Application of a developed high-throughput community analysis toolset to describe the spatial and temporal dynamics of aquatic mycoplankton communities

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Summary

Aquatic fungi are a highly diverse ecological group of organisms adapted to survive in a variety of aquatic habitats ranging from alpine rivers to coral reefs and from arctic lakes to deep sea sediments. Though highly abundant and diverse in many aquatic ecosystems, there are still many gaps in our knowledge and understanding about what is shaping their community structure, distribution, and dispersal patterns. In addition, little is known about their role in the biogeochemical and carbon cycles and their position in the microbial loop and food web structures. A limiting reason was the lack of an adequate toolkit for an easy and high-throughput description of aquatic fungal communities. Therefore, the aim of my dissertation was to develop missing tools such as a primer tool and a pipeline for the analysis of fungal high-throughput 18S rRNA gene sequence datasets and to test them for ecological questions involving the spatio-temporal resolution of mycoplankton communities.

In Chapter I, I present a primer toolkit consisting of a comprehensive list of 18S rRNA gene sequence primer pairs of various characteristics in respect to amplicon length, total fungal coverage, specificity on fungal taxa, and prevention of co-amplification from other eukaryotic groups. This primer list can simplify the choice of the appropriate primer pair, meeting the requirements of future fungal research studies. It can also be applied in qPCR studies. As a part of this toolkit, nu-SSU-1334-5'/nu-SSU-1648-3' (FF390/FR1), together with the use of blocking oligos, is proposed as a suitable Illumina primer pair able to improve potential amplification biases in the investigation of environmental aquatic fungal communities.

In Chapter II, I examine marine mycoplankton communities and their seasonal response to a variety of biotic and abiotic factors by sampling on a weekly basis over a one year period surface water at Helgoland Roads, North Sea (Germany). The Illumina 18S rRNA gene tag-sequencing revealed a highly diverse and dynamic fungal community over the course of the study period. The abundant members of the community were able to be categorized into four distinct groups according to their seasonal frequency patterns. Furthermore, network analyses showed that fungi are connected in multiple ways with planktonic organisms such as phytoplankton and zooplankton, but also within their own communities. The majority of the detected relationships between fungi and the planktonic organisms were negative. In an ecological context, this can be interpreted either as a top-down control of fungi on phyto- and zooplankton or as a role of fungi...
as a food source for zooplankton, or that fungi harmful to other plankton groups are themselves controlled by antagonistic fungi.

In Chapter III, I investigate the spatial community dynamics and underlying assembling processes of aquatic fungi, in a transect line from the island of Helgoland to Lauenburg on the Elbe River (Germany). The sampling region covered a region with marine, estuarine and freshwater bodies. The aquatic fungal communities were divided into three distinct groups, primarily under the influence of local environmental conditions, and to a lesser extent under the effect of spatial control. Different assemblage processes governed the three groups: variable selection in the upstream regions and undominated processes, such as ecological drift in the downstream regions and marine waters. These results offer an important framework that helps to understand the patterns of riverine mycoplankton communities and serves as a ground for future studies so that fungi, as an essential ecological group of organisms, can be incorporated into models like, usage-balance considerations of rivers.
Zusammenfassung


**Abbreviations**

- **ASV**: Amplicon sequence variants
- **Bc**: Barcode coverage
- **bp**: Basepair
- **Bs**: Barcode specificity
- **CARD-FISH**: Catalyzed reporter deposition Fluorescence in Situ Hybridization
- **dbRDA**: Distance-based redundancy analysis
- **DGGE**: Denaturing gradient gel electrophoresis
- **DNA**: Deoxyribonucleic acid
- **dNTP**: Deoxyribonucleotide triphosphate
- **DOC**: Dissolved organic matter
- **DSM**: Deutsche Sammlung von Mikroorganismen
- **DSMZ**: Deutsche Sammlung von Mikroorganismen und Zellkulturen
- **ENA**: European Nucleotide Archive
- **FDR**: False discovery rate method
- **FGG**: FlussGebietsGemeinschaft
- **FIS**: Fachinformationssystem
- **FISH**: Fluorescence in Situ Hybridization
- **HR**: Helgoland Roads
- **IND**: Indeterminata
- **INSDEC**: International nucleotide sequence database collaboration
- **ITS**: Internal transcribed spacer
- **IUPAC**: International union of pure and applied chemistry
- **KSMP**: Kultursammlung mariner Pilze
- **LCA**: Least common ancestor
- **LTER**: Long-term ecological research station
- **M 40 Y**: Medium for osmophilic fungi
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
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<td>NGS</td>
<td>Next Generation Sequencing</td>
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<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>OSD</td>
<td>Ocean Sampling Day</td>
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<td>OTU</td>
<td>Operational taxonomic unit</td>
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<td>PCoA</td>
<td>Principal component analysis</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PSU</td>
<td>Practical salinity units</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>Q-PCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>QPE</td>
<td>Quantitative process estimates</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg, unit for the sedimentation rate as for the 18S rRNA</td>
</tr>
<tr>
<td>SAR</td>
<td>Stramenopiles Alveolata Rhizaria</td>
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<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SSU</td>
<td>Small subunit of RNA</td>
</tr>
<tr>
<td>TGS</td>
<td>Third Generation Sequencing</td>
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<tr>
<td>Tm</td>
<td>Melting temperature</td>
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<tr>
<td>V1-V9</td>
<td>Variable region 1–9 of the 18S rRNA gene sequence</td>
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<tr>
<td>wTO</td>
<td>Weighted topological overlaps</td>
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<tr>
<td>YpSs</td>
<td>Yeast powder soluble starch</td>
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1. Introduction

The kingdom of fungi is a highly diverse group of organisms capable of thriving in nearly all habitats of the planet. It is estimated that a total number of 2.2 - 3.8 million fungal species exist (Hawksworth et al., 2017), with only around 150,000 being formally recognized (Species Fungorum 2020, accessed 30 September 2021). Of these species, a total of 1,901 have been categorized as marine fungi (Marine Fungi, accessed 30 September 2021) and around 3,000 have been recorded to be present in all types of aquatic habitats (Ittner et al., 2018). However, many more species reside in the aquatic biosphere, with it being assumed that at least up to 10,000 species exist in the marine environment alone (Jones et al., 2011, 2015). Aquatic fungi include a group of organisms sharing ecological rather than phylogenetic similarities. The working hypothesis for marine ecosystems, which could also be applied to all aquatic fungi, is that the group includes any members of the fungal kingdom, which can grow, sporulate, adapt, be metabolically active or develop symbiotic relationships with other organisms in aquatic habitats (Pang et al., 2016). Depending on how active, adapted to, and reliant on the aquatic environment they are (Park, 1972), fungi can be characterized as indwellers, periodic or versatile immigrants (Grossart et al., 2019). Though highly abundant and diverse, they are still a relatively understudied group compared to other microorganisms, especially in marine habitats. Little is known about what is shaping their community structure and distribution, about their roles in the biogeochemical and carbon cycles, or their implementation in the microbial loop and food web structures.

1.1 Historical background on marine and freshwater fungal research

Although the first marine fungi were recorded in the mid-19th century (Desmazières, 1849; Durieu & Montagne, 1869), it was only during the 1950s and 1960s that there was progress in the field of aquatic fungi research with the pioneering studies of I.M. Wilson, Samuel P. Meyers, T.W. Johnson, G. Feldman, W. Höhnk, E. Kohlmeyer, J. Kohlmeyer, K. Tubaki, F.K. Sparrow. These early researchers contributed to the field of aquatic mycology by mainly isolating, collecting, and describing many new aquatic fungal species. The first book about marine fungi was published in 1961 by T.W. Johnson and F.K. Sparrow and the authors summarized all the papers published until that point. Later, during the 1970s the books of Gareth
Jones and Jan Kohlmeyer and Erika Kohlmeyer (Jones, 1976; Kohlmeyer & Kohlmeyer, 1979) were another important milestone for aquatic fungal research. The very precise morphological descriptions and physiological characterizations that these books included are still valid and are widely considered in recent studies.

During the 1980s and 90s, based on the application of molecular techniques, a much higher number of aquatic fungal taxa were described from a wide range of different environments and geographic locations (Jones et al., 2009) indicating a more global distribution than previous thought. Prior knowledge from terrestrial species drove researchers of these decades to target mainly aquatic environments analogous to terrestrial habitats like mangrove trees (Hyde & Jones, 1988; Steinke & Jones, 1993), seagrass (Blum et al., 1988) driftwood (Farrant et al., 1985), detritus (Benner et al., 1984), or various animal carcasses (Kohlmeyer & Kohlmeyer, 1979). In following years, with the higher implementation of Sanger sequencing, the focus was expanded to communities rather than single isolates, and often extreme environments such as deep oceans (Bass et al., 2007), deep-sea sediments (Damare et al., 2006), hydrothermal vents (Gadanho & Sampaio, 2005; Le Calvez et al., 2009) and hypersaline waters (Cantrell et al., 2006), were targeted. Progress was also observed in the study of biotic interactions of marine fungi with algae, corals, and sponges, and some first preliminary ideas were developed about their potential role in ecosystem functioning (Zuccaro et al., 2003; Wegley et al., 2007; Gao et al., 2008; Loque et al., 2010). Additionally, the high number and diversity of fungal organisms found in aquatic habitats was connected with a potentially critical role in the biochemical transformation of detritus (Raghukumar, 2004). However, many researchers still considered fungi as non-diverse and low in abundance in marine environments (Kis-Papo, 2005; Massana & Pedros-Alio, 2008; Burgaud et al., 2009; Le Clavez et al., 2009), limiting their ecosystem role to decomposers of woody and herbaceous substrates and animal remains (Mann, 1988; Newell, 1996). Limitations in the available techniques unveiled only a small fraction of aquatic fungi, mainly culturable and fast-growing Ascomycota and Basidiomycota species, similar to the most well-known members prevalent in terrestrial environments. Only over the last two decades with the rise of Next Generation Sequencing (NGS) techniques and the growing interest in natural products with potential biotechnological applications, more NGS-studies targeting aquatic fungal communities became available. This led to the recognition that the majority of aquatic fungal species are so far undescribed and possibly belong to the dark matter fungi (Grossart et al. 2016).
To conclude, over the history of aquatic fungal research, with ongoing technical development, diverse identification and isolation techniques were used. Each technique has its advantages and disadvantages, with the choice for one or the other technique depending highly on the research question. In the next sections, I will discuss each technique in regards to the technical background and the most driving outcomes in the field of aquatic fungal studies. I will begin with the culture-based methods as the most used techniques from a historical perspective.

1.2 Culture-based methods

Although culture-based methods now seem to be surpassed by newer identification techniques, they are still very important to answer unresolved ecological questions. The first insights into the degradation process of a marine *Cladosporium* strain on algal polysaccharides in coastal waters (Cunliffe et al., 2017) and the potential role of *Penicillium* isolates from intertidal zones in nutrient recycling and pollutant degradation (Park et al., 2019) are just two recent examples where culture-based methods shed light on the saprotrophic activity of fungi.

Culture-based methods are based on the sampling of tissue or environmental material, which is transferred to a liquid or solid isolation medium. Different types of isolation media are available and are combined with a variety of plating techniques, dilution methods and adjustments in environmental conditions, which offer an extensive toolset to isolate diverse taxonomic fungi with different life strategies (Yarrow, 1998; Schmit & Lodge, 2005; Kossuga et al., 2012; Nevalainen et al., 2014; Overy et al., 2019; Senanayake et al., 2020). Once, a pure culture of an isolate exists, it can be examined for its macro- and micro- morphology, taxonomically classified based on morphology or molecular markers, and sequenced for its whole genome. Furthermore, fungal material can be collected and used for further biochemical analyses, like screening techniques aiming to identify and produce new enzymes (Velmurugan & Lee, 2012). Besides, diverse experiments can be drawn, such as growth experiments in different environmental conditions and experiments inspecting degradation capacities. Based on which fungal species were isolated in the different marine environments and their particular exhibited characteristics and enzymatic activity, researchers can form new hypotheses and questions about the composition and functionality of fungal communities.
A drawback of this approach, especially when only morphological characteristics are used, is that the correct identification of fungal organisms can be challenging due to their phenotypic plasticity which can be easily triggered by changes in environmental conditions or growth media (Slepecky & Starmer, 2009). Besides, di- or pleiomorphic life cycles, which are very common for fungi, can further prevent correct morphological identification (Begerow et al., 2000; Seifert & Samuels, 2000; Klein & Tebbets, 2007). In the field of community studies a major limitation of these techniques is the great plate count anomaly (Staley & Konopka, 1985) meaning that only a small percentage of fungal organisms can be cultivated, excluding the majority of species for which it is very hard or even impossible to be put into a culture (James et al., 2020). Furthermore, a large amount of novel diversity cannot be easily accessed, because of sampling difficulties since a lot of aquatic fungi exist inside host organisms or in extreme environments such as deep sea sediments and hydrothermal vents (Richards et al., 2012). In addition, when the aim is the description of a habitat-specific community, a certain risk of contamination exists (Raghukumar, 2017). Contamination problems can be minimized to a high extent by following appropriate sampling techniques for each environment, and by following good lab practices regarding the storage and handling of the isolates.

Having a single species isolated in culture offers the possibility to observe and apply diverse biochemical and molecular tests. In the era of -omics having a marine fungal isolate means to have a model organism available to be tested and on which genomic and transcriptomic analyses can be run (Kumar et al., 2018; Pilgaard et al., 2019; Pang, 2020). Additionally, marine fungal isolates have been shown to be a rich source of novel natural products and secondary metabolites with potential anti-bacterial, anti-viral, and anti-cancer applications (Imhoff et al., 2016). Besides, isolation and cultivation of new marine fungal species will assist the progress of molecular studies by improving and enriching sequence databases. Taken together, cultured representatives help us to draw a better picture on individual and community microbial functions validating molecular data through experimentation (van Dorst et al., 2016) and allowing the development of new hypotheses.

**Pros**
- Identification of new species
- Isolates can be used for biochemical analyses and/or genome sequencing
- Isolates can be screened for novel natural products and secondary metabolites
- Enrichment of sequence databases with new sequence data
Cons

- Biases towards cultivable species
- Morphological plasticity can prevent identification
- Only a small percentage of aquatic fungal organisms can be cultivated: great plate anomaly
- Danger of contamination from airborne or water dormant species of terrestrial origin

1.3 Molecular Methods

Molecular tools revolutionized the research of fungal organisms and their applications have had a great impact on fungal phylogeny, taxonomy, and systematics (Shenoy et al., 2007; Jones et al., 2011; Yang, 2011). In aquatic fungal studies, these new methods allowed to detect whole groups of previously undescribed fungi (Jones et al., 2011; Nagahama et al., 2011; Corsaro et al., 2014b; Ishida et al., 2015), to reveal the diversity and structure of environmental communities and to hypothesize about their potential ecological role (Gutiérrez et al., 2016).

The standard tool for molecular taxon identification requires the use of a marker gene for barcoding (Hebert et al., 2003; Dulla et al., 2016). Different aspects have to be taken into account for barcoding depending on the sequencing technique used and the working material such as environmental samples or single isolates. Until now, without the use of a third generation sequencing (TGS) technique (see discussion), it was easier to generate a longer barcode stretch for single fungal isolates than for species within an environmental sample. Sanger sequencing (Sanger et al., 1977) is still the standard method for single taxon identification (Reich & Labes, 2017) but the tide is now turning to long-read sequencing via TGS. Similarly, TGS techniques will also dominate community analyses in the near future (see discussion). For environmental communities, so far, shorter barcode stretches which are applicable for NGS techniques have to be used. NGS can perform sequencing in parallel where thousand to millions of sequences are generated. The most prominent sequencing technique is Illumina (Bennett et al., 2004; Bentley et al., 2008) followed by Ion Torrent (Rusk, 2011).

The starting point in the study of environmental microbial communities under molecular methods is the isolation and extraction of nucleic acids from the target environmental samples. The extracted nucleic acids are amplified for a selected marked gene with polymerase chain reaction (PCR) and then sequenced (Figure 1). Depending on the implemented sequencing technique, a variety of bioinformatics tools are available for the processing and filtering of the
raw data to the desired quality criteria for further analysis. This includes steps such as merging forward and reverse reads, clipping off barcodes, adapters and primers, filtering sequences according to their quality, and identifying chimera sequences. In the case of NGS amplicon data, the next step is the clustering of the sequences into Operation Taxonomic Units according to a defined sequence similarity threshold (Figure 1). For fungal communities, this is still the method of choice as amplicon sequence variants (ASV) do not result in appropriate species-level resolution (Estensmo et al., 2021). OTUs can be taxonomically classified with two main strategies based either on sequence similarity to the sequences of a reference database or on a phylogeny approach by aligning sequence reads and subsequently calculating a phylogenetic tree and/or inserting sequences into a phylogenetic reference tree (Figure 1). In fungal community studies, suitable tools are available for the majority of the described workflow steps but often they are not adapted for aquatic fungi. At the time point, when I started my PhD research project, aquatic fungal community surveys were hindered due to the lack of consensus for a suitable barcode region, optimal primer pairs, blocking oligonucleotides, and phylogenetic reference trees.

**Figure 1:** Workflow suitable for the identification of aquatic fungal environmental communities.
1.4 Fungal DNA barcoding

DNA barcoding is defined as a molecular method using short to longer (dependent on the sequencing technique), variable and easily amplified DNA sequences for identification of organisms at a specific taxonomic level (Hebert, 2003; Velmurugan et al., 2013).

The most commonly used DNA sequence regions for fungal barcoding are the various genetic regions of the ribosomal RNA (rRNA) cluster (Reich & Labes, 2017). The rDNA operon exists in numerous copies in genomes offering a higher amount of DNA template compared to single-copy genes such as protein-coding markers. This makes the region an ideal target for PCR amplification (Lindahl et al., 2013). The various parts of the rDNA cistron, namely the internal transcribed spacer (ITS), a non-translated region, and the 18S rDNA and 28S rDNA coding for a part of the ribosomal small subunit (SSU) and the large subunit (LSU), respectively, are often used as molecular markers for fungi (Figure 2).

![Figure 2: Representation of the primary structure of the rDNA cistron. Red color depicts regions of high variability for 18S and 28S rDNA regions.](image)

1.4.1 ITS

The ITS is composed of two non-coding regions, the ITS1 and the ITS2, situated between the 18S and 28S rRNA gene sequences and interrupted by the conserved 5.8S gene region. The ITS sequence varies significantly in its length among fungal taxa, ranging from around 300bp up to 1200bp (Seifert, 2009). The length limitations of Illumina sequencing allow for the targeting of one of the two ITS regions, either ITS1 (Zhang et al., 2016; Wang et al., 2017) or ITS2 (Retter et al., 2019; Luo et al., 2020). As a non-coding region, the ITS is subjected to a much higher mutation rate than the 18S and 28S rRNA gene regions, thus exhibiting high sequence and length variability on a species level (Lindahl et al., 2013). Therefore, the ITS shows a high resolution
power for many groups in the phyla of Ascomycota and Basidiomycota (Schoch et al., 2012). Another advantage of the ITS as a barcode is the high amount of well-annotated and high-quality sequences in public databases such as the UNITE database (Nilsson et al., 2018). These advantages, together with a highly successful amplification rate led to the proposition of the ITS as the official fungal barcode region (Schoch et al., 2012). However, the recognition of the high quantity of undescribed fungal diversity has changed the opinion of the research community on the usability of the ITS as a barcode for environmental samples (Nilsson et al., 2018), especially in aquatic environments. Besides, it demonstrates low effectiveness on the subphylum Saccharomycotina (Schoch et al., 2012) and some genera of Ascomycota with shorter ITS sequences (~400bp) (Skouboe et al., 1999; Schubert et al., 2007). In addition, it performs poorly on basal fungal lineages (Heeger et al., 2018), which is a clear drawback for studies on marine fungal communities, which are often dominated by zoosporic fungi and yeasts (Richards et al., 2015; Hassett et al., 2020).

The high number of novel diversity in environmental communities makes it necessary to classify taxa in a phylogenetic way. The high length and sequence variability of ITS hinders such an approach and leads to untrustworthy sequence alignments when aligning on a higher taxonomic level than the species/genus (Schoch et al., 2012). Tedersoo et al. (2018a) proposed a method with which community taxonomic data can be mapped on a sequence-based backbone multiple marker phylogenetic tree. However, this approach still does not allow the phylogenetic classification of unknown environmental sequences and does not solve the problems of low resolution among basal fungi groups. In many aquatic fungal surveys when ITS was used as a barcode, up to 80% of the fungal taxa in the community could only be described on a high taxonomic level such as kingdom or phylum (Wang et al., 2014; Jeffries et al., 2016; Wang et al., 2017; Ortiz-Vera et al., 2018; Wahl et al., 2018).

### Pros
- Can resolve to lower taxonomic levels for many groups of Ascomycota and Basidiomycota
- Well annotated and high quality sequences available

### Cons
- Does not perform well for basal fungi lineages
- Does not have phylogenetic power
- May lead to biases for fungi in aquatic habitats where a lot of unknown species reside
1.4.2 28S rDNA

With a length of around 2900 bp, the 28S rRNA gene region is the longest part of the rDNA cistron (Khot et al., 2009; Reich & Labes, 2017). It contains two hypervariable regions the D1 and D2, which are the commonly targeted areas for fungal barcode based surveys (Raja et al., 2017). The domains of D1 and D2 show no length variability and are flanked by relatively conserved regions (Liu et al., 2012; Raja et al., 2017), favoring the alignment of 28S rDNA sequences for phylogenetic analysis and taxonomic placement (Arnold et al., 2009; Öpik et al., 2010). The PCR success on the 28S rDNA is relatively high and many fungal taxa can be identified to species level (Schoch et al., 2012). Compared to ITS, it is considered as a less variable region, although when longer 28S rRNA gene sequences are used, it can outperform ITS (Liu et al., 2012; Porras-Alfaro et al., 2014). 28S rDNA shows high success in the classification of yeasts within the Ascomycota and Basidiomycota and basal fungal taxa (Kurtzman et al., 2011; Schoch et al., 2012; Sumerta & Karti, 2018). On the contrary, the marker exhibits a more mediocre performance for the Ascomycota subphyla of Pezizomycotina and Saccharomycotina (Schoch et al., 2012).

For 28S rRNA gene sequence, high-quality sequence databases such as the RDP (Wang et al., 2007) and SILVA (Quast et al., 2013) exist. However, the major disadvantage of 28S rRNA as a barcode region is that, compared to the ITS and 18S rRNA marker gene, it has a much lower number of sequences available in public databases (Panzer et al., 2015). Thus, in the last available SILVA non-redundant dataset (version 138.1 last accessed 12/10/2021) for the kingdom of Fungi, there are only 1,867 sequences available for 28S rRNA compared to the 9,373 sequences available for the 18S rRNA gene sequence (Quast et al. 2013). In addition, for aquatic fungi, there are very few available studies where the 28 rRNA gene sequence is used as the amplification target barcode region for NGS technology (Wurzbacher et al., 2016; Hasset et al., 2017; Picard et al., 2017). This hinders comparison between studies and the inclusion of datasets into mega-studies, which are urgently required for an overall understanding of the global diversity and dynamics of aquatic fungi.

**Pros**

- Highly promising barcode region
- Can be used as a phylogenetic marker
- Successful in basal fungi lineages identification
Cons

- Limited number of sequences available in public databases

1.4.3 18S rDNA

The 18S rRNA gene region for fungi has a length of around 1,900 bp and is divided into conserved and variable regions with varying degrees of range and variability, which are the target amplification areas for fungal identification and environmental diversity studies (Reich & Labes, 2017). There are nine variable regions (V1-V9), though it has to be noted that the V6 does not possess high variability in eukaryotic organisms (Neefs et al., 1993). Diverse variable regions have been used in fungal community surveys, but there is a lack of specific studies looking into which of the variable regions is the most suitable amplification target. The V4 region is proposed as the best performing one for eukaryotic communities (Hadziavdic et al., 2014). Other proposed candidate regions for eukaryotic biodiversity assessments are the V2 (Hadziavdic et al., 2014) and V9 regions (Amaral-Zettler et al., 2009; Pawlowski et al., 2011; Hadziavdic et al., 2014).

