length as the initial one. This can be observed in the unhybridized RNA samples as well as in the eluted samples indicated by a usually decreasing shoulder towards smaller sized RNAs. Sometimes a high peak at approximately 100 bp appeared in the unhybridized samples of bacterial RNA, which represents most probably tRNAs which were not completely removed during purification steps. Di erences in peak heights of the SSU and LSU peaks in unhybridized samples can be explained by the di erences in RNA losses during the desalting step performed before loading these samples on the Agilent Bioanalyzer chip. Similar shapes of RNA run on a Bioanalyzer system were obtained before in other studies [Bailly et al., 2007]. Nevertheless, despite molecule degradation and losses the unhybridized samples contained in all cases enough intact SSU and LSU molecules. Thus it was possible to identify the origin of the elution peak by simply overlaying the two electropherograms graphically.

The capture e ciency could be further improved by performing the hybridization reaction two times more on the same initial RNA sample. This increase is very important again looking at the applicability of the method for downstream analysis regarding su cient supply of material.

Capture of Fungal and Bacterial SSU rRNA from Pooled Pure-Culture RNA Assays containing pooled fungal and bacterial RNA resulted in a comparable capture e ciency for both probes as expected. Again we could observe a degradation of the RNA used in the sample, but the main fraction of the eluted RNA was found to have the size of a complete rRNA molecule. Also here the approach of identifying the captured RNAs via comparison of lengths in the Agilent Bioanalyzer system was successful. The length di erence of approximately 200 bp was enough to separate the electropherogram peaks and identify the elution peaks via overlapping of the unhybridized and eluted sample graph.

Capture of Fungal and Bacterial SSU rRNA from a Soil-RNA The unhybridized RNA sample of the sampled soil was completely degraded after the multiple hybridization reactions performed. The RNA used was already stored at -80°C for one year before use. This, together with the multiple hybridization reactions can explain the complete degradation. As expected the 2 hybridization reactions carried out with Bac338-TL resulted in 2 elution peaks at the expected position in the electropherogram ($\sim$ 1600 bp). Also the decrease in e ciency indicated by an decrease in peak height and area in the second reaction was expected. In the first fungal capture reaction, no SSU elution peak could be observed. As already mentioned the ratio between bacterial and fungal communities in soil can vary significantly between di erent samples [Fierer et al., 2005]. No capture of fungal SSU rRNA may point to a low abundance of these organisms in the used soil. Surprisingly, in the second fungal capture reaction a elution peak showed up at the same position as the bacterial ones. This might show an unspecific hybridization. Also, the peak is unexpected narrow, since for eukaryotic rRNAs derived from a divers community a broad elution peak was expected due to the higher variance in length for the SSU rRNA sequences in eukaryotes.

Nevertheless, by just looking on length of the captured molecules in this study we will not be able to make scientifically based conclusions on our data. More tests on environmental RNA are necessary and the subsequent identification of the captured communities by sequencing and taxonomical identification of the captured sequences. Also spike-experiments with the artificial addition of bacterial or fungal RNA should be carried

out to investigate the performance of the probes from this study in complex RNA, since co-extracts from nucleic acid extractions from soil samples can cause matrix e ects in biochemical reactions.

We also want to mention that although this study does not target archeal communities a similar approach could be realized for these organisms. We aim here to investigate bacterial and fungal dominated environments like forest top soils, but we know nowadays, that archeal dominated environments do exist and archea can be key players in environmentally important processes [Leininger et al., 2006].

In this study we showed that it is also possible to capture a adequate amount of fungal SSU rRNA using a modified Euk1379 probe, since it was possible to increase the capture e ciency 6-fold. This was a crucial step regarding the final goal to separately isolate fungal and bacterial SSU rRNA molecules from environmental derived samples. Although the method is now working robust with pure-culture RNA, further testing has to be done on the performance with RNA from soil. Taxonomical identification will be key in assessing the specificity of the probes in these assays and drive further development. Combining this new approach with the use of stable isotope probing in soil community studies will lead to new insights in ecosystem functioning and answer questions about the level of participation of fungal and bacterial organisms on relevant environmental processes.

6 Appendix

6.1 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst und keine anderen als die im Literaturverzeichnis angegebenen Hilfsmittel verwendet habe. Ich versichere, dass ich alle wörtlichen und sinngemä en Übernahmen aus anderen Werken als solche kenntlich gemacht habe.
Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

6.2 Statement

I hereby declare that I have written this thesis independently and without assistance and I did not use resources other than those specified in the list of references. I certify that I have indicated all figuratively and literally meanings acquired from other works as such. However, if a violation of the copyright should be disclosed, I request to contact me.

6.3 References

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