The 18S rRNA gene sequence possesses different resolution power for single fungal taxon groups. Thus, it performs very well on yeasts within the Saccharomycotina and the basal fungi lineages but much less well for taxa of the subphylum Pezizomycotina and the majority of Basidiomycota groups (Schoch et al., 2012). One important advantage of the 18S rRNA gene sequence as the aquatic fungal barcode region is the presence of high-quality sequence databases like the RDP (Wang et al., 2007) and SILVA database (Quast et al., 2013) having higher sequence numbers in comparison to the 28S rRNA marker gene (Panzer et al., 2015). Another advantage of this marker region is the existence of a phylogenetic fungal reference tree spanning over the whole fungal kingdom (Panzer et al., 2015; Yarza et al., 2017), allowing for the phylogenetic classification of unidentified taxa.

Pros

- High quality sequence databases and phylogenetic references trees are available.
- Taxonomic classification of unidentified taxa can be done over phylogenetic placement
- High resolution for basal fungal lineages and some yeast groups

Cons

- Can only resolve to higher taxonomic levels for some fungal groups
- Dangers of co-amplification from non-fungal eukaryotes
1.5 Why a phylogenetic approach matters

Over the last few years, the classification of undescribed species has come strongly into focus. Marine fungal community studies based on an ITS-barcoding approach often suffer from the fact that a large part of the detected OTUs cannot be classified further than kingdom level, with the worst case of up to 80% of the OTUs (Jeffries et al., 2016). However, ecological questions can only be adequately addressed if individual subgroups can be tested for their relationships with environmental parameters and then be compared with other studies. This requires a taxonomic name or a form of recognition like a code. Such a coding has recently also been proposed by Tedersoo et al. (2017), who, after phylogenetic placement of several unrecognized fungal clades, provisionally assigned them to informal taxon names.

A phylogenetic-based community analysis allows for undescribed taxa to be classified based on the position they occupy within the phylogenetic tree. Furthermore, clades containing only environmental sequences can receive some form of coding. For example, if a clade formed on the branch of the clade Chytridiomycetes, then it can be called Chytridiomycetes clade 01.

The clades can appear separately in the taxonomic description and can be individually statistically tested, thus allowing a much more detailed analysis of the fungal communities. In addition, phylogenetic information provided by the sequences in the tree could also offer the opportunity to use phylogenetic dependent statistical metrics like UniFrac (Lozupone & Knight, 2005). While the ITS has no phylogenetic power due to its high length and sequence variability among fungi, the 18S and 28S rRNA marker genes can both be used for a phylogenetic-based approach. Generated sequences can be introduced to a phylogenetic reference tree by phylogenetic placement and taxonomically assigned by their specific position on the tree.

1.6 Why the 18S rDNA is a good marker gene for aquatic fungal community analyses

The richness of sequences of a marker gene and the existence of curated databases is an important point for ecological studies. To put my own work into a global context, it is beneficial to compare the taxonomic composition, community dynamics, and key players, with other studies. An example question is how widespread a fungal taxon is across the world's oceans for
which a trait of ecological relevance has been demonstrated in experiments (Cunliffe et al., 2017). Furthermore, aquatic fungi are part of food webs, microbial functional guilds, and often embedded in a network of complex organismal interactions, whose nature can be direct or indirect. Therefore, it is beneficial to put aquatic fungal research into the context of eukaryotic research, which is, as a standard done, on the 18S rRNA gene sequence.

All these above mentioned advantages of 18S rRNA gene sequence led me to select this marker as the amplification target region for my projects. ITS and 28S rRNA gene sequences do not currently have the ability to perform better than the 18S rRNA gene sequence in the description of fungal communities in aquatic environment, where a large proportion of sequences belong to basal fungi lineages or unknown species (Richards et al., 2015; Hassett & Gradinger, 2016; Jeffries et al., 2016; Rojas-Jimenez et al., 2017; Bai et al., 2018; Rojas-Jimenez et al., 2019).

1.7 Primer pair choice

A critical step in the study of fungal communities is the choice of a suitable primer according to the research question, sampled environment, and sequencing technique (Lindahl et al., 2013; George et al., 2019). A considerable variety of 18S rRNA gene sequence primer pairs are available with differences in the targeting of variable regions and amplicon length (White et al., 1990; Gargas & Taylor, 1992; Gargas & DePriest, 1996). Primer pairs designed for terrestrial fungi targeting soil, rhizosphere, or mycorrhiza communities are commonly used without considering the differences in taxa composition with aquatic communities. Global fungal soil diversity shows that terrestrial communities are primarily dominated by Basidiomycota and Ascomycota species (Tedersoo et al., 2014). In contrast, global studies on aquatic fungi reveal that in habitats with atypical salinity regimes, estuarine regions, near-shore coastal waters, and sediments, Chytridiomycota and other basal fungi groups form the dominant part of the total community (Richards et al., 2015; Hassett et al., 2020).

Another problem of the primer pair performance originates from the fact that in the past aquatic fungi were often studied as a portion of the entire eukaryotic community, with the use of general eukaryotic primers targeting the total fragment of eukaryotic life (Bradford et al., 2013;
Bernhard et al., 2014; Edgcomb et al., 2014; He et al., 2014). Until recently, some researchers still preferred to amplify aquatic mycoplankton communities with general eukaryotic rather than fungal specific primers (Hassett et al., 2017; Kettner et al., 2017; Hassett et al., 2019) due to the fact that they were lacking an efficient primer pair.

The use of non-optimal fungal specific primers can produce a relatively low number of target sequences and/or lead to massive co-amplification of non-fungal eukaryotes (Borneman & Hartin, 2000). The latter point is important in aquatic environmental studies as diverse non-fungal eukaryotic organism groups, such as the several lineages of the SAR supergroup (Stramenopiles, Alveolata, Rhizaria), thrive in aquatic ecosystems (Logares et al., 2014; de Vargas et al., 2015; Massana et al., 2015; Bjorbækmo et al., 2019). Due to their high sequence similarity with the primer attachment site in the 18S rRNA gene sequence of fungi, they could immensely increase the co-amplification rate (Chemidlin Prévost-Bouré et al., 2011). Besides, non-suitable primer pairs may be biased towards specific groups, such as the better-studied groups of Ascomycota and Basidiomycota, neglecting a large portion of the environmental diversity. Biased sequencing results may lead to a non-representative image of the true diversity and structure of fungal communities. Despite the disadvantages of sub-optimal primers, researchers often directly adopt primer pairs used in previously published studies, without considering their poor performance and the applicability to their research goals.

An optimal primer aiming to amplify environmental marine fungal communities should be able to target the whole fungal tree of life with a minimum co-amplification of non-fungal eukaryotes as is possible. A part of my work for this thesis was aiming to resolve the problems emerging from the use of suboptimal primers by offering a comprehensive toolkit, reshuffling existing primers into new pairs, and/or designing new ones, and proposing a blocking oligonucleotide approach to improve the sequencing output.
1.8 Aquatic Fungi

1.8.1 Characteristics

There is no fungal synapomorph that includes all fungi but excludes other organism groups (Richards et al., 2017). However, all fungi are characterized, at least in one of their life stages, by the presence of chitin in their cell walls (Richards et al., 2017; Tedersoo et al., 2018a). Based on their vegetative morphology, fungi can be divided into filamentous and unicellular forms which can be further divided into yeast and zoosporic fungi (Raghukumar, 2017; Figure 3). Dimorphic fungi possess the ability to change between unicellular and multicellular forms depending on the environmental conditions (Riquelme et al., 2018). Filamentous fungi form elongated cylindrical-like cells the hyphae, displaying apical or lateral growth (Steinberg et al., 2017, Harris, 2019). In aquatic environment filamentous fungi hyphae branching ability is ideal for colonizing and growing on various organic substrates such as marine aggregates, but not so successful in supporting a freely floating plankton lifestyle in the water column (Richards et al., 2015; Raghukumar, 2017). On the other hand, a yeast form is equally adapted to both living attached to the surfaces of various substrates or being suspended in the water column (Raghukumar, 2017). Zoosporic fungi possess motile zoospores with flagella with which they actively swim and disperse in the water until they finally attach and penetrate their hosts or food source (Barr 2001; Gleason et al., 2011, Figure 3). A unicellular planktonic lifestyle is considered as an advantageous adaptation for liquid habitats while filamentous forms are suggested to be better suited for colonizing solid substrates with high amounts of organic matter (Fell et al., 2012; Richards et al., 2012, 2015; Raghukumar, 2017). In the aquatic environment, fungi can reproduce sexually or asexually, producing spores or conidia adjusted to dispersal in marine conditions (Raghukumar, 2017). Some fungi possess spores with appendages that increase the overall surface area favoring their dispersal (Kohlmeyer & Kohlmeyer, 1979).
Figure 3: Representative depictions of (A) filamentous fungi (B) yeasts, and (C) zoosporic fungi motile fungal zoospores.

1.8.2 Taxonomy

The fungal tree of life is continuously under construction, with frequent updates as a result of the constantly newly available sequencing data holding information about the unknown and unclassified diversity (Tedersoo et al., 2018a; Wijayawardene et al., 2020). The most well phylogenetically-defined group in the fungal tree of life is the group of Dikarya, which includes the phyla of Ascomycota, Basidiomycota, and Entorrhizomycota (Tedersoo et al., 2018a; Wijayawardene et al., 2020). In aquatic environment, numerous hyphomycetes and saprotrophic and parasitic yeast species can be found belonging to the phyla of Ascomycota and Basidiomycota (Grossart et al., 2019). The mainly terrestrial taxa of Entomophthoromycota, Glomeromycota, Entomophthoromycota, Mortierellomycota, and Mucoromycota (Benny et al., 2014, 2016; Grossart et al., 2019), and the taxa including some typical aquatic representatives of Kickxellomycota and Zoopagomycota (Grossart et al., 2019) belong to the basal fungal lineages. Basal fungi also contain the zoosporic groups of, Aphelidiomycota, Blastocladiomycota, Chytridiomycota, Monoblepharomycota, Neocallimastigomycota, and Rozellomycota.
1.8.3 Habitats

Recent studies revealed thriving and diverse fungal communities covering a broad range of the fungal tree of life from a variety of aquatic habitats (Grossart et al., 2019). However, there are still ecosystems such as rivers, estuarine regions, coral reefs, arctic and benthic waters, where only a handful of papers using the power of high-throughput sequencing to shed light on the community composition and diversity of the mycoplankton assemblages exist. Based on currently available studies, representatives of Ascomycota and Basidiomycota are common in pelagic, coastal, and oceanic waters (Wang et al., 2014; Taylor & Cunliffe, 2016, Wang et al., 2018; Morales et al., 2019), deep-sea sediments and hydrothermal vents (Zhang et al. 2016; Li et al. 2016; Xu et al., 2018; Luo et al., 2020), coral reefs (Amend et al., 2012; Góes-Neto et al., 2020) and lake sediments and waters (Tian et al., 2018; Wahl et al., 2018). Chytridiomycota zoosporic fungi were found to be prevalent in pelagic temperate samples close to Arctic regions (Comeau et al., 2016), coastal European sites (Richards et al., 2015), arctic water and sediment samples (Comeau et al., 2016; Hassett & Gradinger, 2016), surface sediment samples (Wang et al., 2017), brackish waters (Rojas-Jimenez et al., 2019), lakes (Wurzbacher et al., 2016) and rivers (Bai et al., 2018; Chen et al., 2020). Sequences belonging to basal fungal lineages and especially to Rozellomycota, were found to be a significant part of the communities in surface water samples from the Baltic and the North Sea (Rojas-Jimenez et al., 2019) urban and Antarctic lake waters (Rojas-Jimenez et al. 2017; Zhang et al., 2018), pelagic waters (Priest et al., 2021) and sediments (Wang et al., 2017).

1.8.4 Abiotic factors

Oscillations in abiotic factors can affect natural environments over multiple organisational scales, driving changes in community structure and ecosystem functioning (Dunson & Travis, 1991; Mouillot et al., 2013; Boyd et al., 2015). During the last decades, global warming and other anthropogenic effects dramatically altered abiotic factors in a relatively short time scale, making it increasingly imperative to comprehend the impact of these changes on organisms (Doney, 2010; Eissa & Zaki, 2011; Türkoglu et al., 2018). It is therefore vital to understand which factors influence the normal dynamics of microorganisms.
A variety of nutrients and physiochemical parameters were addressed as possible factors driving marine fungal environmental communities. Of all the measured physiochemical parameters, salinity, temperature, water depth, and nutrient availability were reported to be the most driving ones, affecting the diversity and composition of marine mycoplankton communities (Taylor & Cunliffe, 2016; Tisthammer et al., 2016; Rojas-Jimenez et al., 2019). Specifically, a significant correlation between fungal assemblages and temperature was described for coastal waters (Taylor & Cunliffe, 2016; Wang et al., 2018), temperate samples close to the Arctic (Comeau et al., 2016) and sediments of subtropical Chinese seas (Li et al., 2016). Besides, temperature was also identified as a critical factor shaping marine oceanic fungal communities, with the highest diversity observed at 8°C (Morales et al., 2019). Regarding salinity, fungal communities seem to be more diverse and abundant in the lower salinities of coastal, arctic, and brackish waters (Taylor & Cunliffe, 2016; Tisthammer et al., 2016; Rojas-Jimenez et al., 2019). The effect of salinity is also reflected in community composition with lower salinity environments dominated mainly by Chytridiomycota and Rozellomycota species in freshwater and brackish habitats (Richards et al., 2015; Wurzbacher et al., 2016; Hassett et al., 2019; Rojas-Jimenez et al., 2019). Water depth sample is another environmental variable showing significant correlations with community composition in open sea habitats, with increased diversity from the surface to deep water samples (Wang et al., 2014; Tisthammer et al., 2016; Morales et al. 2019). A variety of nutrients, such as dissolved organic carbon, total nitrogen, nitrate, phosphate, silicates and sulphide, were also recognized as potential environmental drivers in a series of studies (Orsi et al., 2013; Li et al., 2016; Taylor & Cunliffe, 2016; Tisthammer et al., 2016; Wang et al., 2018; Rojas-Jimenez et al., 2019). Nutrients reflect the availability of marine substrata accessible to fungal organisms, with yeast forms possibly being related to high nutrient concentrations (Raghukumar, 2017). It is hypothesized that decreases in nutrient availability could raise the probability of death for dominant species, facilitating in the same moment the growth of opportunistic species, resulting in an increase in overall community diversity (Wang et al., 2018).
1.8.5 Biotic factors

Marine fungi can develop diverse relations with a plethora of other aquatic organisms such as brown algae (Küpper & Müller, 1999), cyanobacteria (Gerphagnon et al., 2013), diatoms (Hanic et al., 2009), seagrass (Venkatachalam et al., 2015), sponges (Gao et al., 2008), corals (Amend et al., 2012), and crustaceans (Tourtip et al., 2009). The interactions between fungi and the various species of phytoplankton and zooplankton attracted a high level of research interest, with a key point to reveal potential roles of mycobiota in marine food web dynamics. Indeed zoosporic fungi are well recorded as parasites of phytoplankton (Kagami et al., 2011; Frenken et al., 2017). Parasitic fungi exhibit reliance to diatom abundance, and various degrees of specificity in the target host selection, which can both influence the population dynamics and evolution of phytoplankton blooms in the coastal upwelling ecosystem of central Chile (Gutierrez et al., 2016). Similarly, seasonal matching blooms of zoosporic Chytridiomycota and Diatoms were observed in the western English Channel by Taylor & Cunliffe (2016). Besides, other studies in coastal and ocean waters showed positive correlations between diverse phytoplankton groups and the fungal abundance (Comeau et al., 2016; Lepère et al., 2016). A possible control of phytoplankton populations from mycoplankton could be indicative of a top-down control from fungi. Other than top-down control, fungal zoospores could likely serve as a promising food source for diverse zooplankton organisms such as copepods, cladocerans, and rotifers, thereby improving their ability to survive in aquatic ecosystems (Kagami et al., 2007b, 2011; Schmeller et al., 2014; Agha et al., 2016; Frenken et al. 2018). In this way, zooplankton grazing can reduce the numbers of fungal zoospores and potentially alter the fungal community composition and structure (Kagami et al., 2014; Frenken et al., 2020).

Bacterial organisms also exhibit an impact on aquatic fungal communities, with the majority of currently reported interactions in freshwater habitats being of an antagonistic nature (Mille-Lindblom & Tranvik, 2003; Romaní et al., 2006; Baschien et al., 2009; Baudy et al., 2021). Synergistic relations are poorly studied compared to antagonistic ones, with a lack of comparative surveys correlating fungal and bacterial communities with environmental parameters. However, it is proposed that bacteria and fungi could benefit from each other’s breakdown products allowing more efficient organic matter degradation in coastal waters and bathypelagic marine snow (Bochdansky et al., 2017; Cunliffe et al., 2017). Recent experiments showed that Chytridiomycota species are able to modify bacterial communities colonizing...
phytoplankton organisms and particulate organic matter (Roberts et al., 2020; Klawonn et al., 2021).

Except for interactions between fungi and bacteria, there is also a lack of available studies aiming for possible relations between the different members of the fungal community in aquatic environments. Interactions of both competitive and promotional nature were detected between fungi in freshwater ecosystems, suggesting their potential influence in total fungal community structure (Fryar et al., 2001, 2005; Andrade et al., 2016). Members of Rozellomycota are able to parasitize diverse Chytridiomycota, species and behave as necrotrophs, degenerating their cytoplasm (Boddy, 2015). In addition, hyperparasitism of Chytridiomycota species through members of Rozellomycota has been proposed in freshwater ecosystems as one of many examples of intra-fungi relations (Gleason et al., 2014). A similar relationship was also suggested for Rozellomycota species in the coastal waters of the Bohai Sea, where through hyperparasitism, parasitic Chytridiomycota were controlled in favour of the algal bloom (Wang et al., 2018).

1.8.6 Ecological Role

The exact position of marine fungi in various food web dynamics, and their role in nutrient cycling and the microbial pump still remains unclear. Kagami et al., (2007a) was one of the first researchers who tried to integrate fungi into aquatic food web dynamics, proposing the “mycoloop” hypothesis. According to the mycoloop hypothesis, nutrients from large inedible phytoplankton species can be transferred to zooplankton through the grazing of parasitic fungal zoospores. Food web modeling and stable isotope incubation studies showed that around 20% of the photosynthetic carbon could be transferred from phytoplankton to freshwater parasitic Chytrids (Rasconi et al., 2017; Klawonn et al., 2021). Other studies extended the contribution of aquatic fungi to nutrient dynamics, suggesting that, because of the fungal infection, large inedible phytoplankton species can be fragmented and become accessible to zooplankton species (Sime-Ngando, 2012; Gerphagnon et al., 2013; Kagami et al., 2014; Agha et al., 2016). Besides, through parasitism, fungi can modify the palatability of phytoplankton organisms (Frenken et al., 2017). On the other hand, as a result of fungal parasitism, regularly edible phytoplankton organisms could be aggregated and become inedible or sink to lower depths becoming inaccessible to other biota (Kagami et al., 2005, 2006). Except for parasitic chytrids, saprotrophic ones have also the
ability to transfer nutrients from pollen grains to zooplankton through the grazing of their zoospores (Kagami et al. 2017). In addition, there are hints that, through their saprotrophic activity, aquatic fungi participate in the processes of carbon transformation and marine snow formation, influencing the flow of organic matter in the ecosystem (Grami et al., 2011; Bochdansky et al., 2017; Cunliffe et al., 2017). To describe all of these fungal-mediated interactions driving the aggregation or decomposition of organic matter in aquatic environments, Grossart et al. (2019) introduced the term “mycoflux”. The term mycoflux includes our missing knowledge regarding the impact of fungal organisms on the various organic matter pathways and the aquatic carbon pump.

Another important ecological role of fungi is depicted in benthic lake habitats and is related to the mechanism of “benthic shunt” (Grossart et al., 2019). This mechanism refers to the ability of fungi to colonize the benthic organic matter and to enhance the palatability of litter for macrozoobenthos grazers (Attermeyer et al., 2013; Crowther & Grossart, 2015). Macrozoobenthic organisms can transfer organic matter to higher trophic levels, thus improving the functionality of aquatic food networks (Grossart et al., 2019). The three mechanisms of mycoloop, mycoflux and benthic shunt draw a picture in which fungi are an active, dynamic, and functional part of aquatic ecosystems, and therefore influence the biological- and geochemical-driven cycles (Amend et al., 2019; Grossart et al., 2019, Figure 4).
**Figure 4**: Role of aquatic fungi. The figure depicts the three major biochemical processes in which aquatic fungi are involved, namely the mycoloop, mycoflux, and benthic shunt. Adapted from Grossart et al. (2019).

### 1.9 Aims

Understanding the biodiversity and functionality of aquatic fungi is still a black box. Therefore, it was all the more important to develop a toolkit that allows for performing aquatic fungal community analyses with a high-throughput output in a straightforward way. The 18S rDNA primer evaluation and development was the final step to finalize the toolkit developed in our group. The next step was to apply and document the developed toolkit in ecological studies. I did this, by surveying communities in two very different aquatic systems: the marine pelagic surface waters at Helgoland Roads close to the island of Helgoland in the North Sea, and the Elbe River, both in Germany.
1.10 References

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Introduction


Introduction


Introduction


Introduction


Introduction


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Introduction


2. List of publications and declaration of contribution

Chapter I: A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms

Stefanos Banos, Guillaume Lentendu, Anna Kopf, Tesfaye Wubet, Frank Oliver Glöckner, Marlis Reich

*Manuscript published in: BMC Microbiology, 18(1), 190.*
*https://doi.org/10.1186/s12866-018-1331-4*

**Correction to: A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms**

Stefanos Banos, Guillaume Lentendu, Anna Kopf, Tesfaye Wubet, Frank Oliver Glöckner, Marlis Reich

*Manuscript published in: BMC Microbiology, 19(1), 249.*
*https://doi.org/10.1186/s12866-019-1628-y*

Chapter II: Seasonal dynamics of pelagic mycoplanktonic communities: interplay of taxon abundance, temporal occurrence, and biotic interactions

Stefanos Banos, Deisy Morselli Gysi, Tim Richter-Heitmann, Frank Oliver Glöckner, Maarten Boersma, Karen H. Wiltshire, Gunnar Gerdts, Antje Wichels, Marlis Reich

*Manuscript published in: Frontiers in Microbiology, 11, 1305.*
*https://doi.org/10.3389/fmicb.2020.01305*
Chapter III: Mycoplankton biome structure and assemblage processes differ along a transect from the Elbe River down to the river plume and the adjacent marine waters

Yanyan Yang+, Stefanos Banos+, Gunnar Gerdts, Antje Wichels, Marlis Reich

+ These authors contributed equally

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https://doi.org/10.3389/fmicb.2021.640469
Declaration on the contribution of the candidate to a multi-author article/manuscript which is included as a chapter in the submitted doctoral thesis

**Chapter: I.** A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

- Experimental concept and design: ca. 30%
- Experimental work and/or acquisition of (experimental) data: ca. 90%
- Data analysis and interpretation: ca. 85%
- Preparation of Figures and Tables: ca. 90%
- Drafting of the manuscript: ca. 50%

**Chapter: II.** Seasonal dynamics of pelagic mycoplanktonic communities: interplay of taxon abundance, temporal occurrence, and biotic interactions

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

- Experimental concept and design: ca. 15%
- Experimental work and/or acquisition of (experimental) data: ca. 80%
- Data analysis and interpretation: ca. 70%
- Preparation of Figures and Tables: ca. 80%
- Drafting of the manuscript: ca. 50%
Chapter: III. Mycoplankton biome structure and assemblage processes differ along a transect from the Elbe River down to the river plume and the adjacent marine waters

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

- Experimental concept and design: ca. 15%
- Experimental work and/or acquisition of (experimental) data: ca. 50%
- Data analysis and interpretation: ca. 50%
- Preparation of Figures and Tables: ca. 60%
- Drafting of the manuscript: ca. 30%

Date: 18/10/2021

Signature:
3. Chapters

3.1 Chapter I: A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms
A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms

Stefanos Banos1, Guillaume Lentendu2,3, Anna Kopf4, Tesfaye Wubet2,5,7, Frank Oliver Glöckner4,6 and Marlis Reich1*

Abstract

Background: Several fungi-specific primers target the 18S rRNA gene sequence, one of the prominent markers for fungal classification. The design of most primers goes back to the last decades. Since then, the number of sequences in public databases increased leading to the discovery of new fungal groups and changes in fungal taxonomy. However, no reevaluation of primers was carried out and relevant information on most primers is missing. With this study, we aimed to develop an 18S rRNA gene sequence primer toolkit allowing an easy selection of the best primer pair appropriate for different sequencing platforms, research aims (biodiversity assessment versus isolate classification) and target groups.

Results: We performed an intensive literature research, reshuffled existing primers into new pairs, designed new Illumina-primers, and annealing blocking oligonucleotides. A final number of 439 primer pairs were subjected to in silico PCRs. Best primer pairs were selected and experimentally tested. The most promising primer pair with a small amplicon size, nu-SSU-1333-5′/nu-SSU-1647-3′ (FF390/FR-1), was successful in describing fungal communities by Illumina sequencing. Results were confirmed by a simultaneous metagenomics and eukaryote-specific primer approach. Co-amplification occurred in all sample types but was effectively reduced by blocking oligonucleotides.

Conclusions: The compiled data revealed the presence of an enormous diversity of fungal 18S rRNA gene primer pairs in terms of fungal coverage, phylum spectrum and co-amplification. Therefore, the primer pair has to be carefully selected to fulfill the requirements of the individual research projects. The presented primer toolkit offers comprehensive lists of 164 primers, 439 primer combinations, 4 blocking oligonucleotides, and top primer pairs holding all relevant information including primer’s characteristics and performance to facilitate primer pair selection.

Keywords: Fungi, 18S rRNA gene sequence (SSU) primer, Annealing blocking oligonucleotides, Co-amplification, Real-time Q-PCR, Fungal biodiversity, Taxonomic classification, Community survey, FR-1, FF390

Background

Fungi belong to a highly diverse kingdom providing key ecosystem functions. Additionally, their biosynthesis of natural products relevant for biotechnological application renders them of great interest to the research community. Yet, they are a highly understudied group with an estimated species number of up to 3.8 million but only about 120,000 being described [1]. Thus, detection and accurate classification represents one of the critical bottlenecks for fungal research.

Molecular taxon identification is mainly based on marker gene sequencing whose sensitivity, resolution and throughput are controlled by the choice of the marker gene and sequencing platform. While Sanger-sequencing is the standard for single taxon identification, Illumina MiSeq and to a less extent third generation sequencing techniques are the bases for community surveys. Fungal marker genes differ in length, resolution power among...
different fungal groups, phylogenetic power, number of publicly available sequences and available suitable primer sets [2]. The Internal Transcribed Spacer (ITS) region is the proposed barcode for fungi as it has species resolution for a very broad range of fungi compared to other fungal marker genes [3]. However, many fungal taxa recovered by environmental ITS-sequencing can often be identified solely to kingdom or phylum level due to the lack of reference sequences or reference sequences annotated only to high taxonomic levels [4]. One solution is the use of a phylogenetic marker beside the ITS allowing a phylogeny-based assignment of the fungal sequences. Hereby, sequences are inserted into a fungal phylogenetic reference tree to transfer the taxonomic information of the given branch on the query [5]. Thus, sequences originating from unknown fungal taxa can often be assigned to a lower taxonomic level. Such a double-marker gene approach has been shown to be effective in surveys targeting communities mainly composed by undescribed fungal taxa [6, 7]. In the case that the aim of a research project is the analysis of the structure and dynamic of fungal communities rather than the monitoring of known fungal taxon groups, phylogenetic marker sequencing is a promising approach.

Similarly, single taxon identification often depends on multiple markers for a precise classification to a lower taxonomic level. The first step is often a phylogeny-based classification with a pre-marker gene guiding further steps for a taxonomic fine-tuning with a group-specific marker [8].

The most prominent fungal phylogenetic markers are the 28S and the 18S rRNA gene sequences [9]. Though the 28S rRNA gene often resolves to a lower taxonomic level, most of the publicly available sequence data are 18S rRNA gene sequences [10]. In the last decades, several 18S rRNA gene sequence primers have been designed as fungi-specific, however, characteristics, overall fungal and group-specific coverage rate, and possible co-amplification with non-fungal eukaryotic taxa are rarely reported [11, 12] and comparisons among primer pairs are generally lacking.

The presented primer toolkit aims to systematically simplify the choice of the correct primer pair dependent on the research aim (community survey versus isolate classification), sequencing platform, and fungal target group. The analysis included an intensive literature research, compilation of primer, primer and annealing blocking oligonucleotides design, followed by in silico and empirical evaluation of the primer performance. The outcome is a toolkit comprising of most comprehensive lists of primers (pairs) reporting characteristics, referenced annealing position, coverage of variable regions, overall and subphyla-specific coverage rate, and co-amplification rate for a total of 164 primer, 439 primer pairs and four annealing blocking oligonucleotides.

Results

In silico evaluation of fungi-specific 18S rRNA gene sequence primer pairs

The literature research revealed a total of 164 fungi-specific 18S rRNA gene sequence primers. 100 exhibited a fungal coverage rate of ≥50% with one mismatch. The highest fungal coverage rate observed among primers was 95.9% for zero and 98.1% for one mismatch, respectively (Additional file 1). Out of the 100 single primers, 436 different primer pairs were formed including pairs already proposed elsewhere (for example [11, 13, 14]). Amplicon products mainly spanned the V4 and V5 region of the 18S rRNA gene sequence targeting the other variable regions to a much less extent. None of the primer pairs matched the V9 region (Fig. 1). However, 89% of those primer pairs were excluded from further analyses as their overall fungal coverage rate fall below the acceptable threshold (Additional file 2).

A final number of seven primer pairs fulfilled the evaluation criteria. Three primer pairs were identified suitable for Illumina or Ion Torrent sequencing (Group S), two for Sanger sequencing (Group M), and two for third-generation techniques like PacBio (Group L). None of them exceeded the value of 82.3 and 92.7% fungal coverage rate with zero and one mismatch, respectively (Table 1). All primer pairs targeted Dikarya sequences with a minimum of 70.5% under the condition of zero mismatches while the coverage rate of other phyla varied with the primer pair. However, group-specific coverage rates exceeded in general 70% under a one-mismatch-stringency with the exceptions of Cryptomycota, Entomophthoromycotina and Zoogomycota. Co-amplification of non-fungal eukaryotic sequences was low and ranged between 0.2–2.9% with zero mismatches. Thus, group-specific coverage rates of non-fungal eukaryotes stayed below 0.5% with some exceptions (Additional file 3).

Amplification conditions and success of the proposed best primer pairs

The PCR conditions and the primer performance of the proposed seven best primer pairs were experimentally evaluated. The optimal annealing temperature for the different primer sets lay within the range of 42 to 45 °C (Additional file 4). Application of the primer-specific annealing temperature led to a successful amplification of the template DNA of 12 distant fungal taxa. The success was independent from the number of PCR replicates (Additional file 5).

Design of fungi-specific primers generating a short amplicon (Group S)

The program ecoPrimers [15] suggested 20 candidate primer pairs, which were subjected to further in silico analysis with the TestPrime tool [16]. Only three primer pairs passed the evaluation criteria, all targeting the V4 and V5 region of the 18S rRNA gene sequence. Their overall fungal
coverage rate ranged between 83.4 to 86.5% and 91.1 to 94.8% for zero and one mismatch, respectively. Fungal phyla and subphyla were homogeneously targeted with a coverage rate of ≥70% under the condition of zero mismatches except Zoopagomycotina, Mucoromycotina and Entomophthoromycotina. Co-amplification of non-fungal eukaryotic sequences was high reaching 16.3 to 34.1% with zero mismatches. The highest co-amplification rate was reported for the genus *Telonema* being targeted with a minimum of 84.5% by the newly designed primers. Similarly, co-amplification caused by sequences of Stramenopiles and Alveolata exceeded for all primer pairs the coverage value of 50% (Additional file 6).

**Group-specific primer pairs**

We have screened our dataset for primer pairs applicable for the classification of fungal isolates through Sanger sequencing. In total, 15 primer groups were defined, of which three showed high group specific coverage rate at the phylum level, namely for Blastocladiomycota, Cryptomycota and Chytridiomycota, and 12 at the subphylum level. The latter group included primers specific to the three ascomycete subphyla (Pezizomycotina, Saccharomycotina, Taphrinomycotina), three of the four basidiomycete subphyla (Agaricomycotina, Pucciniomycotina, Ustilagomycotina), all three mucoromycete subphyla (Glomeromycotina, Mortierellomycotina, Mucoromycotina), and the three zoopagomycete subphyla (Entomophthoromycotina, Kickxellomycotina, Zoopagomycotina). For most of the taxonomic groups, five promising primer pairs were identified and at least one of the primer pairs exhibited a group-specific coverage rate of 85% with zero mismatches. For Cryptomycota, Entomophthoromycotina, Kickxellomycotina and Zoopagomycotina only two, one, four and two primer pairs, respectively, were meeting the evaluation criteria matching sequences of the specific taxon group with a minimum of 70%. The majority of all primer pairs covered with their amplicons the V4 and V5 regions.

![Primer pairs covering the different variable regions of the fungal 18S rRNA gene sequence.](image)
region of the 18S rRNA gene sequence. All relevant primer information can be found in the Additional file 7 including suggested annealing temperatures.

**Design of annealing blocking oligonucleotides**

To reduce co-amplification, four different annealing blocking oligos were designed, targeting sequences of Stramenopiles, Alveolata, Rhizaria (SAR group), or Telonema, respectively. Blocking oligos are modified “primers” overlapping with the primer binding sites of co-amplifiable organisms and prevent elongation through a 3'-end modification. The designed oligos for Rhizaria and Telonema targeted the attachment site of the forward primer nu-SSU-1333-5', while the oligos specific to Stramenopiles and Alveolata targeted the one of the reverse primer nu-SSU-1647-3'. A maximum of 77.1% target sequences were matched by the specific blocking oligos while the coverage rate for non-fungal eukaryotic sequences lay within the range of 0.2–18.7%. Overall fungal coverage rate was negligible with ≤0.1% and specific fungal groups were matched with a maximum of 0.8%. Solely, the Alveolata-specific blocking oligo covered 4.3% of the Zoopagomycotina sequences (Table 2, Additional file 8).

**Fungal community survey with the primer pair nu-SSU-1333-5'/nu-SSU-1647-3' (FF390/FR-1)**

A total of 1,840,354 sequence reads were generated for nine libraries (samples HR48, OSD28 and OSD36, with and without blocking oligonucleotides; three samples exclusively with blocking oligos) and assigned to 897,076 fungal sequences (Additional file 9). Amplification of the libraries with the primer pair nu-SSU-1333-5' and no blocking oligos resulted in high co-amplification rates of non-fungal eukaryotic sequences. They made up to 88.2 and 22.1% of the relative sequence abundance in the samples HR48 and OSD36, respectively. Co-amplified products belonged mainly

### Table 2

Characteristics of the best blocking oligonucleotides complementing the primer pair nu-SSU-1333-5'/nu-SSU-1647-3' (FF390/FR-1)

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>ComPrim</th>
<th>Amplicon (nt)</th>
<th>Variable regions covered</th>
<th>Fungi (%) (0 M/1 M)</th>
<th>Co-Amplif. (%) (0 M/1 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolata</td>
<td>gtcgtcctacggattga</td>
<td>nu-SSU-1647-3'</td>
<td>16</td>
<td>V7, V8</td>
<td>80.4/92.7</td>
<td>0.2/5.0</td>
</tr>
<tr>
<td>Rhizaria</td>
<td>ttaacgaacgagacctgga</td>
<td>nu-SSU-1333-5'</td>
<td>15</td>
<td>48.9</td>
<td>63</td>
<td>0.9</td>
</tr>
<tr>
<td>Stramenopiles</td>
<td>tcgcacctacggttga</td>
<td>nu-SSU-1647-3'</td>
<td>14</td>
<td>48.3</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Telonema</td>
<td>gacgctaccactaagtgta</td>
<td>nu-SSU-1333-5'</td>
<td>4</td>
<td>48.1</td>
<td>0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Fungal and non-fungal eukaryotic sequence coverage rate tested by in silico analysis

ComPrim: Sequence complement to the indicated primer

# nt: Number of identical nt's shared by primer and blocking oligo sequence

Tm: Annealing temperature

Fungi (%): Coverage rate for fungal sequences

Alv. (%): Coverage rate for Alveolata sequences

Rhiz. (%): Coverage rate for Rhizaria sequences

Stram. (%): Coverage rate for Stramenopiles sequences

Tel. (%): Coverage rate for Telonema sequences

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to the members of the Stramenopiles, Rhizaria and Alveolata. Amplification with blocking oligos increased fungal read abundance from 11.8 to 72.6% and from 77.9 to 84.3% in these two samples, while reducing the amount of non-target amplification (Fig. 2a). The fungal community of sample HR48 was dominated by Chytridiomycota (58.5%), Ascomycota (38.1%) and to a less extent by Basidiomycota and Cryptomycota, and showed a rich taxon composition on a lower taxonomic level. In contrast, the fungal community of sample OSD36 was mainly structured by taxa of the Chytridiomycota (93.1%) and to a very low extent by Ascomycota, Basidiomycota, Zoopagomycotina and Cryptomycota. In sample OSD28, Ascomycota was the dominating group with 97.1% of the relative sequence abundance and only few taxa of Basidomycota and Chytridiomycota were detected (Fig. 2b).

UniFrac permutation tests revealed no significant effect of the blocking oligos on the fungal taxa composition ($p > 0.05$) when datasets were subsampled. For the non-subsampled dataset, only sample pair HR48/HR48_BO showed a significant difference for both Weighted and Unweighted UniFrac metrics ($p < 0.05$) (Additional file 10) being the sample with the highest reported co-amplification of 88%. Co-amplification rates up to 22% like for OSD36 did not affect the description of the fungal assemblages (Fig. 2b, Additional file 10).

Comparison of the results obtained from a fungal and eukaryotic 18S tag sequencing and metagenomics approach

Within the Ocean Sampling Day (OSD) initiative 2014, the microbial communities of sample OSD28 and OSD36 were analyzed by a PCR-independent metagenomics and a general eukaryotic primer approach [17, 18]. The fungal sequence data thereby generated were compared to the fungal sequence dataset obtained with the primer set nu-SSU-1333-5’/nu-SSU-1647-3’ (FF390/FR-1). For OSD36, the fungal 18S tag sequencing detected all fungal taxa identified with the two other approaches being exclusively Chytridiomycetes. The fungal community amplified with the fungi-specific primers was composed of four additional classes. However, Chytridiomycetes clearly dominated the fungal 18S tag community with 90% of the relative sequence abundance matching the trend observed in metagenomics and eukaryotic 18S tag sequence data. Similarly for OSD28, the fungal primer approach detected all fungal taxa identified by the two other approaches. While the metagenomics dataset was exclusively composed of Dothideomycetes and Saccharomycetes sequences, common classes of the 18S tag datasets were Dothideomycetes, Eurotiomycetes, Sordariomycetes, Saccharomycetes, Exobasidiomycetes, and Agaricomycetes. Three additional classes were solely detected by the fungal
specific primer approach. Saccharomycetes dominated communities of metagenomics and eukaryotic 18S tag sequencing while the fungal 18S tag sequencing dataset was dominated by Dothideomycetes sequences (Fig. 3).

Primer performance on environmental samples
The fungal primer set nu-SSU-1333-5’/nu-SSU-1647-3’- was further tested on samples from diverse habitat types, namely brackish water, freshwater, and marine sediment. Sequences were assigned to the five phyla of Ascomycota, Basidiomycota, Chytridiomycota, Cryptomycota and Mucoromycota and classified into 26 fungal classes/subgroups. The primer set captured the variations of the community structure over the three different habitat types. The brackish and freshwater samples were dominated by diverse clades of the Chytridiomycetes but clade-composition and abundance differed between these two sample types. Conversely the marine sediment sample was dominated by Dothideomycetes and Saccharomycetes, while Chytridiomycetes ranged on third position showing similar relative abundance values as Sordariomycetes and Cryptomycota. Seven fungal classes were uniquely identified in the marine sediment sample but all with relative sequence abundance below < 0.5% (Additional file 11).

Discussion
A list of seven top primer pairs for fungal community surveys
A final number of seven primer pairs were nominated to be the best performing one for fungal community surveys based on results of the in silico analysis (Additional files 2 and 3) and experimental testing (Additional file 5). However, none of the pairs exceeded the overall fungal coverage rate of 83% under a zero-mismatch-stringency (Table 1). The coverage rate is a crucial value for the power of PCR-based biodiversity assessments. Total universality is difficult to reach with a single primer pair especially for taxon rich kingdoms. Thus, similar coverage values were reported for proposed best primer pairs specific for bacteria, archaea [19] and eukaryotes [20]. The coverage rate depends on the discrimination power of the genetic region covered by the amplicon. For fungi, the variable regions V1, V4, V5, and V9 are the most discriminating ones [2]. Interestingly, the two best proposed primer pairs of the Group S covered the variable regions V7/V8 and V8 (Fig. 1) outperforming other primer pairs by targeting all major fungal groups and showing low co-amplification (Additional files 2 and 3).

Among the seven best primer pairs (Table 1) only primer pair nu-SSU-1333-5’/nu-SSU-1647-3’ (FF390/FR-1) was already introduced as primer combination [21, 22], while all others are newly composed pairs. In contrast, the

Fig. 3 Comparison of three detection methods. Subsampled fungal communities were described by a eukaryote-specific primer (TAReu454FWD1/ TAREu454REV3_modified), a fungi-specific primer (nu-SSU-1333-5’/nu-SSU-1647-3’ (FF390/FR-1)), and a metagenomics approach. Taxonomic composition as relative sequence abundance of sample a OSD36 and b OSD28. Colored shadows between bars show which proportion (approximated) the fungal sequences detected by the eukaryote-specific and metagenomics approach represent in the non-subsampled community of the fungi-specific approach. Others in a: Dothideomycetes, Sordariomycetes. Others in b: Eukaryote-specific approach: Agaricomycetes. Fungal approach: Arthoniomycetes, Lecanoromycetes, Pezizomycetes, Agaricomycetes
combination of nu-SSU-0817-5′-24/nu-SSU-1200-3′ (old names: nu-SSU-0817-5′/nu-SSU-1196-3′) [11], currently one of the most prominent pairs used for fungal biodiversity assessments [23–25], failed to be included into the list of the top primer pairs as it did not target any sequence of the Entomophthoromycotina. Similarly, the primers nu-SSU-0214-5′ (EF4) and nu-SSU-1770-3′ (NS8) of the prominent combinations nu-SSU-0214-5′/nu-SSU-1729-3′ (EF4/EF3, [12]) and nu-SSU-0038-5′-19/nu-SSU-1770-3′ (NS1/NS8, [13]) were already discarded during the first evaluation step exhibiting too low overall fungal coverage rate (Additional file 1).

The in silico analysis revealed a taxonomic bias towards Dikarya for all the top primer pairs (Additional file 3). One problem is the overrepresentation of those groups in public sequence databases based on the fact that species of Dikarya form ~70% of the described fungal species [26] and Ascomycota is the species-richest fungal phylum [27]. Additionally, most of the fungi-specific 18S rRNA gene sequence primers were designed in the nineties or the beginning of the millennium (Additional file 1). Since then, sequence number of public databases constantly increased [28] including high numbers of sequences generated from environmental samples. The new sequence information led to the discovery of so far undiscovered fungal clades [29, 30] and the refinement of fungal taxonomy [31]. However, most existing 18S rRNA gene sequence primer pairs were not tested on updated sequence databases. Our results demonstrate that already a reshuffling of existing primers into new pairs increased their performance (Additional file 2) and underline the necessity of a regular reevaluation of existing primer sets.

Another step of our study was the design of primer pairs on a sequence database that included all recent submissions. Hereby, we focused solely on primer pairs generating a short amplicon (Group S) suitable for the sequencing techniques widely used for fungal community surveys. Several design strategies were tested leading to a final number of three primer pairs fulfilling the evaluation criteria. They exhibited an overall fungal coverage rate slightly higher than the ones identified by the literature research and covered the different fungal taxon groups more homogeneously. In contrast, the non-fungal eukaryotic co-amplification of the three primer pairs was not acceptable and co-amplification targeted many more eukaryotic groups compared to the top primer pairs (Additional files 3 and 6). These results indicate the limitations of primer design for a relative conserved marker like the 18S rRNA gene aiming to target the great majority of sequences of a taxon-rich group as Fungi. When a certain threshold of fungal sequence coverage was reached, co-amplification rate quickly increased and vice versa. One possibility to overcome this limitation is the use of more than one primer set, which may, however, negatively affect comparability and semi-quantification.

Co-amplification is regularly reported for rRNA sequence-based fungal community surveys [7, 11, 12] and is caused by the conflict to find primer pairs possessing a low Shannon entropy [32] among fungal taxa but a high one against non-fungal eukaryotic groups. The position of the mismatch with non-target taxon groups is hereby very important as it influences the PCR sensitivity [33, 34]. Degenerated primers might be particularly prone to mismatching as the permuting position results in different binding energies to the nucleotides of the template DNA [35]. Three primer pairs of the top primer pair list have up to two wobbles. Surprisingly, their non-fungal eukaryotic co-amplification rate stayed below 3% with zero mis-matches outperforming the newly designed primer pairs having no wobbles (Additional files 3 and 6).

A compiled primer list for the amplification of fungal isolates by sanger sequencing

Fungi are a rich and promising source of novel biotechnological and medical agents. Compounds discovery often follows the classical discovery approach using culture based isolation techniques to screen isolates in bio-assays [2]. The taxonomic classification of the isolated strains is based on a multiple-marker gene approach with the 18S rRNA gene sequence being one of the prominent markers [36]. Unfortunately, the 18S rRNA is reported to be the fungal marker with the highest PCR failure rate among markers of the rRNA [3]. The primer pair influences beside other parameters the PCR success. Thus, in case of amplification failure, a solution can be the change of the primer pair towards a pair with a high coverage rate for the target group. However, only few group-specific fungal 18S rRNA gene sequence primers have been designed [37–39] and in general, no information on coverage rates on lower taxonomic levels is provided for primers in literature. Thus, the selection of an appropriate alternative can be highly time-consuming.

In this study, we compiled an additional list with primer pairs independent of their overall fungal coverage rate but adequate for the amplification of diverse fungal phyla or subphyla. For each pair, amplicon length, variable regions covered, and a proposed annealing temperature was documented. As for the top primer list, the large majority of primer pairs were newly combined pairs (Additional file 7) outperforming existing pairs, which were specifically designed for a single taxon group [37].

Performance of primer pair nu-SSU-1333-5′/nu-SSU-1647-3′ (FF390/FR-1) on environmental samples

Based on the in silico analysis, the primers nu-SSU-1333-5′ and nu-SSU-1647-3′ (FF390 and FR-1) were proposed as best performing pair for the Group S. This primer pair is prominent for DGGE analysis [21, 40, 41] and less used for high-throughput sequencing [22, 42, 43]. In this study,
we evaluated its performance with focus on taxonomic bias, co-amplification, and different source material. Fungal communities of four habitat types were analyzed. The results indicated a habitat-specific composition of the communities. Thus, Chytridiomycetes dominated nearly all aquatic fungal communities but differed in abundance and clade composition (Fig. 2, Additional file 11). Zoosporic fungi are known to significantly shape marine and freshwater communities being often highly abundant and playing important roles in the ecosystems [44–47]. In contrast, the fungal community of the sediment sample was more diverse being composed of 21 fungal classes (Additional file 11). Similar values have been reported for fungal soil communities [48, 49]. Additionally, many of the fungal classes detected with this primer set are among those one that dominate soil communities on a global scale [50]. These results attest for a good performance of the primer set nu-SSU-1333-5′/nu-SSU-1647-3′, when applied to environmental samples independent from the habitat type, fungal diversity or composition. It further contrasts with the results of the in silico analysis, which showed a taxonomic bias towards Dikarya (Additional file 3). The primer set was able to capture the various fungal compositions even when the fungal community was dominated by non-Dikarya taxa (Fig. 3b, Additional file 11). This kind of discrepancy between results of in silico analyses and empirical tests is a well-known issue and emphasizes the necessity to use both approaches to validate a primer pair for environmental studies. A careful selection of the primer set and the use of adequate PCR conditions further assist in receiving a reflection of the true picture of natural microbial communities [51].

In a final step, the results obtained for samples OSD28 and OSD36 through a fungal and eukaryotic 18S tag sequencing and PCR-free metagenomics approach were compared. In spite of certain statistical differences, the fungal primer approach detected all fungal taxon groups found by the two others but led to a deeper resolution of the fungal community, even when being subsampled (Fig. 3). Fungal sequences in marine metagenomics and eukaryotic 18S tag sequence datasets are generally represented by a small amount of the total sequence reads [52, 53]. Thus, for OSD28, only one OTU of the most abundant Dothideomycetes OTUs detected by the fungal primer approach was also detected by the eukaryotic 18S tag sequencing. Additionally, abundance values can significantly change when the same community is sequenced by different marker genes or (variable) regions [54]. Here, the V4 and V7/V8 of the 18S rRNA gene sequence was targeted by the eukaryotic and fungal 18S primer set, respectively. Metagenomics can recover a somehow similar taxonomic overview but suffer from the inability to infer fungal OTUs and from high uncertainty in identification [54] explaining the low fungal diversity detected in the two OSD samples (Fig. 3).

**Co-amplification of non-fungal eukaryotic sequences**

Our results revealed that the matching of the primer sequence with the few co-amplified groups of the in silico PCR with 0.2 and 5% of relative sequence abundance under a zero and one mismatch-stringency, respectively, became a relevant problem in environmental surveys. Up to 88% of sequence reads of the marine samples were non-fungal co-amplified products (Fig. 2b). One possibility to reduce co-amplification of non-target organisms is the use of blocking oligos [55, 56]. In this study, four types of blocking oligos were designed targeting Stramenopiles, Alveolata, Rhizaria, and *Telonema* (Table 2).

The addition of these blocking oligos to the PCR and blocking of other non-fungal eukaryotic groups (Additional file 8) in the fungal community surveys resulted in a relevant reduction of the co-amplified sequences while none of the fungal groups were lost (Fig. 2b). UniFrac permutation tests confirmed no effect of the blocking oligos on the description of fungal assemblages (Additional file 10). Thus, the observed differences in abundance of fungal classes (Fig. 2b) were not caused by the presence of distant taxa in the two communities but by different abundance values of taxa being present in both assemblages. Anyhow, these differences were not significant. However, the high amount of co-amplified sequences biased the view on the fungal assemblage as shown for sample HR48 (Additional file 10), why the use of blocking oligos is recommended for samples risking high co-amplification. Nevertheless, the use of blocking oligos cannot guarantee a complete reduction of the target organisms (Fig. 2b). The SAR group consist of the most diverse protistan supergroups with more than 25,000 morphospecies of Stramenopiles and 10,000 of Alveolata and Rhizaria being described [8] and new clades being continuously discovered [57, 58]. The design of a single blocking oligo covering all sequences of such large and diverse target groups is not possible (Table 2). So far, only few studies were reporting the application of blocking oligos for environmental sequencing using up to two blocking oligos in the same PCR [59, 60]. We could show that the simultaneous use of four blocking oligos was effective. However, it is unclear if there is a limitation for the number of blocking oligos used in a single PCR, especially when "universal" blocking oligos are used which may co-effect each other and lead to uncontrolled co-blocking of sequences.

The primer pair nu-SSU-1333-5′/nu-SSU-1647-3′ (FF390/FR-1) has further been proposed as the candidate for quantifying fungal biomass by real-time Q-PCR [61]. The authors validate their results with a cloning/Sanger-sequencing step of fungal soil communities detecting no co-amplified products. They concluded that the primer pair is suitable for quantification of soil fungi and remark that non-fungal eukaryotic groups with a risk of
co-amplification like Alveolata and Stramenopiles do not occur in soil. However, these groups display an abundant part of the diverse eukaryotic fractions in marine realms such as ocean surface water [53], deep sea and hydrothermal vents [62], and freshwater systems [63] and can reflect a significant portion of non-target amplification products (Fig. 2b) [7, 64]. Consequently, the application of the primer pair nu-SSU-1333-5′/nu-SSU-1647-3′ (FR-1/FF390) is not suitable for aquatic samples as realized by Taylor and Cunliffe [65] without a careful check of co-amplified groups by a sequencing step. Failure to do so can lead to sample amplicons being dominated by co-amplified non-fungal products leading to wrong fungal biomass estimations.

**Conclusions**

The choice of primers is an essential step in the workflow of fungal taxonomic classification controlling the specificity of amplification. Most often, primer pairs are chosen based on comparable research studies, although they may not be the best choice in terms of efficiency and target specificity. This study revealed a high variation among 18S rRNA fungal specific primers and their characteristics, which reflects the variety of research issues and techniques for which, and the time point when, primers were designed. Thus, primer pairs highly differed in their (total) fungal coverage rate on higher as well as on lower taxonomic levels and in their non-fungal eukaryotic co-amplification. The total fungal coverage rate was for most of the primer pairs even too low to be recommended for the description of fungal communities. Only seven of the 439 tested primer combinations fulfilled the evaluation criteria. Surprisingly, six of them were new primer combinations of existing primers. Besides, some other primer pairs were identified as suitable candidates for the phylogenetic classification of isolates as they exhibit high coverage rates of specific fungal taxon groups. This illustrates the necessity for a careful selection of primer pairs and PCR strategies, which will differ dependent on the research question.

The in silico analysis attested that all primer pairs have very small rates of non-fungal eukaryotic co-amplification. These values are in the range of fungal primers in general, which are often neglected as they do not cause problems for the sequencing output. By contrast, co-amplifying groups were represented by high numbers of generated sequences in some samples of our study. For the primer pair nu-SSU-1333-5′/nu-SSU-1647-3′ (FF390/FR-1), this may be of special importance when applied to marine samples. Although our designed blocking oligos effectively reduced co-amplification, it may be necessary to adapt and/or design new blocking oligonucleotides for different type of sample and habitats. Most important, these results emphasize that “fungi-specific” 18S rRNA primers cannot directly be used for fungal biomass assessment by real-time Q-PCR without a prior assessment of the PCR specificity by a sequencing step.

The selection of the right primer pair adapted to the research issue and sequencing technique is often time-consuming. To remedy this issue, we developed this primer toolkit which provides the gap by providing in-depth information on fungal primers. The primer toolkit further complements the already existing (fungal specific) 18S rRNA gene sequence tools. In combination, they allow now an easy and straight-forward (phylogeny-based) classification of fungal query sequences in a user-friendly manner.

**Methods**

**Compilation of a comprehensive primer list**

A comprehensive literature research on fungi-(group)-specific 18S rRNA gene sequence primers was conducted in March 2015. Search engines like “Web of Knowledge” [66], “Google Scholar” [67] and “Google” [68] were browsed with keywords including “fungi”, “primer”, “SSU” or “18S”, “fungal community”, “environmental sample”, and names of fungal phyla/subphyla. If needed, the sequence format of the identified primers was adjusted to the IUPAC wobble system [69]. Primer-specific characteristics were calculated including the GC-content, basic and salt adjusted melting temperature ($T_m$) using the program OligoCalc [70]. Positions of the primers were referenced against the non-redundant SSU Ref database version r126 allowing zero or one mismatch using the TestProbe 3.0 tool [16]. The fungal coverage is defined as the percentage of fungal sequences from the total number of fungal sequences being matched by the primer. Only primers covering at least 50% of the fungal sequences with one mismatch were used for further analyses. Primers were assembled into pairs whenever the respective melting temperatures showed $< 5 \, ^\circ C$ difference. The resulting primer pairs were divided into three groups according to the expected amplicon size: (i) Group small (Group S) with a generation of fragments ≤600 bp, (ii) Group middle (Group M) generation of fragments between 600 to 1,000 bp, and (iii) Group large (Group L) generation of fragments > 1,000 bp.

Primer pairs were subjected to in silico evaluations to analyze co-amplification, overall and fungal phyla/subphyla coverage rate using the same settings and sequence dataset as described above but using TestPrime
1.0 as evaluation tool [16]. Fungal taxonomy of the underlying SILVA dataset was manually adjusted to the new fungal taxonomy for zygomycete fungi [31]. Variable regions covered by the amplicon, amplicon length and start/end position was noted for each primer pair. For biodiversity assessments, only primer pairs meeting the following criteria were further shortlisted: (i) ≥65 and ≥75% fungal coverage with zero and one mismatch, respectively, (ii) targeting all major fungal phyla and subphyla, and (iii) < 20% co-amplification of non-fungal eukaryotic organisms with the parameter of one mismatch. Primer pairs were ranked based on the highest number of fungal coverage together with the lowest non-fungal eukaryotic co-amplification with special focus on groups reported to be highly problematic in marine samples [7, 64], namely Stramenopiles, Alveolata, Rhizaria, and Telonema.

To define best primer pairs suitable for classification of specific fungal phyla/subphyla, only primer pairs with < 20% co-amplification under a zero-mismatch-stringency and < 30% co-amplification under a one-mismatch-stringency were further analyzed. From those, up to five best primer pairs were recorded for each subphyllum where the subphylum-specific coverage exceeded 70%. This search was solely conducted for the primer pairs belonging to the Group M as Sanger sequencing is the method of choice for classification of fungal isolates.

**Primer design for the amplicon category < 600 bp**

It was further tested if a new primer pair for the Group S can be designed that outperforms the best primer pairs recognized by the above mentioned approach. Primer design was performed by the ecoPrimers program v 1.0 [15] using the manually curated high-quality 18S rRNA gene sequence alignment containing 12,870 fungal nearly full-length sequences [10]. To evaluate possible co-amplification, a non-fungal eukaryotic sequence reference database was prepared. Therefore, eukaryotic non-fungal sequences of the National Center for Biotechnology Information (NCBI) non-redundant nucleotide sequence Genbank database, release 213 [73] were used and enriched by sequences from the SILVA database being not redundant to the first one resulting into a final non-fungal eukaryotic 18S rRNA gene sequence number of 101,067. Different design strategies were tested changing parameters (0.5 < sensitivity quorum < 0.8; 0.5 < strict matching quorum < 0.8; 0.1 < false positive quorum < 0.3), target groups (all fungal groups; each fungal group separately), and databases (all fungi and outgroup sequences; only fungi; only basal fungi, i.e. excluding Dikarya and Glomeromycotina). The primer pairs were further filtered with the following parameters: (i) targeting all fungal groups, (ii) ≤ 20% co-amplification, (iii) ≤ 600 bp amplicon generation, (iv) primer length between 18 to 21, (v) most specific primer in the pair with the lowest Tm, and (vi) ≤ 10 °C Tm difference between both primers in the pair. Next, the 20 primer pairs with the highest minimum barcode coverage ($B_{min}$, the proportion of target species amplified in-silico) and barcode specificity ($B_{s}$, the proportion of species in-silico amplified which are unambiguously identified) value as well the lowest co-amplification rate were selected. Detailed match of the selected primers with the databases sequences were produced with ecoPCR program v 0.8 [74], allowing until 3 mismatches. When a primer matched the target groups with multiple variants, a consensus primer with degenerated nucleotides was built in order to improve the coverage of target groups. In a final step, consensus primer pairs were subjected to the same in silico evaluation approach described in the paragraph "In silico evaluations".

**Annealing blocking oligonucleotide design**

Group-specific blocking oligonucleotides were designed for the eukaryotic SAR group and Telonema species targeting the annealing region of one of the two primers nu-SSU-1333-5′ and nu-SSU-1647-3′ (FF390/FR-1), [21, 22] identified to form the best performing primer pair within the Group S. In a first step, the SILVA database was amplified with the best primer pair of the Group S using the ecoPCR program with the setting of a maximum of one mismatch per primer. Next, the in silico amplified sequences including the primer sequences at both ends were split among the different taxonomic groups and replicated. Dictionaries of 18- to 25-mer blocking oligos with at least 3 nt overlap with one of the two primers were created for each co-amplified outgroup. Finally, candidate blocking oligos were selected among those with the best coverage for the target group, the smaller cumulative coverage of fungal groups while having a similar $T_m^*$ to the best fungi-specific primer pair of the Group S.

**Fungal cultures**

As the selection of the best primer pairs was based on the in silico analysis, the next step was the proof of successful in vitro amplifications. Primer pairs were tested to amplify template DNA derived from various taxonomic fungal groups. Fungal taxa were selected to cover the major part of the fungal tree on higher taxonomic level. Thus, for each subphyllum of the Dikarya and for three distant phyla of the Fungi *Incertae sedis* a representative taxon was chosen. Six of the 12 strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH (Braunschweig, Germany), namely *Ustilago maydis* (DSM 4500, Ustilagomycotina, Basidiomycota), *Leucosporidium scottii* (DSM 4636, Pucciniomycotina, Basidiomycota), *Wallemia sebi* (DSM 5329, Wallemiomycotina, Basidiomycota), *Taphrina deformans* (DSM 4398, Taphrinomycotina, Ascomycota), *Coenansia erecta* (DSM 6933, Kickxellomycotina, Zoopagomycota), *Cladosporium herbarum* (DSM 6933, Zoopagomycotina, Zoopagomycota), *Acremonium strictum* (DSM 40888, Plectomycetidae, Basidiomycota), *Bipolaris oryzae* (DSM 20799, Ustilaginomycotina, Ustilaginomycota), and *Entomophthora muscae* (DSM 5621, Entomophthoromycota, Entomophthoromycota).
and *Allomyces arbuscula* (DSM 955, Blastocladiomycota). Two additional strains were isolated during an excursion to the Jadebusen (Germany, 53.441293, 8.295822) by Dr. Marlis Reich on the 11th of September 2013, namely *Davidiellaceae* sp. (CB2, Pezizomycotina, Ascomycota) and *Didymellaceae* sp. (CA1, Pezizomycotina, Ascomycota) from the seawater and surface sediment, respectively. Fungal strains were grown for 3 weeks at 18 °C in the dark on Malt Extract Peptone Agar (30 g/l malt extract, 3 g/l soya peptone, 15 g/l agar) (strain DSM 4500), Potato Dextrose Agar (20 g/l glucose, 15 g/l agar solved in infusion of potatoes) (strains DSM 4636, DSM 4398), YpSs Medium [75] (strain DSM 955), M 40 Y medium (400 g/l sucrose, 20 g/l malt extract, 5 g/l yeast extract, 20 g/l agar) (strain DSM 5329), or Czapek-Dox medium [76] (strains CB2, CA1). Six small colonized agar pieces were transferred to liquid medium, grown for 4 weeks at 18 °C in the dark on a Pro- max 2020 shaker (Heidelberg, Karlsruhe, Germany) at 110 rpm. Biomass was harvested over a 3 μm particle retention round filter (Grade 389, Sartorius, Goettingen, Germany) and stored at –20 °C for further treatment.

Cell material of four other fungal strains were provided by Prof. Dr. Imhoff from the KSMP (Kultur Sammlung Mariner Pilze) culture collection (GEOMAR, Kiel, Germany), namely *Candida mesenterica* (MF249, Saccharomycotina, Ascomycota), *Pichia anomala* (LF964, Saccharomycotina, Ascomycota) and *Mucor fragilis* (KF737, Mucoromycotina, Mucoromycota). A fruiting body of *Agaricus bisporus* (Agaricomycotina, Basidiomycota) was sampled from a compost heap in Bremen (Germany, 53.104635, 8.895263) by Dr. Marlis Reich on the 15th September 2015. It was cut under sterile conditions in pieces and the inner stem tissue directly below the carpophore was sampled and stored at –20 °C.

Finally, the genomic DNA was extracted from 0.5 g of freeze-grinded tissue of each fungal species using the innuPREP Bacteria DNA kit (jenaAnalytica, Jena, Germany) following the manufacturer’s instructions.

**PCR efficiency of the best primer pairs for biodiversity assessments**

In a first step, the optimal annealing temperatures for the best primer pairs of each amplicon-size group were defined in a gradient PCR approach: for each primer pair a range of eight different annealing temperatures was tested using the lower T_m of both primers within a pair as middle value. Subsequently, with steps of 0.5 °C three temperatures lower and four higher than the middle temperature were tested.

The PCR reactions were conducted in 20 μL volumes containing 1/10th volume of 10x Dream Taq DNA Buffer (Thermo Fischer Scientific, Darmstadt, Germany), 1 μM Bovine Serum Albumin (GeneON, Ludwigshafen, Germany), 200 μM dNTPs (Fermentas Thermo Fischer Scientific, Pittsburgh, PA USA), 0.2 μM of each primer (Eurofins Genomics, Ebersberg, Germany), 0.5 U Dream Taq DNA polymerase (Thermo Fisher Scientific) and 50 ng/μL of the template DNA on a peqSTAR 2x double block thermocycler (peqlab Biotechnologie GmbH, Erlangen, Germany). Genomic DNA of *Taphrina deformans* and *Agaricus bisporus* served as template DNA. Each PCR was repeated three times independently.

The PCR conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, calculated annealing temperatures for 60 s, extension at 72 °C for 90 s and a final extension step at 72 °C for 10 min. Successful amplification was checked on a 2% agarose gel, stained with ethidium bromide and visualized with a UVP Benchtop 2UV Transilluminator (UVP, LLC, Upland, USA). The best annealing temperature for a primer pair was defined as temperature where for both template strains the strongest band intensity was observed. In the case of having DNA bands of the same intensity, the median of the optimal temperature was chosen.

Finally, the in vitro performance of the four primer pairs was tested on the above mentioned 12 fungal species following the same PCR conditions and using the proposed best annealing temperature for each primer pair.

**Fungal community analysis**

The best primer pair of the Group S (nu-SSU-1333-5′/nu-SSU-1647-3′) and its corresponding blocking oligos were tested for their performance in diverse fungal biodiversity assessments. Surface water biomass of three marine, one brackish water and one freshwater samples (all 0.2–0.5 m depth), as well as biomass of one sediment sample served as DNA templates. Two of the three marine samples were taken during the OSD campaign on the 21st of June 2014. Sample OSD28 originated from a back reef environment at Belize in the Caribbean Sea (16.8025, –88.0816) and sample OSD36 from the Woodland Beach of Delaware, USA at the North Atlantic (39.3322, –75.4699) (for more information see [18]). The third marine sample was taken at the HR station (54.1833, 7.9) on the 7th August 2015 kindly provided by Dr. Gunnar Gerdts and Dr. Antje Wichels of the Alfred-Wegener-Institute Helgoland (AWI). The brackish (53.9817, 8.405) and freshwater (53.4744, 9.9837) samples were obtained at two stations of a transect from the island of Helgoland to the Elbe river (Germany) during a cruise with the research vessel Uthörn on the 5th of August 2015. In all cases, a maximum of two liters of water was filtered on a Sterivex membrane (0.2 μm pore size, hydrophilic PVDF Durapore membrane, Merck, Darmstadt, Germany), stored at –20 °C until DNA extraction with the Power Water DNA Isolation Kit (MoBio, Carlsbad, CA, USA) following the manufacturer’s instructions. The sediment sample was obtained from a 5 m long gravity core (HE443–010-3; 54.0865, 7.9701) which was collected on the RV HEINCKE, cruise HE443
on 30th of April 2015 kindly provided by Prof. Dr. Sabine Kasten. 5 g of sediment sample was collected at regular depth intervals and DNA was extracted from 1 g of sediments as described in Oni et al. [77].

For each of the three marine surface samples, two sequencing libraries were prepared: one solely with the fungi-specific primer pair and one including additional the four different group-specific blocking oligos. The libraries of the other three samples were all prepared with the addition of the blocking oligos. Library preparation and sequencing were performed at LGC Genomics GmbH, Berlin, Germany. All sequencing reactions were based upon an Illumina MiSeq chemistry following the manufacturers’ instructions. Sequence data can be obtained from INSDEC with accession number PRJEB25747. Generated community data was compared to data obtained by two further approaches including a general eukaryotic primer based (TAReuk454FWD1/TAReuk-REV3_modified, [78]) and a PCR-independent metagenomics approach [17].

Sequence processing analysis
Generated sequence reads were delivered in an already demultiplexed form from which adapter and primer sequences were removed. Further sequence processing followed the OSD’s protocol for 18S rRNA gene sequence data [17] including a merging, length and quality trimming step. Next, quality-checked sequence reads were clustered into operational taxonomic units (OTUs) and taxonomically assigned by the SILVAngs pipeline v 1.6 [16] based on the SILVA non-redundant database 123 using the default parameters but setting the sequence similarity threshold to 98%. OTUs represented by less than five sequence reads and/or no taxonomic assignment were discarded. Finally, sequences were subsampled using the sample with the smallest read output as a reference over the sub-sample function in Mothur v1.25.0 [79]. Sequence processing and assignment of the eukaryotic and metagenomics libraries followed the same conditions.

Statistical analysis
To test for an effect of blocking oligos on fungal taxon groups, a UniFrac pairwise significance test was run [80]. For each of the samples HR48, OSD28, and OSD36, four datasets were compiled. They were composed of subsampled and non-subsampled community data generated solely with primers or primers and blocking oligos. The latter case aimed to test if the amount of co-amplified sequences affected the community structure. Tests were run with the program PyCogent 1.9 [81] using unweighted and weighted UniFrac metrics permuting 1000 times. Fungal assemblages of samples were defined to be significant different with a Bonferroni corrected $p$-value of $P < 0.05$.

Additional files

- **Additional file 1:** List of the 164 fungi-specific primers detected by a literature research. For each primer, performance, characteristics and source literature are provided. (XLSX 36 kb)
- **Additional file 2:** List of the 436 fungi-specific primer pairs tested for their performance by in silico PCR. Primer pairs were grouped according to the expected amplicon size into three groups; $S$ for small ($\leq 600$ bp), $M$ for medium ($600$–$1000$ bp), and $L$ for large size ($> 1000$ bp). (XLSX 124 kb)
- **Additional file 3:** List of the seven most promising primer pairs for different sequencing methods dependent on the expected amplicon size. Sequence coverage rate of diverse fungal and non-fungal eukaryotic groups as revealed by in silico PCR. (XLSX 15 kb)
- **Additional file 4:** Annealing temperatures empirically evaluated for the most promising primer pairs. Two fungal strains, one of the Basidiomycota and one of the Ascomycota, served as template DNA. Intensity of the color indicates the strength of the amplification product detected by ethidium bromide staining. Red, template DNA from Taphrina deformans; Green, template DNA from Agaricus bisporus; $*$, optimal annealing temperature. (XLSX 60 kb)
- **Additional file 5:** Performance of the most promising primer pairs empirically tested on 12 fungal strains. (XLSX 10 kb)
- **Additional file 6:** List of the three newly designed primer pairs passing the evaluation criteria. Primer performance was tested as for other primer pairs. Characteristics and sequence coverage rates of fungal and non-fungal eukaryotic groups are given. (XLSX 12 kb)
- **Additional file 7:** Primer pairs suitable for the amplification of specific fungal phyla/subphyla. Characteristics of the primer pair and sequence coverage rate of the target group is indicated. (XLSX 19 kb)
- **Additional file 8:** List of the designed annealing blocking oligonucleotides for the eukaryotic groups Stramenopiles, Alveolata, Rhizaria and Telosoma. Characteristics and sequence coverage rates of fungal and non-fungal eukaryotic groups are given. (XLSX 11 kb)
- **Additional file 9:** Information on sequence generation and downstream analysis process. (XLSX 9 kb)
- **Additional file 10:** UniFrac pairwise permutation test. The effect of blocking oligos (BO) and amount of co-amplified sequences on fungal community structure was tested. Bonferroni-corrected $p$-values are reported. Source datasets were the subsampled and non-subsampled community data of samples OSD28, OSD28_BO, OSD36, OSD36_BO, HR48, HR48_BO. *Significant difference of fungal assemblage ($p < 0.05$). (XLSX 10 kb)
- **Additional file 11:** Taxonomic composition of three environmental samples. Bar chart indicates relative sequence abundance of the different fungal classes/subgroups amplified by the primer pair nSSU-1333-5’nu-SSU-1647-3’ (FF390/FR-1). Others: Blastocladiales, Glomeromycetes, Monoblepharidomycetes, Pucciniomycota_Incertae sedis. (PDF 192 kb)

Abbreviations

- Bc: Barcode coverage; $bp$: Basepair; Bs: Barcode specificity; DGGE: Denaturing gradient gel electrophoresis; DNA: Deoxyribonucleic acid; dNTP: Deoxyribonucleotide triphosphate; D5SA: Deutsche Sammlung von Mikroorganismen; D5SA2: Deutsche Sammlung von Mikroorganismen und Zellkulturen; HR Helgoland Roads; INSDC: International nucleotide sequence database collaboration; ITS: Internal transcribed spacer; IUPAC: International union of pure and applied chemistry; KSMAP: Kultursammlung mariner Pilze; M 40: Y: Medium for osmophilic fungi; mnr: Minute; NCBI: National center for biotechnology information; nt: Nucleotide; OSD: Ocean Sampling Day; OTU: Operational taxonomic unit; PCR: Polymerase chain reaction; PFGE: Polyvinylidenefluorid; Q-PCR: Quantitative polymerase chain reaction; RNA: Ribonucleic acid; rRNA: Ribosomal ribonucleic acid; S: Svedberg, unit for the sedimentation rate as for the 18S rRNA; SAR: Stramenopiles Alveolata Rhizaria; sec: second; SSU: Small subunit of ribosomal ribonucleic acid; Ult, $T_m$: Melting temperature; V1-V9: Variable region 1 of 18S rRNA gene sequence; YpSs: Yeast powder soluble starch.
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The authors are grateful to Joanna Hoinis for supporting the literature research of primers and Yanyan Yang for technical support. We further thank the captain and the crew of the RV UTHÖRN, the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Dr. Gunnar Gerds and Dr. Antje Wichels to provide us with a water surface sample taken at the Helgoland Roads Station, Germany. We would like to thank the captain and the crew of the RV HEINCKE, the AWI, Prof. Dr. Michael W. Friedrich and Prof. Dr. Sabine Kasten for providing sediment sample from the Helgoland Mud Area, Germany. We further acknowledge Prof. Dr. Johannes F. Imhoff contributing fungal cell material.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article (and its additional file(s)). The sequence datasets generated during the current study are available over the INSDC with the accession number PRJEB25747. Eukaryotic amplicon and metagenome sequences can be downloaded over the accession numbers ERR867765, ERR771018 and ERR867749, ERR771012 for sample OSD28 and OSD36, respectively.

Authors’ contributions

AK, MR, TW and FOG discussed, planned and designed the study. Data analyses: SB, GL. Writing of the paper: SB, MR. All authors contributed to the revision of the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

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Consent for publication

Not applicable.

Competing interests

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References


Correction to: A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms

Stefanos Banos¹, Guillaume Lentendu²,³, Anna Kopf⁴, Tesfaye Wubet²,⁵,⁷, Frank Oliver Glöckner⁴,⁶ and Marlis Reich¹*

Correction to: BMC Microbiol

https://doi.org/10.1186/s12866-018-1331-4

Following publication of the original article [1], we have been notified that three of the primer names identified as most promising candidates for fungal community surveys were incorrectly renamed following the primer nomenclature system proposed by Gargas & DePriest [2]. Their positioning on the reference sequence had to be shifted 1bp towards the 3'-end (see Table 1 for the correct naming). The same error occurred in some primer names listed in the additional files (see attached Supplementary information).

As consequence, the number of identical nucleotides shared by the most promising primers and the newly designed blocking oligo sequences changed (see Table 2).

In this correction, the revised supplementary materials are included.

### Supplementary information

**Additional file 1.** List of the 164 fungi-specific primers detected by a literature research. For each primer, performance, characteristics and source literature are provided.

**Additional file 2.** List of the 436 fungi-specific primer pairs tested for their performance by in silico PCR. Primer pairs were grouped according to the expected amplicon size into three groups: S for small (<600 bp), M for medium (600–1000 bp), and L for large size (>1000 bp).

**Additional file 3.** List of the seven most promising primer pairs for biodiversity assessments identified by in silico PCR. Primer pairs are suitable for different sequencing methods dependent on the expected amplicon size. Sequence coverage rate of diverse fungal and non-fungal eukaryotic groups as revealed by in silico PCR.

**Additional file 4.** Annealing temperatures empirically evaluated for the most promising primer pairs. Two fungal strains, one of the Basidiomycota and one of the Ascomycota, served as template DNA. Intensity of the color indicates the strength of the amplification product detected by ethidium bromide staining. Red, template DNA from *Taphrina deformans*; Green, template DNA from *Agaricus bisporus*; *, optimal annealing temperature.

**Additional file 5.** Performance of the most promising primer pairs empirically tested on 12 fungal strains.

**Additional file 6.** Primer pairs suitable for the amplification of specific fungal phyla/subphyla. Characteristics of the primer pair and sequence coverage rate of the target group is indicated.

**Additional file 7.** List of the designed annealing blocking oligonucleotides for the eukaryotic groups Stramenopiles, Alveolata, Rizaria and Telorhiza. Characteristics and sequence coverage rates of fungal and non-fungal eukaryotic groups are given.

**Additional file 8.** Taxonomic composition of three environmental samples. Barchart indicates relative sequence abundance of the different fungal classes/subgroups amplified by the primer pair nu-SSU-1334-5/nu-SSU-1648-3 (FF390/FR-1). Others: Blastocladiomycetes, Glomeromycetes, Monoblepharidomycetes, Pucciniomycotina_Incertae sedis.

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Table 1 Characteristics and in silico performance of the best primer pairs. Primer pairs were grouped according to the expected amplicon size into three groups: S for small (≤600 bp), M for medium (600 – 1,000 bp), and L for large size (>1,000 bp). Fungal and non-fungal eukaryotic sequence coverage rates tested by in silico PCR. Individual primer sequence and characteristics are listed in the Additional file 1. For primer pairs see Additional file 2.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Old name</th>
<th>Amplicon (nt)</th>
<th>Var. regions covered</th>
<th>Fungi (%)</th>
<th>Co-Amplifi. (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0M/1M</td>
<td>0M/1M</td>
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<td><strong>Group S</strong></td>
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<td></td>
<td></td>
<td></td>
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<td>nu-SSU-1334-S'/nu-SSU-1648-3'</td>
<td>FF390/FR-1</td>
<td>348</td>
<td>V7, V8</td>
<td>80.4/92.7</td>
<td>0.2/5.0</td>
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<td>SR14R/FR-1</td>
<td>235</td>
<td>V8</td>
<td>76.8/86.0</td>
<td>0.8/2.5</td>
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<td>V1, V2, V3</td>
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<td>1.5/8.0</td>
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<td>75.8/86.2</td>
<td>0.5/4.5</td>
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<tr>
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<td>Basid 3/FR-1</td>
<td>904</td>
<td>part of V4, V5, V6, V7, V8</td>
<td>68.3/80.8</td>
<td>2.9/14.7</td>
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<td></td>
</tr>
<tr>
<td>nu-SSU-0068-S'/20/nu-SSU-1648-3'</td>
<td>Fun18S1/FR-1</td>
<td>1615</td>
<td>all except V9</td>
<td>82.3/90.3</td>
<td>2.3/6.8</td>
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<td>1133</td>
<td>V4, V5, V7, V8</td>
<td>73.1/88.4</td>
<td>0.9/2.0</td>
</tr>
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</table>

Amplicon (nt), length of generated amplicon
Fungi, coverage rate of fungal sequences with zero (0M) and one (1M) mismatch
Co-Amplifi., non-fungal eukaryotic co-amplification

Table 2 Characteristics of the best blocking oligonucleotides complementing the primer pair nu-SSU-1334-S'/nu-SSU-1648-3' (FF390/FR-1). Fungal and non-fungal eukaryotic sequence coverage rate tested by in silico analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>ComPrim</th>
<th>#nt</th>
<th>T_m, (°C)</th>
<th>Fungi (%)</th>
<th>Alv. (%)</th>
<th>Rhiz. (%)</th>
<th>Stram. (%)</th>
<th>Tel. (%)</th>
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</thead>
<tbody>
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<td>gtcgctcctaccgattga</td>
<td>nu-SSU-1648-3’</td>
<td>12</td>
<td>50.3</td>
<td>0.08</td>
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<td>6.3</td>
<td>0.9</td>
<td>3.3</td>
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<td>Rhizaria</td>
<td>ttaacgaacgacctcga</td>
<td>nu-SSU-1334-5’</td>
<td>16</td>
<td>48.9</td>
<td>0</td>
<td>0</td>
<td>24.3</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Stramenopiles</td>
<td>tcgcacctaccgattagaa</td>
<td>nu-SSU-1648-3’</td>
<td>13</td>
<td>48.3</td>
<td>0</td>
<td>0.5</td>
<td>0.3</td>
<td>77.1</td>
<td>1.7</td>
</tr>
<tr>
<td>Telonema</td>
<td>gacctaacctacataagta</td>
<td>nu-SSU-1334-5’</td>
<td>5</td>
<td>48.1</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>39.2</td>
</tr>
</tbody>
</table>

ComPrim, sequence complement to the indicated primer.
#nt, number of identical nt’s shared by primer and blocking oligo sequence
T_m, annealing temperature
%Fungi, coverage rate for fungal sequences
%Alv., coverage rate for Alveolata sequences
%Rhiz., coverage rate for Rhizaria sequences
%Stram., coverage rate for Stramenopiles sequences
%Tel., coverage rate for Telonema sequences

References
Additional File 1: List of the 164 fungi-specific primers detected by a literature research.
https://ndownloader.figstatic.com/files/18610463

Additional File 2: List of the 436 fungi-specific primer pairs tested for their performance by in silico PCR.
https://ndownloader.figstatic.com/files/18610472

Additional File 3: List of the seven most promising primer pairs for biodiversity assessments identified by in silico PCR.
https://ndownloader.figstatic.com/files/18610484

Additional File 4: Annealing temperatures empirically evaluated for the most promising primer pairs.
https://ndownloader.figstatic.com/files/18610490

Additional File 5: Performance of the most promising primer pairs empirically tested on 12 fungal strains.
https://ndownloader.figstatic.com/files/18610502

Additional File 6: List of the three newly designed primer pairs passing the evaluation criteria.
https://ndownloader.figstatic.com/files/13623851

Additional File 7: Primer pairs suitable for the amplification of specific fungal phyla/subphyla.
https://ndownloader.figstatic.com/files/18610511

Additional File 8: List of the designed annealing blocking oligonucleotides for the eukaryotic groups Stramenopiles, Alveolata, Rhizaria and Telonema.
https://ndownloader.figstatic.com/files/18610517
Additional File 9: Information on sequence generation and downstream analysis process.

https://ndownloader.figstatic.com/files/13623884

Additional File 10: UniFrac pairwise permutation test.

https://ndownloader.figstatic.com/files/13623728
Additional file 11: Taxonomic composition of three environmental samples. Barchart indicates relative sequence abundance of the different fungal classes/subgroups amplified by the primer pair nu-SSU-1334-5'-a/nu-SSU-1648-3’ (FF390/FR-1).

Others: Blastocladiomycetes, Glomeromycetes, Monoblepharidomycetes, Pucciniomycotina_Incerta sedis.
3.2 Chapter II: Seasonal dynamics of pelagic mycoplanktonic communities: interplay of taxon abundance, temporal occurrence, and biotic interactions
Seasonal Dynamics of Pelagic Mycoplanktonic Communities: Interplay of Taxon Abundance, Temporal Occurrence, and Biotic Interactions

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Marine fungi are an important component of pelagic planktonic communities. However, it is not yet clear how individual fungal taxa are integrated in marine processes of the microbial loop and food webs. Most likely, biotic interactions play a major role in shaping the fungal community structure. Thus, the aim of our work was to identify possible biotic interactions of mycoplankton with phytoplankton and zooplankton groups and among fungi, and to investigate whether there is coherence between interactions and the dynamics, abundance and temporal occurrence of individual fungal OTUs. Marine surface water was sampled weekly over the course of 1 year, in the vicinity of the island of Helgoland in the German Bight (North Sea). The mycoplankton community was analyzed using 18S rRNA gene tag-sequencing and the identified dynamics were correlated to environmental data including phytoplankton, zooplankton, and abiotic factors. Finally, co-occurrence patterns of fungal taxa were detected with network analyses based on weighted topological overlaps (wTO). Of all abundant and persistent OTUs, 77% showed no biotic relations suggesting a saprotrophic lifestyle. Of all other fungal OTUs, nearly the half (44%) had at least one significant negative relationship, especially with zooplankton and other fungi, or to a lesser extent with phytoplankton. These findings suggest that mycoplankton OTUs are embedded into marine food web chains via highly complex and manifold relationships such as parasitism, predation, grazing, or allelopathy. Furthermore, about one third of all rare OTUs were part of a dense fungal co-occurrence network probably stabilizing the fungal community against environmental changes and acting as functional guilds or being involved in fungal
INTRODUCTION

The marine biological pump plays a central role in Earth’s ecosystems. The core of this biological pump is the carbon cycle. In this process, carbon is drawn down from the atmosphere into the ocean by phytoplankton-driven photosynthesis. The generated organic carbon is largely degraded and taken up by microorganisms and subsequently transferred to the ocean’s depths or to higher trophic levels via the food web (Turner, 2015). The current valid framework of the microbial loop includes bacteria and archaea as well as eukaryotic protists (Worden et al., 2013). Remarkably, marine fungi are still largely excluded, despite the increasing evidence that they can assimilate and decompose essential amounts of phytoplankton-derived organic matter (Gutierrez et al., 2011; Cunliffe et al., 2017), influence phytoplankton population dynamics (Gutierrez et al., 2016), or may act as trophic link between phytoplankton and zooplankton via a marine mycoloop (Amend et al., 2019) analogous to the one described for freshwater systems (Kagami et al., 2014).

Marine microbial plankton, including fungi, are exposed to a highly fluctuating environment, which is reflected in changing diversity and structure (Gilbert et al., 2012; Lucas et al., 2016; Taylor and Cunliffe, 2016; Teeling et al., 2016; Duan et al., 2018). The dynamics within marine microbial communities can be described by different patterns (Needham et al., 2013), ranging from relatively small variations around an average to rapid increases and decreases within a very short time. These dynamics can result in dramatic changes in the overall microbial abundance. Nevertheless, despite these fluctuations, the functionality of the microbial community seems to be maintained (Wittebolle et al., 2009; Werner et al., 2011). Although it has long been assumed that solely the abundant species of a community are relevant for maintaining its functionality and stability, we now know that the rare fraction is also involved (Huber et al., 2007; Hernandez-Raquet et al., 2013; Logares et al., 2014) and can even occupy a disproportionally important role in biogeochemical cycles (Peter et al., 2011).

Given the strong variation in microbial community dynamics, it is highly likely that different factors control the dynamics of individual taxa, and thus, the way they are involved and embedded in the marine processes of microbial loop and food webs. Fungi, and their suite of different nutritional modes, are expected to be major players controlling taxon dynamics. Marine fungi are saprotrophs or symbionts, including pathogenic and mutualistic lifestyles (reviewed by Jones, 2000; Jobard et al., 2010). However, the biotic relationships of marine fungi go beyond direct interactions. For example, they can be members of cross-feeding networks, compete with other taxa for resources, and can produce allelopathic substances. It has also been shown that fungal tissue and spores constitute an important food source for a diversity of marine organisms (Newell et al., 1977; Cleary et al., 2016). In short, the role of fungi in the marine environment is extremely complex, and little understood.

Not only do we know little about the role of marine fungi, but we also do not understand their temporal dynamics. As visual identification of marine fungi is tedious and time consuming, there is a great need for molecular identification techniques to investigate the interplay of OTU dynamics and abundance (established as OTU-reads), and potential biotic interactions to increase our understanding on the impact these have on the planktonic community at large and even larger-scale ecosystem processes. We hypothesize that biotic interactions significantly influence the dynamics of individual OTUs and thus have effects on the entire mycoplankton community. Due to the diversity of possible biotic interactions we expect mycoplankton OTU dynamics to show very different patterns. We further assume that OTUs with similar abundance and temporal patterns have similar ecological niches, formed by biotic interactions. To test these hypotheses, we sampled seawater at the station Helgoland Roads in the German Bight (Wiltshire, 2004) over the course of 1 year on a weekly basis, correlated environmental data with Next Generation Sequencing-based fungal community data and calculated co-occurrence networks.

MATERIALS AND METHODS

Sampling Scheme

Seawater samples were collected at the Long-Term Ecological Research (LTER) station “Kabeltonne” at Helgoland Roads (Wiltshire et al., 2010), located about 70 km off the mainland in the German Bight (Germany, 54° 11.3′ N, 7° 54.0′ E). Water samples were taken once a week (with the exception of days with adverse weather conditions) from July 2015 to June 2016, resulting in a total of 43 samples. Samples were collected on board of the research vessel Aade, with an impeller pump (Jabsco, United States) from ~1 m-depth into a sterile 10 L bottle (NalgeN, Germany) and were directly processed in the laboratory of the Biologische Anstalt Helgoland. Two liters of the water was filtered directly without pre-filtration onto a Sterivex...
GP filter unit (0.22 μm PES membrane, Merck, Darmstadt, Germany) using a peristaltic pump (Verder, Germany) and stored at −20°C until further treatment.

The environmental data used to explain the dynamics patterns of the mycoplanктon are part of the long-term monitoring program of the Biologische Anstalt Helgoland, collected at the same time point and water depth as samples for mycoplanктon analysis (Wiltshire, 2004). This incorporated data on nutrients (SiO₂, NO₂, NO₃, NH₄, and PO₄), salinity, dissolved organic matter (DOC), surface water temperature, water pH (Supplementary Table S1), seven phytoplankton groups, namely Dinophyceae (dinoflagellates), Coccocilithophoridiae INDeterminata (IND), flagellates, Bacilariophyceae (penate diatoms), Bacillariales (penate diatoms), Biddulphiales (centric diatoms), and Dictyochophyceae (silicoflagellates), as well as the total phytoplankton cell counts (Wiltshire et al., 2015; Wiltshire, 2016; Sarker and Wiltshire, 2017) (Supplementary Table S2). Zooplanктon sampling was carried out 3 days/week (for details, see Greve et al., 2004; Wiltshire et al., 2008) and counts of 42 different zooplanктon groups were provided (Boersma et al., 2017).

**DNA Extraction, PCR and Sequencing**

DNA was extracted using the Power Water DNA Isolation kit (MO BIO Laboratories, Dianova, Hamburg, Germany). After filtration, the Sterivex GP-filter unit was opened and the filter was transferred into an extraction vial. DNA was extracted following the manufacturer’s instructions. PCRs were performed using the fungi-specific primer pair nu-SSU-1334-5′/nu-SSU-1648-3′ (CGATAACGACGAGACT/ANCCATTCAATCGGTANT) (Vainio and Hantula, 2000), recently shown to be the most promising primer for marine fungal community analysis in environmental samples (Banos et al., 2018). Fungi-specific 18S rDNA gene sequence primers risk to co-amplify specific non-fungal eukaryotic organisms groups. Therefore, the PCR protocol included the addition of four annealing blocking oligonucleotides. The blocking oligos with a 3′-amo linker C6 modification were specific to taxa within Stramenopiles (sequence of oligo: TCGCACCCTACGTTGGA), Alveolata (GTGCCTCCTACGTTGGA), Rhizaria (TTAAGCACGACCTCGA) and Telonema (GACCTTAACTTACTAAATAGTTA) (Banos et al., 2018). PCR, library preparation and sequencing were performed at LGC Genomics GmbH, Berlin, Germany. All sequencing reactions were based upon an Illumina Miseq chemistry following the manufacturer’s instructions.

**Bioinformatics**

Sequence reads were received in a demultiplexed form from which adapters and barcodes were already trimmed. Primers were removed using the software cutadapt v1.14 accepting an error tolerance of 16% (Martin, 2011). Forward and reverse reads were joined using PEAR v0.9.8 with the default settings (Zhang et al., 2013). The quality of sequence reads was checked by a 4-base wide sliding window approach embedded in the program Trimmomatic v 0.36 (Bolger et al., 2014). Sequences were excluded whenever the window average quality was lower than 20. Sequence reads shorter than 280 bp, or having more than 6 bp of homopolymer stretches, or more than four ambiguous symbols were deleted with Mothur v1.36.1 (Schloss et al., 2009).

Finally, the cd-hit-dup tool of the software CD-HIT v4.7 (Li and Godzik, 2006; Fu et al., 2012) was used under the default settings to dereplicate the remaining sequence dataset and to detect chimeras with the de novo algorithm.

The sequence classification was based on a phylogenetic approach. In the first step, the dereplicated dataset was aligned to the non-redundant SILVA database SSURef_NR99_128 (Quast et al., 2013) using the SINA aligner v1.2.11 (Pruesse et al., 2012) with the default settings. Based on the position of query sequences in the alignment, the SINA aligner classified the query sequences following the least common ancestor (LCA) rule. Only sequence reads classified as fungi under a 95% sequence threshold were extracted from the dataset for further analyses. Next, the extracted sequences were clustered into OTUs based on 98% sequence similarity using the CDHIT-EST tool within the CD-HIT software. Singletons defined to be present only in a single sample with less than ten sequence reads were removed from the dataset. The final phylogenetic classification was done by phylogenetic placement of the sequences into the fungal phylogenetic reference tree (Yarza et al., 2017) using the Maximum Parsimony Algorithm, the standard algorithm of the ARB program v6.0.3 (Ludwig et al., 2004). Prior to the classification of the here generated sequences, the phylogenetic tree was further enriched by reference sequences of so far unrecognized soil-inhabiting order-level clades identified within the work of Tedersoo et al. (2017). The phylogenetic tree was then inspected for “novel fungal diversity” becoming obvious as newly formed clades. The clades were used as a working hypothesis to find different behavior patterns within the “novel diversity”. Thus, clades were manually assigned by transferring the taxonomic name of the tree branch but adding the word “clade” and increasing numeration in the case that more than one clade was found (e.g., Chytridiomycota Clade 01).

**Statistics**

We were interested to understand if the patterns and responses to environmental factors of OTUs differed, depending on their abundance and temporal occurrence. Therefore, in addition to the dataset with information on all OTUs, we further created subsets of the community matrix based on OTU abundance and sample distribution (e.g., abundant, rare, resistant, transient OTUs): The dataset for abundant OTUs contained information of all OTUs, whose relative sequence abundance summed up to 90% of the one of the total community. The rare community fraction was formed by OTUs that were not included in the abundant community fraction. OTUs were defined as persistent OTUs if they were present in at least 25 out of the 43 samples independent from their sequence abundance. In contrast, OTUs were defined as transient, if they occurred in ≤5 samples and represented at least 2% of the relative sequence abundance in the sample. Another sub-dataset contained information on all OTUs classified as zoosporic fungi. Zoosporic fungi exist as a motile spore using a flagellum for motility, containing information on all OTUs classified as zoosporic fungi.
locomotion in at least one life stage, the so-called zoospore. Zoosporic fungi are discussed to play crucial roles in the dynamics of phytoplankton in oceans (Gutierrez et al., 2016; Lepere et al., 2016; Hassett et al., 2019). This is the reason why we investigated them as a group in more detail. Prior to any calculation, total OTU counts were Hellinger transformed (Bhatatcharya, 1943) and correlating environmental data were standardized using Z-score transformation (Clark-Carter, 2014). Generalized UniFrac distances (Chen et al., 2012) were calculated on community data and ordinated by principal component analysis (PCoA), the standard ordination with UniFrac-values (Lozupone and Knight, 2005). To test significance of the community response in a multivariate model, we first checked the candidate parameters for collinearity using a spearman correlation test. Parameters that had a correlation value higher than | for more than ten other factors were excluded from the analysis. Next, a distance-based redundancy analysis (dbRDA) was performed on generalized UniFrac distances. dbRDA was chosen as it can deal with the high variability found in microbial community datasets (for detailed information we refer to Legendre and Anderson, 1999). Important variables were detected using a forward selection method (function “ordistep” in vegan, see below). Analysis of variance was performed using the “anova.cca” command and permuting 999 times. Pearson correlation tests were used to test for possible interactions of the most abundant zoosporic fungal OTUs with the phytoplankton groups. The resulting p-values were adjusted for multiple testing with the false discovery rate (FDR) method (Benjamini and Hochberg, 1995).

All statistical analyses were carried out within the “R environment” v3.4.4 (R Core Team, 2013) using the packages “GUniFrac” v1.1 (Chen et al., 2012), “phyloseq” (McMurdie and Holmes, 2013), and “vegan” (Oksanen et al., 2013). The graphical representation of results was realized using the R package “ggplot2” (Wickham, 2016) and the program “GIMP2” version 2.8.22.

**Co-occurrence Network Analyses**

Co-occurrence network analyses were calculated with the weighted topological overlap (wTO) measure (Ravasz et al., 2002; Zhang and Horvath, 2005; Carlson et al., 2006). In contrast to a correlation-based approach, this measure allowed us to take all shared correlations between a pair of OTUs/parameters into account and to normalize it. The wTO measure was modified by Nowick et al. (2009) to accommodate both positive and negative interactions. Later, it was further modified to remove links incorporated by randomness by bootstrapping (Gysi et al., 2018). Thus, the wTO value is much more reliable for detecting links between a pair of OTUs/parameters and the nature of the interaction (±) than a correlation based network approach. As our data were based on a time series, the Blocked Bootstrap re-sample strategy was additionally applied. This strategy identifies blocks of high auto-correlations often occurring in the data distribution of time series by building an empirical distribution for each of the links (Efron and Tibshirani, 1993). This identifies blocks of highly auto-correlated time measures and builds an empirical distribution for each of the links. The wTO can be computed using this re-sampling method within the R package wTO (Gysi et al., 2018). All networks were constructed using the Pearson correlation, 1,000 bootstraps, the lag – autocorrelation window for the blocks – was defined using the autocorrelation function, and fixed in 4 weeks for all co-occurrence networks and in two for the inter-fungal interaction network. Networks were filtered for Benjamini and Hochberg adjusted P < 0.001 and links were kept if their wTO weight were higher than |0.15|, minimum of the empirical quantile estimated within the “wTO” R package. We would like to point out that the wTO-values cannot be read as correlation values due to the different calculation methods applied. The temporal series underlying the dataset of this study, in which many of the fungal OTUs and/or environmental parameters do not exist in all time points, can cause as a side effect lower wTO-values. However, due to the robustness of the wTO approach, node interactions with lower wTO values are valid links that are included in the network calculation.

Observed differences in the interaction patterns (Fungus-Fungus, Fungus-Metadata, Fungus-Biota) and relationships (positive/negative) were tested for significance using the Chi-squared test and 1-sample proportion test, respectively.

**RESULTS**

**Mycoplanktonic Community Composition**

After quality control, we retained 786,478 sequence reads classified as fungal. Three samples were removed from the dataset due to very low sequence output (time-points: 28.08.15, 17.09.15, 17.12.15). Fungal clusters were clustered into 3,314 OTUs belonging to seven phyla (including undefined Basal fungi), 11 subphyla, 22 classes and 59 orders (Supplementary Tables S3, S4). Manual inspection of the phylogenetic tree allowed the identification of eight additional clades, three on the branch of the basal fungal lineages and three falling into the group of Cryptomycota (Rozellomycota) sensu lato, one Ascomycota, and one Basidiomycota clade (Supplementary Figure S1). None of the newly formed clades included a reference sequence of clades defined as novel diversity by Tedersoo et al. (2017). However, the closest neighbors to the Basal Fungi Clade 03 were Cryptomycota sequences of the branch 2 and GS1 clade.

Ascomycota was the dominant phylum representing 24–88.5% of the relative sequence abundance for 31 out of the 40 samples. The majority of the Ascomycota sequences were phylogenetically assigned to unclassified Pezizomycotina, whilst a few samples were more represented by Saccharomycetes and Dothideomycetes. Chytridiomycota (primarily Chytridiomycetes) dominated the fungal community in six samples with 39–70% of the relative sequence abundance, and were otherwise only represented by 0–17% of the relative sequence abundance with a few exceptions. Basidiomycota were the most abundant phyla only in two samples, with 59
and 63% of the relative sequence abundance formed mainly by Agaricomycetes and Malasseziomycetes sequences. The time-point 16.07.15 was dominated by Mucoromycota sequences (47%), which exhibited, in general lower relative sequence abundance values (<5%) with few exceptions. Zoopagomycota and Cryptomycota sensu lato sequences were represented by less than 5% but increased at specific time-points with their relative sequence abundance up to 23% (Figure 1A).

The abundance-based classification of the OTUs identified 128 abundant OTUs (3.9% of all OTUs). Most of them were assigned to Ascomycota (58 OTUs) and Basidiomycota (41 OTUs). Chytridiomycota, Cryptomycota sensu lato, Mucoromycota, and Zoopagomycota were represented with 13, 7, 6, and 3 OTUs, respectively. While the majority of Ascomycota were filamentous groups, the majority of Basidiomycota were classified as (dimorphic) yeasts. Dimorphic fungi have morphologically diverse life cycles, often including a yeast and a filamentous stage (Esser, 2014). The rare community contained a total of 3,186 OTUs (96.1% of all OTUs).

Regarding the occurrence of OTUs over the course of the sampling period, 28 OTUs were defined as persistent taxa (0.84% of all OTUs), from which three were part of the rare community. 19 persistent OTUs were classified as Ascomycota, seven as Basidiomycota, one as Chytridiomycota, and one as Mucoromycota. Out of the Dikarya OTUs, eight belonged to (dimorphic) yeast taxa and the rare persistent taxa were all filamentous Ascomycota.

The group of transient OTUs was composed of eight Basidiomycota, eight Chytridiomycota, six Ascomycota, two Zoopagomycota, and one OTU of the Basal Fungi Clade 03 forming a total of 25 OTUs (0.75% of all OTUs). Six of the transient OTUs were also categorized as highly abundant OTUs including three Chytridiomycota, one Zoopagomycota, one Basidiomycota, and one Ascomycota. Zoosporic fungi were represented with a total of 300 OTUs (9.1% of all OTUs) (Supplementary Tables S3, S5).

### Environmental Factors Structuring the Mycoplankton Community

The mycoplankton community structure was connected to diverse environmental factors (n = 34, P < 0.05, dbRDA, forward selection). Three abiotic factors were identified as significant in the analysis: temperature, NO₃, and pH. Among the biotic factors, fish eggs, the larval stage of Echinodermata, the copepod Centropages spp., and the nauplius stage of copepods were described as factors connected with mycoplankton dynamics over a year (Table 1).

Additionally, the PCoA shows a certain influence of seasonality on the phylogenetic structure of the mycoplankton community, especially for the samples taken in autumn (03/09/15-26/11/15) and winter (03/12/15-24/02/16) (Supplementary Figure S2).

### Patterns of Fungal OTU Dynamics

We investigated the patterns of the abundant fungal OTU dynamics in more detail, as they are the product of the interplay of the taxon's life style and the relationship with environmental factors. Rare OTUs had to be excluded from this specific analysis, as their sequence abundance was often close to the detection limit. Based on the plotted read counts of individual fungal OTUs against time, four different categories of fungal OTU dynamics were identified and defined (Figure 1B): (I) The Boom-bust like type that is characterized by one to three peaks with a plateau phase lasting a maximum of 2 weeks. (II) The Frequent peaking type, which has numerous peaks (>3) and/or has a unique plateau phase of at least 3 weeks. (III) The Steady type which shows a continuous presence with fluctuations around a stable mean. (IV) The Long-lasting type is defined by at least two peaks with plateau phases of 2 weeks and longer. Many OTUs in this category showed distinct plateau phases of up to 6 weeks. 35 OTUs fell into the first category (I) while 38, 12, and 43 OTUs belonged to the category II, III, and IV, respectively. Only 50% of the persistent OTUs fell into the category III, which was solely formed by persistent OTUs. All transient OTU dynamics patterns were represented in category I. None of the abundant zoosporic OTUs were assigned to the category III (Steady type) (Supplementary Table S5).

For the majority of sampling time points (70%) there were more than ten abundant fungal OTUs with a clear abundance peak, defined as an abundance value greater than the average value over all sampling time points for the specific OTU. Exceptions were samples taken on the 02.07.15, 09.07.15,
The Fungus-Phytoplankton Relationships
Thirty out of the 128 abundant fungal OTUs (23%) were significantly correlated to one and up to several phytoplankton groups (pearson correlation, \(n = 40\), FDR-adjusted \(P < 0.05\), \(r > |0.4|\) including diverse Basidiomycota, Ascomycota, zoosporic fungi, and one Zoopagomycota OTU. From the seven tested phytoplankton groups, Bacillariales and Dinophyceae exhibited the highest number of correlations with fungal OTUs (with 7 and 6%, respectively) (Supplementary Table S5).

As for a large majority of zoosporic fungi, a parasitic life style has been described (Esser, 2014), we investigated the relationship among zoosporic OTUs’ dynamics and significantly correlated phytoplankton groups (\(n = 40\), \(P < 0.05\), \(r > |0.4|\)) in more detail, using the most abundant OTUs. Examples are the three Chytridiomycota incertae sedis OTUs OtMZZ147, OtMZZ452 and OtMZZ26. The OTU OtMZZ147 was significantly negatively correlated to Dictyochophyceae (\(P = 0.006; r = -0.43; r^2 = 0.18\)) showing a first smaller peak during the Dictychophyceae bloom and a second time-retarded much larger peak. The peak observed for OTU OtMZZ452 matched the final stage of the significantly correlated Bacillariales bloom (\(P < 0.001; r = 0.85; r^2 = 0.72\)). The dynamic of the OTU OtMZZ26 was correlated with both Dinophyceae (\(P < 0.001; r = 0.42; r^2 = 0.18\)) and Flagellates (\(P < 0.001; r = 0.61; r^2 = 0.38\)). The double peak observed for the fungal OTU fell into the bloom phase of both phytoplankton partners (Figure 2B).

The Fungus-Zooplankton Relationships
Thirty out of the 42 zooplankton groups were significantly related with fungal OTUs (network analyses, Benjamini and Hochberg adjusted \(P < 0.001; \text{wTO} > |0.15|\)). The number of observed relationships differed highly among zooplankton groups but always included zoosporic fungi and transient OTUs with few exceptions (Figure 3). The links between copepods and fungi were mainly negative (83%). The fungal assemblage differed greatly for different copepod genera/orders. Prominent fungal interaction partners were OTUs assigned to the zoosporic Chytridiomycetes incertae sedis and Cryptomycota sensu lato Clade 01, and the filamentous group of Pleosporales. Up to eight different fungal orders were negatively associated with a copepod group (Figure 4).

The majority of relationships to other zooplankton groups were negative (59%). The highest fungal diversity with 13 different fungal orders/undefined groups was observed with fish eggs, all being negative. Fungal interaction partners differed with zooplankton groups but no trend toward a phylogenetic group became visible (Supplementary Table S6).

Signals of Potential Antagonistic Inter-Fungal Relationships
Thirty-seven percentage of the marine fungal OTUs showed potential antagonistic relationships with other fungi (network analysis, Benjamini and Hochberg adjusted \(P < 0.001; \text{wTO} > |0.15|\)). Most of the significant negative associations were inter-phyla relationships dominated by Ascomycota and Basidiomycota. In contrast, all negative associations of zoosporic fungal OTUs showed exclusively an inter-phyta nature dominated by relationships to mainly Pezizomycotina and Agaricomycotina. Only 2% of all associations detected for the zoosporic fungi were between Cryptomycota and Chytridiomycota OTUs. The Basal fungi Clade 03 was represented by 1% of the negative associations related to Pezizomycotina and Agaricomycotina with only one exception. This kind of community membership had no impact on inter-fungal relationships (Figure 5).

Co-occurrence Network
The network calculated for the whole fungal community, containing the environmental data, comprised 1,797 significant interactions formed by 937 fungal OTUs and 17 environmental factors (represented as nodes in the network, Benjamini and Hochberg adjusted \(P < 0.001; \text{wTO} > |0.15|\)). With 95.2%, the large majority of interactions were significantly positive (1-sample proportion test, \(P < 0.05\)). The correlations were significantly dominated by fungus-fungus interactions (96.1%, Chi-Squared test, \(P < 0.05\)). In 53%, inter-phyta interactions were observed while the remaining 47% were intra-phyta interactions. Intra-phyta interactions were dominated by connections of Dikarya and, to a lesser extent, connections of other fungal phyla/clades. Among the nodes guaranteeing the network stability, members of Dikarya, Chytridiomycota and Cryptomycota were represented. They belonged, with a few exceptions, to the rare community. Nine out of 28 persistent OTUs and ten out of 25 transient OTUs were part of the network but did not hold a prominent position within the network. The majority of nodes representing environmental factors were part of the main module except for fish eggs, Cirripedia, and the larvae of Echinodermata. The majority of interactions found between environmental data and fungal OTUs were negative (82.5%) (Figure 6 and Supplementary Table S6).

DISCUSSION
Fungal Dynamics
This project aimed to identify potential marine fungal ecological relationships that shape fungal abundance patterns. The high diversity found among marine fungi requires investigations at a low taxonomic level, as this allows information on OTU abundance and temporary occurrence to be included in the analysis. Only if we understand the mechanisms that drive species dynamics, will we be able to decipher the mechanisms maintaining and modifying marine fungal community diversity. Since this study is based on Illumina tag sequencing data, it is difficult to estimate to what extent the signals are caused
FIGURE 2 | Continued
by dispersing spores, dormant structures, or by active members of the community. However, given (i) that the mycoplankton community was dominated by diverse phyla during the sampling period, (ii) that within the fungal OTU dynamics the increase/decrease of the sequence reads was sequential, (iii) that the patterns have been divided into only four categories due to similar temporal succession curves, and (iv) that these resemble already published patterns of aquatic microbes (Gerphagnon et al., 2013; Needham et al., 2013), we suppose that the majority of the sequence data is derived from taxa that actively respond to their environment.

About one-third of all fungal OTUs at Helgoland Roads were part of a dense co-occurrence network significantly dominated by positive relationships. Dense co-occurrence networks of microbial communities are normally advantageous for their members, as they generate an environment, in which specific
biosynthetic pathways can be activated. This leads to an increase of the chemical diversity, which in return, stabilizes the community (Jousset et al., 2017; Oppong-Danquah et al., 2018). Rare OTUs mainly formed the observed co-occurrence patterns presumably acting as the backbone of the mycoplankton community. Such a backbone has large advantages, as the subcommunity formed represents a diversity reservoir that can respond quickly to environmental change on a temporal scale (Logares et al., 2014). Since ocean water is a highly dynamic system, strong fluctuations of environmental parameters occur over the course of the year. This could explain why roughly similar frequencies of inter- and intra-phyla relations were reported. Niche preferences are mainly visible at higher taxonomic levels (Philippot et al., 2010) and inter-phyla co-occurrence is favored under more constrained environmental conditions (Cao et al., 2018). Thus, both factors, inter-phyla relations and the high number of rare OTUs involved in forming the community backbone, can probably explain the mycoplankton diversity observed in this project.

The ecology of rare microbial subcommunities can sometimes differ substantially from that of more abundant microbes (Lynch and Neufeld, 2015), which may explain, why the abundant and persistent OTUs played a minor role for the overall network cohesion. This was also reflected in the dynamics patterns: 50% of all persistent OTUs fell into category III (Steady type), which in turn was formed only from persistent OTUs exclusively assigned to filamentous Ascomycota and (dimorphic) yeasts of the Dikarya. Marine yeasts are ubiquitous and the upper ocean layer can be inhabited by up to thousands of cells per liter of water (Meyers et al., 1967; Gerdts et al., 2004; Kutty and Philp, 2008), in which they can assimilate a large proportion of dissolved organic material (Suehiro et al., 1962). Both groups, (dimorphic) yeasts and filamentous Ascomycota, can be attached to larger

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**FIGURE 4** | Stacked barcharts showing the taxonomic diversity of significantly related fungal partners with seven different copepod groups. Percentages are based on the counts of significant negative wTO links obtained from network analyses observed between fungal OTUs and copepods (Benjamini and Hochberg adjusted $P \leq 0.001$; wTO $> 0.15$).
FIGURE 5 | Chorddiagramm representing potential antagonistic interactions among fungi. The results are based on all significant negative correlations (Benjamini and Hochberg adjusted $P \leq 0.001$; $w_{TO} > |0.15|$) identified by network analysis using the dataset that comprised solely fungal OTUs. The thickness of the connecting lines signifies the number of correlated OTUs assigned to the two connected fungal taxa.

Particles (Jones, 2000; Bochdansky et al., 2017). Furthermore, it has recently been demonstrated that Malassezia (yeast) and Cladosporium (filamentous Ascomycota) taxa can use laminarin as food source (Cunliffe et al., 2017). Most marine Dikarya possess a wide spectrum of exo-enzymes (Raghukumar, 2008), so they are expected to be able to degrade various organic material of different decomposition stages and ages. These characteristics may explain the pattern of the type category III (Steady type) suggesting a saprophytic life style.

In contrast, all other OTUs, regardless of their taxonomy and morphology, showed dynamics pattern characterized by at least one very strong increase in read abundance. Only three of the nine abiotic factors, namely temperature, pH, and nitrate, were significantly associated with the dynamics of the fungal community. These factors are among those shaping mycoplankton assemblages independent of the sampling site in marine systems (Taylor and Cunliffe, 2016; Tisthammer et al., 2016; Duan et al., 2018). Zooplankton and phytoplankton groups, in contrast, were linked to fungal OTUs in numerous significant interactions.

Correlation tests and co-occurrence networks are important tools for the development of hypotheses on how marine fungi are embedded into marine food webs and microbial loops. However, highly complex data or data from time series may result in diverse errors in the detection of true relationships. We addressed these two points by using a wTO-based network analysis rather than a correlation-based one and by calculating time windows of possible data autocorrelation prior to the network analyses. Despite these tailored analyses, a side effect may be relative low wTO values, which is further enhanced if there are no replicates per sampling time-point. To identify the correct threshold for filtering the wTO values, the empirical distribution was used, computed by blocked bootstrapping and using the quantiles for the real data. A general drawback of correlation and network analyses is that it is unclear whether the detected relationship actually exists in reality and what its exact nature is. Correlations/wTO links can also simply occur because two species favor similar environmental conditions without any interaction. Nevertheless, strong real patterns of interactions are usually detected by these approaches (Krabberod et al., 2017).
Biotic interactions can be direct or indirect, and positive or negative. Positive associations may indicate that the interaction partners are organized in functional guilds or that interspecies cross-feeding exists. Negative associations are interpreted as antagonistic interactions, such as grazing, competition, predatory/pathogenic relations, or allelopathy. Biotic interactions can have marked effects on marine processes such as decomposition, nutrient cycling or energy flow between different trophic levels (Newell et al., 1977; Meunier et al., 2016). For this reason, we subsequently discuss the signals in our dataset for potential fungal interactions with phytoplankton, zooplankton, and between marine fungi in more detail.

**The Fungus-Phytoplankton Relationships**

Phytoplankton blooms have a strong impact on the ecosystem because of succession and the fact that general growing conditions change dramatically over short periods of time. Due to the introduction of fresh organic material, one hypothesis would be that fungi, as heterotrophic organisms, benefit greatly from this and were seen to react as many OTUs with increased abundance. However, our data showed an opposite trend, so that the number of fungal OTUs with a strong read abundance peak was lowest during the spring phytoplankton bloom at Helgoland Roads. A similar trend was described by Duan et al.
Potential Antagonistic Inter-Fungal Relationships

Thirty-seven percent of the fungal OTUs were significantly negatively related with another fungal OTU, which may indicate negative inter-fungal interactions as described for many fungal taxa (Laughlin and Spatafora, 2014). In the context of antagonistic relations, a negative association may indicate mycoparasitism, competition, resource partitioning, or allelopathy. The patterns of potential negative associations differed among Dikarya and Basal Fungi in our study: Thus, the dimorphic and yeast groups of Dikarya were mainly involved...
in negative intra-phyla associations, a pattern that Ruszkiewicz-Michalska (2010) has described for mycoparasitic Dikarya. For dimorphic taxa, the filamentous stage is often the one that infects and explores the host’s resources while the yeast stage feeds saprotrophically (Sampaio, 2004). The antagonistic nature of several yeasts within the Saccharomycetales and Tremellales is different: They secret fungalid protease-sensitive toxins (Golubev, 1998). The effect on the counterpart is highly specific and depends for example on its sexual, chemotaxonomic and physiological characteristics (Golubev and Nakase, 1997; Golubev, 2013).

In contrast to the Dikarya, the negative interactions of the Basal Fungi were nearly exclusively of an inter-phyla nature. Thus, the related partners of several OTUs of the Cryptomycota sensu lato Clade 01 were mainly Dikarya or Chytridiomycota. Cryptomycota are parasites of all kinds of organisms (Jones et al., 2011). For example, some Rozella species infect diverse Chytridiomycota and act as necrotrophs, degenerating their cytoplasm for nutrient uptake (Boddy, 2015). Other notable relationships involved the taxa assigned to the Basal Fungi Clade 03. This clade branched on the basal part of the fungal phylogenetic reference tree. Phylogenetic relations among Basal Fungi are still under debate and inclusion into the phylum of Microsporidia has been proposed (Bass et al., 2018). Microsporidia are obligate and highly efficient parasites and include mycoparasites. They sporulate abundantly when cultured with their hosts (Ahrendt et al., 2018). In our dataset, most of the possible antagonists exhibited category I (Boom-bust like type) or III (Steady type) dynamic patterns. Our hypothesis for the occurrence of these opposite patterns is that antagonistic fungi have different ranges of host specificity or that the interaction is strongly influenced by external environmental parameters.

CONCLUSION

Our data suggest that biotic interactions are important for the niche formation of fungi in marine surface water. The kind of interaction seems to be related to fungal OTU abundance and its temporal occurrence. Our results further suggest a strong antagonistic relationship between fungi and other planktonic organisms. In an ecological context this may mean (i) a top-down control by fungi on phytoplankton or zooplankton populations with consequences for the microbial loop and food web structure; (ii) that fungi transfer organic material and nutrients to other planktonic organisms by acting as food source (one possible scenario may be a marine mycoloop); and (iii) that the proliferation of individual fungal species harmful to other planktonic organisms may be limited by other fungal species.

To further build upon this work, one to one interactions between individual fungal OTUs and plankton organisms should be analyzed by modern fluorescent microscopy to decipher the true nature of interactions and OTU dynamics. This approach should be accompanied with a genetic analysis in vitro culturing in order to unravel the mechanisms which are essential to the interaction.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Sequence data can be obtained from the European Nucleotide Archive (ENA) with the accession number PRJEB33370. The following corresponding metadata is published in PANGAEA: The count data of mesozooplanktonic individuals can be accessed over (Boersma and Renz, 2016; DOI: 10.1594/PANGAEA.864586) and (Boersma and Renz, 2017; DOI: 10.1594/PANGAEA.870666) for the years 2015 and 2016, respectively. The cell count data of the nine phytoplankton groups for the year 2015 can be accessed over (Wiltshire, 2016; DOI: 10.1594/PANGAEA.862909). Exceptions are the data of the hydrochemistry and DOC as well as the phytoplanktonic cell counts for the year 2016, which have not been submitted yet and can thus be accessed over the Supplementary Tables S1, S2, respectively. The fully annotated OTU table can be found as Supplementary Table S3 and the related representative sequences in Supplementary Table S4.

AUTHOR CONTRIBUTIONS

MR, GG, AW, and FG planned and designed the study. KW provided and interpreted physiochemical and phytoplankton biodiversity data. MB provided and interpreted DOC and zooplankton biodiversity data. SB, MR, and TR-H analyzed the fungal biodiversity data. DG run and interpreted the co-occurrence networks. SB and MR wrote the manuscript. All authors have reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01305/full#supplementary-material

FIGURE S1 | Positioning of the newly identified clades within the fungal phylogenetic tree used for the classification of fungal sequences. The newly formed clades are only composed by sequences generated in this study (clades are colored in red). Clades were collapsed at the branch, which defines them. The phylogenetic tree is based on the fungal reference tree of Yarza et al. (2017).

FIGURE S2 | Principal coordinate analysis (PCoA) of mycoplankton community data. A generalized UniFrac distance matrix was generated revealing phylogenetic differences of fungal assemblages over sampling time-points. The color code defines the four seasons as described in Lucas et al. (2015). In short, spring (1 March–31 May), summer (1 June–31 August), autumn (1 September–30 November), winter (1 December–29 February).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Figure 1: Positioning of the newly identified clades within the fungal phylogenetic tree used for the classification of fungal sequences. The newly formed clades are only composed by sequences generated in this study (clades are colored in red). Clades were collapsed at the branch, which defines them. The phylogenetic tree is based on the fungal reference tree of Yarza et al. (2017).
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Supplementary Figure 3: Dynamics of phytoplankton abundance over the course of a year (summer 2015 to summer 2016).
Supplementary Table S1: Hydrochemistry and DOC content at Helgoland Roads as measured for the different sampling time points.

Supplementary Table S2: Phytoplankton cell counts at Helgoland Roads for the different sampling time-points.

Supplementary Table S3: Fully annotated fungal OTU table (all OTUs) with read abundance values over the different sampling time-points.

Supplementary Table S4: Fasta-file with representative sequences of all annotated OTUs found in Supplementary Table S3.

Supplementary Table S5: Characteristics of fungal OTUs (without rare OTUs).

Supplementary Table S6: Significant node relations as identified by network analyses.

All Tables can be downloaded in the following link:

https://ndownloader.figstatic.com/files/23454140
3.3 Chapter III: Mycoplankton biome structure and assemblage processes differ along a transect from the Elbe River down to the river plume and the adjacent marine waters
Mycoplankton Biome Structure and Assemblage Processes Differ Along a Transect From the Elbe River Down to the River Plume and the Adjacent Marine Waters

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Rivers are transport systems and supply adjacent ecosystems with nutrients. They also serve human well-being, for example as a source of food. Microorganism biodiversity is an important parameter for the ecological balance of river ecosystems. Despite the knowledge that fungi are key players in freshwater nutrient cycling and food webs, data on planktonic fungi of streams with higher stream order are scarce. This study aims to fill this knowledge gap by a fungi-specific 18S ribosomal RNA (rRNA) gene tag sequencing approach, investigating mycoplankton diversity in the Elbe River along a transect from shallow freshwater, to the estuary and river plume down to the adjacent marine waters (sections of seventh stream order number). Using multivariate analyses and the quantitative process estimates (QPEs) method, questions (i) of how mycoplankton communities as part of the river continuum change along the transect, (ii) what factors, spatial and environmental, play a role, and (iii) what assembly processes, such as selection or dispersion, operate along the transect, were addressed. The partitioning of mycoplankton communities into three significant distant biomes was mainly driven by local environmental conditions that were partly under spatial control. The assembly processes underlying the biomes also differed significantly. Thus, variable selection dominated the upstream sections, while undominated processes like ecological drift dominated the sections close to the river mouth and beyond. Dispersal played a minor role. The results suggest that the ecological versatility of the mycoplankton communities changes along the transect as response, for example, to a drastic change from an autotrophic to a heterotrophic system caused by an abrupt increase in the river depth. Furthermore, a significant salinity-dependent occurrence of diverse basal fungal groups was observed, with no clade found exclusively in marine waters. These results provide an important framework to help understand patterns of riverine mycoplankton communities and serve as basis for a further in-depth work so that fungi, as an important ecological organism group, can be integrated into models of, e.g., usage-balance considerations of rivers.

Keywords: aquatic fungi, estuary, QPE analysis, salinity gradient, Chytridiomycota, shallow freshwater, dispersal, habitat
INTRODUCTION

Rivers are unique ecosystems that strongly influence adjacent terrestrial ecosystems and coastal waters through their nutrient input. Furthermore, they serve human well-being by acting as food source, drinking water reservoirs, or waterways, among other things. It is therefore all the more important that human use does not disturb the ecological balance of the river (Friberg et al., 2011). Different scenarios are often modeled for usage-balance considerations (Regier and Kay, 1996; Schöl et al., 2014; Schiemer et al., 2020). Beside physical properties or topology, microbial diversity is a non-negligible parameter, as microorganisms are key players of riverine carbon and nutrient cycles and stand at the beginning of food web chains. Numerous inventories of riverine bacterioplankton communities exist targeting rivers and river sections of different stream order numbers (Selje and Simon, 2003; Read et al., 2015; Huber et al., 2020). In contrast, the existing studies on fungi mainly focused on streams of lower order numbers and/or focused on individual fungal groups (Wood-Eggenschwiler and Bárlöcher, 1983; Lecerf and Chauvet, 2008), eukaryotic communities (Simon et al., 2015; Lu et al., 2020; Shi et al., 2020), or habitats other than the water body (Liu et al., 2015; Chen et al., 2020). This is a major contradiction to the knowledge that mycoplankton are key components of ecosystem functioning in all freshwater systems. For example, pelagic fungi possess an extremely diverse enzymatic repertoire (Zemek et al., 1985; Christmas and Cunliffe, 2020), are involved in various nutrient cycles, and exert top–down control on phyto- and zooplankton (Barron, 2004; Rasconi et al., 2011; Frenken et al., 2017). Grossart et al. (2019) outlined the following two cycles as important aquatic ecosystem services of pelagic fungi: Mycoflux, which describes the fungal decomposition and provision of organic material for other organisms; Mycoloop, in which zoosporic fungi represent an important trophic link between phytoplankton and zooplankton; by infecting large, grazing-resistant phytoplankton species, the fungus can use the host's nutrients and organic material to form new zoospores, which can then be grazed by zooplankton.

Lotic systems differ from other freshwaters in that they form a continuum of time and space during the transition from the upper course of the river to the river mouth, which finally fades out in the river plume. During the passage of the river, the water masses are exposed to different influences. For example, the adjacent landscape structure, anthropogenic activities or physical streams have an effect on the site-specific water chemistry, residence time, and flow velocity (Friberg et al., 2011; Geerts et al., 2017). Thus, the biota occurring in the water body are exposed to physiochemical and hydrological gradients. This phenomenon of a downstream gradient that biota have to cope with is referred to as the “river continuum concept” (Vannote et al., 1980). One of the strongest gradients around the river mouth is salinity, which influences the presence and abundance of many organisms (Attrill, 2002; Telesh et al., 2011), including fungi (Livermore and Matess, 2013). In addition to environmental gradients, the water transport along a given longitudinal direction also influences the composition of the biotic communities. The dispersal strategy is therefore an important variable for the occurrence of individual taxa. Aquatic fungi possess very different dispersal strategies: Early diverging lineages display morphological adaptations in the form of zoospores or amoeboid spores, while James and Berbee, 2012; Powell and Letcher, 2014; Letcher et al., 2015) some ascomycetes form spore appendages, with which they can attach to particles (Jones, 2006). Other possibilities include resistant spores, fragments of hyphae, the whole thallus, and as passenger on/in particles or host tissue (Kohlmeyer and Kohlmeyer, 1979; Gleason and Lilje, 2009).

Given that fluvial fungal communities are important players in ecological processes (Krauss et al., 2011), can hold human–pathogenic members (Ulf et al., 1997), but also are source for the discovery of new antibiotics or biotechnologically relevant natural products (Svahn et al., 2012; Grossart and Rojas-Jimenez, 2016), it is of critical importance to gain a better understanding of the spatial patterns of fluvial fungal diversity, their main driving forces, and their persistency in the face of changing environmental conditions. Therefore, we addressed here the following questions: (i) To what extent do mycoplankton communities change, as part of the river continuum, from shallow freshwater through the estuary down to the Elbe River plume and adjacent marine waters? (ii) What are the driving forces? (iii) Which assemblage processes affect the mycoplankton communities, and do they differ along the sampled transect? (iv) Since the transect spans a sharp salinity gradient, how does this affect the fungal distribution and abundance with eye mark on basal fungal lineages. We hypothesized that communities are segregated along fresh, brackish, and marine water types and due to high heterogeneity in environmental parameters across the transect that communities are composed primarily by variable selection.

MATERIALS AND METHODS

Sampling and Measurements of Environmental Parameters

The study was conducted over a progressively increasing salinity gradient spanning from the shallow freshwater area of the Elbe River into the Elbe estuary, the river plume, and the transition zone with marine water in the North Sea. The curved transect line, following the course of the river and lying within the seventh Horton–Strahler order stream section of the Elbe (Schmidt et al., 2020), traversed in total 24 sampling stations starting at the city of Lauenburg (Germany, 53°22′11.60″N, 10°33′8.37″E) and ending close to the island of Helgoland in the German Bight (Germany, 54°09′06.1″N, 7°53′30.1″E) (Figure 1 and Supplementary Table 1). The total length of the curved line transect was 217.8 km, measured as the cumulative water channel distance. Sampling was carried out from the August 4–6, 2015 during the tidal water runoff to keep sampling conditions stable and avoid influence from incoming North Sea water. Surface water was sampled beside the fairway at a water depth of ~1 m into a sterile 10-L bottle (Nalgene, Germany) from onboard of the research vessel Uthörn or offboard from the coast line. Two liters of the water was filtered on a 0.22-μm polyethersulfone (PES) membrane (Merck, Darmstadt, Germany) using a peristaltic pump and stored until further treatment at −20°C.

At each of the 24 sampling points, additional water was collected to measure 10 environmental parameters, namely,
FIGURE 1 | Sampling sites and local environmental conditions. The environmental heterogeneity between sites was analyzed with similarity profiles. Sample positions encircled with the same color in the map indicate similar environmental conditions ($P < 0.05$). Mycoplankton communities from individual sampling sites that show similar phylogenetic composition as identified by principal component analysis (PCoA)/permutational multivariate ANOVA (PERMANOVA) ($P < 0.05$, see Figure 2B), have the same color for the sampling point. Notably, the grouping differed from the water type zonation of fresh-, brackish, and marine waters. In the Elbe River, the end member of the freshwater can shift depending on the strength of the water runoff. Thus, to test a water-type-specific distribution of the basal fungi (see Figure 5), only samples that fall into one water type throughout the year were used. “X” indicates samples that were excluded from further analyses due to low sequence output.

drainage salinity, pH, water temperature, nitrate, nitrite, ammonium, phosphate, silicate, chlorophyll $a$, and dissolved organic matter (DOC) (Supplementary Table 1; PANGAEA dataset: Reich et al., 2021). A detailed description of the sampling procedure and analyses can be found by Lucas et al. (2016). Incomplete data fields for salinity (samples 1-8) were filled up by searching the appropriate values on the data portal “Fachinformationssystem (FIS)” of the FGG (FlussGebietsGemeinschaft, Magdeburg, Germany) Elbe (Flussgebietsgemeinschaft (FGG) der Elbe, 2020) if possible. Furthermore, based on literature values, which confirmed the measured ones, samples were grouped into the three water types of fresh, brackish, and marine waters following the definition of Remane and Schlieper (1972) with $< 0.5$, $0.5 - < 30$, and $> 30$ practical salinity units (PSU), respectively, as water-type specific salinity range. Not all samples could be considered in this classification because the freshwater boundary of the Elbe shifts depending on the season and the associated intensity of water runoffs (Dähnke et al., 2008). In summer, it can be located at the level of Hamburg (145 km to the river mouth) or, in the case of strong water runoffs, at the level of Glückstadt (85 km to the river mouth). For this reason, we used samples whose position lay within one water type over the whole year (Bergemann, 1995; Amann et al., 2012). Thus, samples 1-5, 11-17, and 20-24 were considered as fresh, brackish, and marine water samples, respectively. Furthermore, waterway deepening in rivers has significant impact on river ecosystems (Manap and Voulvoulis, 2016). To measure a possible indirect impact of dredging activities on the fungal community composition, we took the depth of the sampling stations as proxy. Incomplete data fields (samples 1-16) were filled by using the digital relief model of the river, which is the DGM-W 2010 Unter-und Auß enelbe data (Digitales Geländemodell des Wasserlaufes) provided by the Zentrales Datenmanagement (ZDM), Küstendaten, of the Wasserstraßen- und Schifffahrtsverwaltung des Bundes (DGM-W_Unter_und_Außenelbe, 2010) (Supplementary Table 1).

DNA Extraction, Sequencing, and Bioinformatics

DNA was extracted using the Power Water DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, United States) according to the manufacturer’s instruction. PCR reactions were performed using the fungi specific 18S ribosomal RNA (rRNA) primer pair nu-SSU-1334-5′/nu-SSU-1648-3′ (Vainio and Hantula, 2000), recently shown to be the best performing primer pair on aquatic fungal assemblages (Banos et al., 2018, 2019). Additionally, four annealing blocking oligonucleotides with a 3′-amino linker C6 modification were added to the PCR reaction to prevent coamplification of the abundant eukaryotic groups of Alveolata, Rhizaria, Stramenopiles, and Telonema (Banos et al., 2018). PCR, library preparation, and sequencing was performed at LGC Genomics GmbH (Berlin, Germany). All sequencing reactions were done with the IlluminaMiseq Reagent Kit v3 for 2 × 300 bp reads (Illumina, Berlin, Germany) following the manufacturer’s instructions.
Sequences were analyzed with a phylogeny-based approach following the pipeline of Banos et al. (2020). Shortly, quality-controlled sequence reads were passed on for classification. In a first step, reads were incorporated into the backbone alignment of the non-redundant SILVA database SSURef_132 (Quast et al., 2013) using the SINA aligner v1.2.11 (Pruesse et al., 2012) with the default settings. This step includes the classification of the query sequences by using the 10 most similar sequences as provided by the alignment and applying the least common ancestor rule (LCA) with a 95% sequence similarity threshold. Only sequences that were classified in this way as fungi were further clustered into operational taxonomic units (OTUs) based on a 98% sequence similarity using the CH-HIT-EST tool within the CD-HIT software v4.6 (Li and Godzik, 2006; Fu et al., 2012). The final classification of the OTUs’ reference sequences was carried out by inserting them over phylogenetic placement into the fungal phylogenetic reference tree (Yarza et al., 2017). Prior to the insertion, the fungal reference tree was enriched by 254 fungal 18S rRNA gene sequences of the SILVA dataset SSURef_128 not yet present in the tree, by 210 reference sequences of so-far unrecognized soil-inhabiting order-level dataset SSURef_132 not yet present in the tree, by 20 sequences was carried out by inserting them over phylogenetic placement into the fungal phylogenetic reference tree (Yarza et al., 2017). To identify which mycoplankton communities from the different sampling sites own a similar phylogenetic composition, a distance-based principal component analysis (PCoA) was run using generalized Unifrac (GUniFrac) distance values as input. The significance of the observed sample clustering in the PCoA was tested by permutational multivariate ANOVA (PERMANOVA) (FDR adjusted P < 0.05). The correlation of the environmental variables with the first two axes of the PCoA was calculated by the Pearson correlation coefficient with default settings between sample scores on each axis and each of the environmental variables (FDR, adjusted P < 0.005, R² > 0.5, score > 0.7). All these steps were calculated with the R packages phyloseq (McMurdie and Holmes, 2013) and GUniFrac v1.1 (Chen et al., 2012) and pairwiseAdonis (Martinez Arbizu, 2020).

To identify samples with a similar environmental profile, a non-hierarchical clustering based on k-means and coupled to similarity profile test (SIMPROF) was performed using PRIMER v7 (Primer-e, 2017) on the basis of the z-transformed environmental factor matrix. The significance level for SIMPROF was set to 5% and performed with 999 permutations to define the optimal number of k-groups (between 2 and 10) to describe the clustering of the samples, which is based on maximizing R.

To check if an impact of the geographical distance on the phylogenetic dissimilarity exists, a distance-decay analysis was carried out applying linear regression (n = 231; P < 0.05) using the “lm” function of R. As this showed to be significant, the power of control of spatial factors on the fungal community variation was further tested. Thus, a spatial eigenfunction was carried out using the “distance-based Moran’s eigenvector maps” (dbMEM) function of the R package adespatial (Dray et al., 2017) to calculate eigenfactor and eigenvalues. As our sampling was not a standard sampling situation (e.g., not a straight transect line), the truncated distance matrix was generated following the example of Brind’Amour et al. (2005) but not looping the sample sites at the end. Eigenfunctions with a positive eigenvalue

Statistics

If not differently stated, the statistical analyses were carried out within the R environment v4.0.2 (R Core Team, 2015). Rarefaction curves were generated with the “iNEXT” function of the R package iNEXT (Hsieh et al., 2016). Next, prior to any calculation, OTU counts were subjected to Hellinger transformation (Bhattacharyya, 1943) and contextual data to z-scoring transformation (Clark-Carter, 2014). Environmental factors were checked for collinearity using a Spearman rank correlation test and adjusting the P values with the false discovery rate (FDR) method (Benjamini and Hochberg, 1995), and highly correlating factors were removed (Supplementary Table 2). To identify which mycoplankton communities from the different sampling sites own a similar phylogenetic composition, a distance-based principal component analysis (PCoA) was run using generalized Unifrac (GUniFrac) distance values as input. The significance of the observed sample clustering in the PCoA was tested by permutational multivariate ANOVA (PERMANOVA) (FDR adjusted P < 0.05). The correlation of the environmental variables with the first two axes of the PCoA was calculated by the Pearson correlation coefficient with default settings between sample scores on each axis and each of the environmental variables (FDR, adjusted P < 0.005, R² > 0.5, score > 0.7). All these steps were calculated with the R packages phyloseq (McMurdie and Holmes, 2013) and GUniFrac v1.1 (Chen et al., 2012) and pairwiseAdonis (Martinez Arbizu, 2020).

To identify samples with a similar environmental profile, a non-hierarchical clustering based on k-means and coupled to similarity profile test (SIMPROF) was performed using PRIMER v7 (Primer-e, 2017) on the basis of the z-transformed environmental factor matrix. The significance level for SIMPROF was set to 5% and performed with 999 permutations to define the optimal number of k-groups (between 2 and 10) to describe the clustering of the samples, which is based on maximizing R.

The sample groups well separated in the PCoA and identified as significantly different by PERMANOVA were further inspected. Thus, for each group, average OTU richness (Chao1) and diversity (Shannon) was calculated using the “estimate_richness” function in the package phyloseq (McMurdie and Holmes, 2013). Additionally, phylogenetic diversity (PD) was calculated using the program PHYLOGCOM v4.2 (Webb et al., 2008). Significance in α-diversity between the different sample groups was tested with the Tukey’s post hoc test [Tukey honestly significant difference (HSD)] with the default settings in the package states (Gleditsch and Ward, 1999). Correlations of the abundant OTUs with environmental factors were tested by Pearson rank order correlations. Furthermore, PERMANOVA was applied to test if the salinity value of 8 PSU significantly separates fungal communities.

To check if an impact of the geographical distance on the phylogenetic dissimilarity exists, a distance-decay analysis was carried out applying linear regression (n = 231; P < 0.05) using the “lm” function of R. As this showed to be significant, the power of control of spatial factors on the fungal community variation was further tested. Thus, a spatial eigenfunction was carried out using the “distance-based Moran’s eigenvector maps” (dbMEM) function of the R package adespatial (Dray et al., 2017) to calculate eigenfactor and eigenvalues. As our sampling was not a standard sampling situation (e.g., not a straight transect line), the truncated distance matrix was generated following the example of Brind’Amour et al. (2005) but not looping the sample sites at the end. Eigenfunctions with a positive eigenvalue
were tested for significance ($P \leq 0.05$) by distance-based redundancy analysis (dbRDA)-based forward selection [function “ordisres” in the R package vegan (Oksanen et al., 2013)]. Based on a scologram using the Moran’s I coefficient as ordinate, significant distance-based Moran’s eigenvector map (dbMEM) eigenfunctions were defined to different scaling submodels. Next, to explain the partitioning of the observed variation in the mycoplankton community between the two components, spatial and environmental, a dbRDA-based model was also built on the environmental factors using forward selection. Finally, the different spatial submodels and the first three best models on the environmental parameters were independently used as input for variation partitioning analysis (VPA) by dbRDA using the “varpart” function in the R package vegan (Oksanen et al., 2013).

To estimate which ecological processes influence the fungal community at the different sampling sites, the statistical framework of Stegen et al. (2015) incorporated in the quantitative process estimates (QPEs) method was applied. This method considers five different assembly processes, namely, variation selection, homogenizing selection, dispersal limitation, homogenizing dispersal, and undominated processes, which are a lack of dominance between selection and dispersal. This approach requires significant phylogenetic signals, which are used for interpretation. First, the phylogenetic turnover of communities between the diverse sites was calculated as β-mean nearest taxon distance (MNTD) metric. Next, a null expectation was tested, meaning that ecological selection was not the primary factor of compositional differences by randomly reshuffling the OTUs over the tips of the phylogenetic tree. Significance was evaluated via the nearest taxon index (β-NTI) expressing the differences between the observed β-MNTD and the mean of the null distribution in units of SD. In the case of βNTI > |2|, a significant deviation from the null expectation exists, and variable (βNTI > 2) or homogenizing (βNTI < -2) selection is responsible for the differences observed. If the observed βNTI is not due to selection (| βNTI | < 2), it can be due to low or high rates of dispersal or undominated process (Stegen et al., 2012). To distinguish among these possibilities, the Raup–Crick metric ($\beta_{RC_{bray}}$) (Chase et al., 2011) was calculated with $\beta_{RC_{bray}} > 0.95$ and $\beta_{RC_{bray}} < -0.95$, indicating dispersal limitation and homogenizing dispersal, respectively, while $\beta_{RC_{bray}} < |0.95|$ reflects an undominated process. All calculations were done with the R code provided by Stegen et al. (2013) in GitHub1. Significant differences in the assembly processes among the sampling groups were calculated by Tukey HSD.

Another important question arose from the strong salinity gradient that spans the river section under investigation. The water’s salinity seems to be an important factor in the distribution and occurrence of aquatic basal fungi (Livermore and Mattes, 2013) (basal fungi include Chytridiomycota, Rozellomycota, and novel diversity clades on the branch of basal fungi). Samples 1–5, 11–17, and 20–24 were considered as above described as freshwater, brackish, and marine samples, respectively. (Note that in some cases, this grouping is not equal to the sample grouping found by PCoA/PERMANOVA and has solely been used to identify salinity-driven water-type-specific occurrence of the basal fungal lineages). Significant distribution (using the frequency) of the diverse basal fungal lineages over the three water types was tested with the Tukey HSD.

RESULTS

Taxonomic Community Composition

Fungal sequence reads (647,568) were generated and clustered into 913 fungal OTUs. Prior to further analyses, the two samples, 6 and 22, were removed due to low sequence output. All rarefaction curves levelled off reaching a plateau, indicating sufficient sequencing depth to capture most of the mycoplankton diversity (Supplementary Figure 2). OTUs were phylogenetically classified into six fungal phyla, 15 subphyla, 32 classes, and 55 orders. The phylogenetic approach led to the recognition of novel diversity forming 16 new clades, seven within the Chytridiomycota, seven within the Rozellomycota, and two branched along the basal fungi (Supplementary Figure 1). Three of the Chytridiomycota clades and five of the Rozellomycota clades clustered together with reference sequences of novel diversity groups recognized by Tedersoo et al. (2017). Additionally, abundant OTUs within the Chytridiomycetes clade 01 and Basal Fungi clade 02 had supporting BLASTn hits with sequences of Chytridium polysiphinae and an uncultured Rozellomyctota, respectively (Supplementary Table 3).

From the shallow freshwater area of the Elbe River to the marine environment, the relative abundance of Chytridiomycota decreased from 84.6% (sample 1) to 0% (sample 24), while Dikarya OTUs showed an opposite pattern, increasing from 7.5% (sample 1) to 94.7% (sample 24). Rozellomycota taxa were represented in all samples except one with up to 34.8% (sample 19) of the relative sequence abundance and were mainly accounted for by Rozellomyctota clade 01 (Figure 2A).

Nineteen OTUs were defined as abundant, from which 13 were classified as Chytridiomycota, 5 as Ascomycota, and 1 as Basal Fungi clade 02. In the BLASTn analysis, all of them showed high sequence similarity to sequences annotated to species level. For all of the matched sequences except one, literature screening allowed the identification of the taxon’s nutritional mode (Supplementary Table 3). The most abundant OTU was OTU SMBZZZZ9 from the order Zygoophlyctidales, representing up to 86.2% of the relative sequence abundance in the samples and being present in all samples with two exceptions (samples 20 and 23) (Supplementary Tables 3, 4). All Ascomycota OTUs showed highest frequency in the sample 24 located already beyond the river plume in marine waters (sample 24: position most close to the island of Helgoland). In contrast, nine of the other OTUs were highly frequent in the shallow freshwater area upstream of Hamburg (samples 1–5), but all were also present in the downstream area of Hamburg (samples 7–24). Here, two different pattern types were observed. The first one was a continuous OTU presence over numerous adjacent sampling sites (> 4) with sometimes further occurrence at other sampling sites but then usually with a lower frequency (84.2% of all abundant OTUs). The second pattern was defined by OTUs with presence at sampling sites with often high frequency.

1https://github.com/stegen/Stegen_etal_ISME_2013
FIGURE 2 | Partitioning of the mycoplankton communities. (A) Bar chart showing the relative sequence abundance of mycoplankton classes and novel diversity clades of samples taken on a transect of the lower reaches of the Elbe River including samples from the shallow, freshwater (samples 1–3) through the estuary (samples 4–17), to the river plume (samples 18–19), and the adjacent marine waters of the North Sea (samples 20–24). For information on taxonomic classification, see Supplementary Figure 1. (B) Principal component analysis (PCoA) ordinating mycoplankton communities based on their phylogenetic composition (Generalized UniFrac). Communities clustered in three significant different groups (PERMANOVA, P values see graph). Group I included samples of the shallow, freshwater zone of the Elbe transect; group II, samples taken at locations with pronounced river bed depth due to dredging activities; and group III, samples of the outer river plume. PSU, practical salinity unit.
but not in more than four adjacent sampling stations (15.8%) (Supplementary Figure 3).

Fungal Community Partitioning and Impact of Environmental Factors

PCoA ordinated the samples based on their phylogenetic diversity into three different sample groups with axis 1 explaining 34.4% and axis 2 14.4% of the observed separation (Figure 2B). Samples 1–5 grouped together (group I) and included samples of the shallow, freshwater water region upstream of Hamburg, with two samples taken at sites with tidal influence. Samples 7–19 formed a distant group (group II), which comprised samples of sites with fairway adjustment through dredging. Hereby, both freshwater and brackish water samples were represented. Sample group III was formed by samples 20–24, which were defined as marine based on the salinity values. The three sample groups differed significantly from each other (PERMANOVA (F value, $R^2$, FDR-adjusted $P$ value), groups I and II: 9.3, 0.37, 0.003; groups I and III: 57.5, 0.89, 0.006; groups II and III: 30.1, 0.67, 0.003) (Figures 1, 2B).

Environmental parameters were tested for collinearity using the Spearman rank order correlations identifying strong collinearity (FDR adjusted $P < 0.05$, $R^2 > 0.5$) mainly between NO$_2$ and NH$_4$ and between salinity, depth, and temperature (Supplementary Table 2). Prior to any analysis, we excluded NO$_2$ and NH$_4$, as the values of NO$_3$, NO$_2$, and NH$_4$ depend in their dynamics on each other in the Elbe River (Amann et al., 2012; Sanders et al., 2018), while the impact of salinity, depth, and temperature was analyzed separately (for example in different models). Pearson’s correlation coefficient identified salinity ($P = 0.001$, $R^2 = 0.79$), DOC ($P = 0.001$, $R^2 = 0.62$), and depth ($P = 0.001$, $R^2 = 0.52$) as explaining environmental variables for the sample separation along the axis 1, and PO$_4$ ($P = 0.001$, $R^2 = 0.66$), NO$_3$ ($P = 0.001$, $R^2 = 0.66$), temperature ($P = 0.001$, $R^2 = 0.76$), and chlorophyll a ($P = 0.001$, $R^2 = 0.70$) as the one for axis 2 (Figure 2B and Table 1). The sampling strategy had no impact on observed community differences (Tukey HSD, $P > 0.05$). Similarity profiles on environmental factors identified a wide environmental heterogeneity over the different sampling sites clustering the samples into eight distant groups ($P < 0.05$). Compared to the sample groups defined by PCoA and PERMANOVA, sample group I falls into regions with two different overall environmental conditions, sample group II into five, and only the marine sample group III showed relative homogeneous environmental conditions over the four sample locations (Figure 1).

Pronounced differences were observed in the correlations among the abundant OTUs of Ascomycota and Chytridiomycota with environmental parameters. Most Chytridiomycota OTUs showed negative correlations with depth and salinity and positive ones with temperature, chlorophyll a, and silicates. DOC was also among the positive correlated factors but less pronounced. Ascomycota OTUs, however, were negatively correlated mainly with silicate and nitrate ($P < 0.05$) (Supplementary Table 5).

As the value of 8 PSU is stated in the literature as the value separating organismic communities along salinity gradients, it was tested with PERMANOVA if a PSU of 8 also applies for the mycoplankton assemblage in this study. Indeed, when tested only for this factor, fungal communities significantly separated at the 8 PSU with samples 1–12 and 13–22 in the two different groups ($F$ value = 10.04, $R^2 = 0.33$, FDR-adjusted $P < 0.001$).

The comparative analysis as PCoA including communities of this study and the one of Banos et al. (2020) monitoring mycoplankton communities at Helgoland Roads over a year showed a grouping of samples 20–24 (marine samples) with the samples of Banos et al. (2020), while the sample group structure of groups I and II remained intact and well separated from the rest (Supplementary Figure 4).

α-Diversity

Sample group I stood out with the highest OTU richness and phylogenetic diversity among all identified sample groups with significant higher Chao1 and PD values of 395.9 ± 138.7 and 12.3 ± 2.7, respectively (Tukey HSD, permutations = 999, $P < 0.001$). Sample group III showed lowest Chao1 and PD values with 37.5 ± 35.4 and 1.8 ± 1.3, respectively. In contrast, no significance was found in Shannon diversity among sample groups ranging from 2.1 to 1.9 (Table 2).

Impact of Spatial and Environmental Factors on Community Assemblage

The phylogenetic dissimilarity among fungal communities increased gradually with distance of the sampling sites. The relationship was significant as shown by linear regression ($n = 231$, $P < 0.001$, $r^2 = 0.339$) (Supplementary Figure 5). dbMEM identified 21 eigenvectors, from which seven were positive along Moran I. Forward selection attested four out

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**Table 1** | Environmental factors with significant correlation with the first two principal component analysis (PCoA) axes, as tested by Pearson correlation coefficient analysis showing the FDR-adjusted $P < 0.005$ and $R^2 > 0.5$ for one of the two axes.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Axis 1</th>
<th>Axis 2</th>
<th>$R^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO$_4$</td>
<td>0.25</td>
<td>−0.96</td>
<td>0.66</td>
<td>0.002</td>
</tr>
<tr>
<td>NO$_3$</td>
<td>0.69</td>
<td>−0.71</td>
<td>0.65</td>
<td>0.002</td>
</tr>
<tr>
<td>Chl a</td>
<td>0.44</td>
<td>0.89</td>
<td>0.69</td>
<td>0.002</td>
</tr>
<tr>
<td>Salinity</td>
<td>−0.98</td>
<td>−0.16</td>
<td>0.79</td>
<td>0.002</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.59</td>
<td>0.80</td>
<td>0.76</td>
<td>0.002</td>
</tr>
<tr>
<td>DOC</td>
<td>0.83</td>
<td>−0.56</td>
<td>0.62</td>
<td>0.002</td>
</tr>
<tr>
<td>Depth</td>
<td>−0.97</td>
<td>−0.22</td>
<td>0.51</td>
<td>0.003</td>
</tr>
</tbody>
</table>

In bold, factors significantly related with one of the two axes.

**Table 2** | α-Diversity for the three identified samples groups (see Figure 2).

<table>
<thead>
<tr>
<th>α-diversity</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Significant difference ($P &lt; 0.001$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chao1</td>
<td>395.9 ± 138.7</td>
<td>109.1 ± 53.8</td>
<td>37.5 ± 35.4</td>
<td>GI–GII, GI–GIII</td>
</tr>
<tr>
<td>Shannon</td>
<td>2.0 ± 0.58</td>
<td>2.1 ± 0.58</td>
<td>1.9 ± 0.6</td>
<td>GI–GII, GI–GIII</td>
</tr>
<tr>
<td>PD</td>
<td>12.3 ± 2.7</td>
<td>4.7 ± 1.8</td>
<td>1.8 ± 1.3</td>
<td>GI–GII, GI–GIII</td>
</tr>
</tbody>
</table>

Taxon richness (Chao1), diversity (Shannon), and phylogenetic diversity (PD) was calculated for each group ($±$ SD), and significance between groups was tested with the Tukey’s post hoc test ($P < 0.001$).
of all positive eigenvectors a significant impact on the fungal assemblage (Supplementary Table 5). Based on the scalogram of Moran’s I, two different submodules (small and broad local scale) were defined (dbMEM1 and 2 as broad-scale factors; dbMEM3 and 5 as small-scale factors) (Supplementary Figure 6).

Due to collinearity among some of the environmental variables (Supplementary Table 2), three distant models were tested. The best model identified salinity ($F = 9, P < 0.001$) and PO$_4$ ($F = 3.5, P < 0.01$) as most important environmental factors, which were then used as input for VPA. For both spatial models applied in VPA, environmental parameters explained more of the observed variability than spatial factors. The model with a broad-scale effect identified 49.2% of the variations to be under environmental control, but the larger part (73%) of the environmental-dependent variation was shared with spatial factors pointing toward a spatial control of the environment. Only 1.8% of the observed variation among fungal communities was under spatial control alone, while 49% of the observed variation stayed unexplained. In the small-scale model, 49.2% were explained by the environment from which 10% were under spatial control. Spatial factors alone explained only 3.1% of the observed variations; 47.7% stayed unexplained (Figure 3). The two other environmental models, with (I) chlorophyll a ($F = 4, P = 0.001$), DOC ($F = 6, P = 0.001$), and NO$_3$ ($F = 3, P = 0.016$), or (II) temperature ($F = 7, P = 0.002$) and PO$_4$ ($F = 6, P < 0.001$) as most driving factors, were tested with the spatial models but showed similar trends. Thus, environmental factors explained always a higher percent of the observed variation than spatial factors (Supplementary Figure 7).

**Distant Assemblage Processes Dominate Different Sampling Sites**

QPE identified five different assembling processes acting on the fungal communities, namely, variation selection, homogenizing selection, dispersal limitation, homogenizing dispersal, and undominated processes. While variable selection dominated with 50% of the assembly processes in the upstream regions (group I) of the Elbe, undominated processes became dominant (60.3%) in the sample group II (dredged section of the Elbe) and group III (outer rive plume and beyond, 83.3%). All but homogenizing selection showed significance between samples group I and III (Tukey HSD, $P < 0.05$), while variable selection and homogenizing dispersal differed significantly among sample groups II and III ($P < 0.01$) and dispersal limitation and undominated processes among sample groups I and II ($P < 0.05$) (Figure 4).

**Salinity as Driving Factor for Distribution of Basal Fungi Within the Water Types of Marine, Brackish, and Freshwater**

Significant differences were observed in the frequency of the basal fungal taxa over the three water types of marine, brackish, and freshwater. The majority of Chytridiomycota taxa (12 taxa, 92%) were most frequent in freshwater. Among the eight Rozellomycota clades, seven were most frequent in brackish water. None of the taxa were only present in the marine environment. Most of the observed significant differences were found between marine and freshwater water types, to a less extent between fresh- and brackish waters, and only one between marine and brackish waters (Tukey HSD, $P < 0.05$) (Figure 5).

**DISCUSSION**

In order to develop sustainable concepts for river systems, where human use harmonizes with the stability of the ecosystem, it is important to have knowledge on the diversity and dynamics of central ecosystem key players. Pelagic fungi occupy a central role in the carbon cycle and food webs of freshwaters (Wurzbacher et al., 2010; Grossart et al., 2019). In contrast to other lotic systems (Krauss et al., 2011; Monchy et al., 2011; Duarte et al., 2015; Lepere et al., 2019), the mycoplankton of streams with higher order number is a poorly studied group of organisms. In this study, for the first time, mycoplankton communities were surveyed with fungus-specific primers and high-throughput sequencing over a transect encompassing the shallow freshwater area up to the river plume of the Elbe River and beyond to the marine area. This study aims to provide an initial

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**FIGURE 3** | Variation partitioning analysis attested that environmental factors (Env.) had a greater impact on the observed differences in mycoplankton assemblages than spatial factors. This holds true under two spatial scenarios: (A) broad scale and (B) small scale. Spatial scenarios are based on a scalogram using the Moran’s I coefficient as ordinate, where significant distance-based Moran’s eigenvector map (dbMEM) eigenfunctions were defined to the two scaling submodels (see Supplementary Figure 6).
FIGURE 4 | Different assemblage processes within the three sample groups as defined by quantitative process estimates (QPEs) method. Significant differences in the underlying assemblage processes between sample groups was tested by Tukey honestly significant difference (HSD) (\(^* P < 0.05; ** P < 0.01; *** P < 0.001\)).

FIGURE 5 | Relative frequencies of basal fungal taxon groups within the three water types of fresh, brackish, and marine waters. Partial sketch of the phylogenetic tree showing the relationship of the basal fungal lineages (for further details on the naming of the environmental clades, see Supplementary Figure 1, and for the original tree, see Supplementary Material 2). Colored branches indicate the presence of the specific taxon group in the given water type; black branch indicates absence. Significant differences in frequency between water types based on Tukey honestly significant difference (HSD) \( P \) values \((P < 0.05)\). Neighbor taxa to the clades in the phylogenetic tree, not originating from the transect, are shown in gray. The column “Match with other clades/sequences” indicates the accession number of sequences with high BLASTn scores with sequences of in this study newly identified environmental clades and/or indicates that representative sequences from Tedersoo et al. (2017) identified clades were phylogenetically placed on the branch of the environmental clades identified in this study.
picture on mycoplankton community pattern and underlying assemblage processes in order to implement more targeted studies, in which seasonality and different degrees of water runoff should be included. However, despite the short sampling time, the results reflected the site-specific conditions of the investigated Elbe section: Thus, in the Elbe, the water body is exposed to strong environmental changes during its passage through the riverbed, which many of them are (indirectly) associated with anthropogenic activities (Schöl et al., 2014; Geerts et al., 2017; Sanders et al., 2018). Thus, the effect of the most structural variable, the salinity gradient, was partly superimposed by secondary effects of Elbe deepening works, especially downstream of the city of Hamburg. As a consequence, the patterns of mycoplankton communities deviated from a clear grouping into marine, brackish, and freshwater communities as shown for bacterioplankton in other river systems (Selje and Simon, 2003; Henriques et al., 2006). Mycoplankton communities formed three distant biomes, in which Dikarya and basal fungi showed opposite distribution patterns with a dominance of Chytridiomycota in the upstream and estuary sections of the Elbe toward a dominance of Ascomycota in the end members of the river plume and in marine waters. In addition to environmental parameters, however, spatial parameters can also be important drivers of microbial community structures. River systems, for example, support passive organismic dispersal, from which pelagic microorganisms with small body size benefit much more than larger, multicellular organisms (Soininen et al., 2013). In this study, the phylogenetic similarity of mycoplankton communities declined significantly with increasing distance between sampling sites. However, the VPA determined that environmental factors, to some degree under spatial control, had the greatest influence on the mycoplankton community structure. Thus, it may be concluded that the process of fungal assemblage across the sampled transect depended largely on niche-based rather than neutral processes. However, niche-based processes cannot always be explained by environmental filtering alone, as selection pressure increases or decreases along the environmental gradient (Stegen et al., 2012). In the latter case, other processes such as low dispersal rates can still lead to a high taxon turnover between sites and evoke separation of communities (Stegen et al., 2013; Vass et al., 2020). This shows that multiple processes can simultaneously govern ecological systems. Especially for a naturally nested system like rivers, it is even more important to distinguish between assemblage processes resulting from a combination of variable selection linked to low or high dispersal rates, which is why the ecological framework of Stegen et al. (2015) was applied in this study. This distinguishes between five different assemblage processes, namely, variable selection, homogenizing selection, dispersal limitation, homogenizing dispersal, and undominated process like ecological drift. Applied to the studied Elbe transect, significant differences were observed: Variable selection dominated in the upstream samples, whereas the proportion of undominated processes, where neither selection nor dispersal dominated, increased toward the river mouth and beyond.

Sample group I comprised all samples of the shallow freshwater area upstream of the city of Hamburg, which is a highly saturated eutrophic system, characterized by strong phytoplankton growth with a seasonal mean of 150 µg chlorophyll a L⁻¹ and an oxygen oversaturation of the water (Schöl et al., 2014). The strong growth and high diversity of phytoplankton are a source for a rich plankton community forming complex food webs (Kerner et al., 2004). The mycoplankton communities within group I were dominated with up to 84.6% of the relative sequence abundance by Chytridiomycota. Taxa in this phylum are saprotrophs, or pathogens infecting phytoplankton, zooplankton, insects, and other fungi (Gleason et al., 2008). Both nutrition modes were found among the detected Chytridiomycota OTUs. Their ecological versatility makes them key players in food web dynamics affecting primary producers, predators, grazers, and degraders (Gleason et al., 2014; Kagami et al., 2014). The high taxon richness and phylogenetic diversity detected in this study suggest that they occupy diverse ecological niches and are involved at various trophic levels of the food webs established in this Elbe section. The often specific interactions among host–parasite or prey–grazer (Holfeld, 1998; Pinto et al., 2001; Ibélings et al., 2004; Vargas et al., 2006) may be among the reasons why variable selection was the dominant assemblage process in this sample group. Dispersal limitation was the second most important one. A major factor is certainly the weir at the level of Geesthacht, which prevents tidal upstream mixing of water between samples 3 and 4. Factors causing a dispersal limitation of microorganisms have been further identified on a microscopic level (Peay et al., 2010; Stegen et al., 2013). This is the case when local conditions inhibit subsequent colonization from one niche to another, such as reported for cells from particle-associated biofilms inhibited to colonize the surrounding water (Martiny et al., 2011). The extent to which such barriers play a role in the assembly of mycoplankton within the studied Elbe River transect needs to be investigated in further works.

Interestingly, the mycoplankton communities found downstream from the city of Hamburg differed significantly from sample group I, although some sample sites still had a salinity value within the freshwater range. From the port of Hamburg, the environmental conditions change drastically due to a sudden increase in depth to a maximum of 15 m caused by dredging (Geerts et al., 2017). The consequence is light limitation and strongly reduced oxygen content, while the net phytoplankton growth becomes negative and the system changes from an autotrophic to a heterotrophic one (Amann et al., 2012; Schöl et al., 2014). The significant separation of sample groups I and II, which were both dominated by Chytridiomycota, was partly due to abundance shifts of existing OTUs together with a strong reduction in phylogenetic diversity. However, one-third of the observed beta-diversity among sites could be attributed to taxon turnover by variable selection. The presence of numerous OTUs over larger sections of the estuary and the increase in abundance of various OTUs at different sample locations suggest a niche-specific growth and rules out that these OTUs are only inoculum-like resting spores. This observation also speaks against the possibility that the abundant OTUs are sediment-inhabiting taxa flushed up by turbulences. The residence time of the water body during summer time with low discharge is up to 11 days in the first 30 km downstream from Hamburg and even up to 35 days in the areas further downstream.
to the mouth of the Elbe, which is also a consequence of the Elbe dredging (Bergemann et al., 1996; Amann et al., 2012). This may give the mycoplankton communities time to grow in their specific niche and/or react to site-specific conditions. Some of the most abundant Chytridiomycota OTUs in the Elbe estuary were identified as saprotrophs and showed significant correlation with DOC. In the estuary of the Elbe River, there are various inputs of organic substrate, from external sources, including marine-, terrestrial-, and river-derived algae detritus and wastewater, and in situ estuarine sources (Middelburg and Herman, 2007). Chytridiomycota are among the important players of freshwater degradation (Sparrow, 1960; Wurzbacher et al., 2010) and have been reported to decompose smaller particles or benefit directly from DOC (Wurzbacher et al., 2014). By considering the frequency at sampling sites and the distribution pattern along the estuary of individual Chytridiomycota OTUs, three different scenarios can be deduced. First, the potential saprotrophic taxa possess a broad substrate specificity (Gleason et al., 2011) and/or are actors within functional guilds. Second, they may occupy specific niches that are maintained over longer stretches of the estuary through a long residence time. This may especially hold true for sample locations 8–13, which are situated in a river section with high particulate organic carbon (POC) concentrations (Amann et al., 2012). These first two scenarios may be reflected in continuous OTU abundance over four and more adjacent sampling sites as reported for several abundant OTUs. Third, taxa with pronounced abundance at single sampling sites may have proliferated quickly because the appropriate food source, such as labile organic material, was available. The percentage of labile POC in rivers increases, for example, through the input of sewage (Etcheber et al., 2007), as it occurs in the Elbe estuary due to the strong anthropogenic activities of the Hamburg city area. Beside variable selection, mycoplankton communities in group II were largely assembled by ecological drift, a process, where moderate dispersal rates are coupled with low selection pressure. Ecological drift causes taxon abundances to vary, lowering diversity within communities and increasing differences among otherwise similar communities. One process affecting drift in natural systems are indirect multispecies interactions (Gilbert and Levine, 2017), and their level of complexity has a profound impact on the assembly of eukaryotic microbial communities (Bock et al., 2020). According to Read et al. (2015), microbial networks are particularly complex in downstream sites because the river water is older and contains a planktonic community that is in a later stage of ecological succession. Banos et al. (2020) recently showed that a large proportion of taxa in marine pelagic mycoplankton taxa interact with each other in manifold ways such as in competition or in potential functional guilds. Applied on the Elbe transect, this situation may be particularly true for the areas around the mouth of the river with the longest water residence times.

Sample group III included samples in the area, where the river plume faded out and conditions became marine. In this area, the Elbe River outflow mixes with ocean waters due to western incoming currents, and the hydrographic conditions change to oceanic (Callies and Scharfe, 2015). Lucas et al. (2016) showed that this leads to a significant difference between bacterioplankton communities in the inner German Bight and those in the open North Sea. The authors assumed Helgoland to be the eastern boundary of the main current direction in the German Bight. Relative to the mycoplankton communities, the lowest phylogenetic diversity and taxonomic abundance was found in group III. Additionally, communities were now dominated by Ascomycota. A comparison with the mycoplankton communities monitored over a full year at the nearby long-term research station Helgoland Roads (Banos et al., 2020) showed large agreement with the communities of samples 20-24. In the mentioned study, mainly Ascomycota dominated the mycoplankton communities. The observed significant taxonomic shift between groups II and III may, thus, have resulted from the interplay of different converging processes: (I) Growth and reproduction of pelagic Chytridiomycota seems to be favored in environments with lower salinities compared to the open oceans (Hassett and Gradinger, 2016; Hassett et al., 2019). Bouvier and del Giorgio (2002) reported a similar abrupt change between salt-tolerating and less tolerant bacterioplankton communities relating it to organismic salt tolerance resulting into cell inactivation or cell death. (II) The negative response of some phytoplankton species to marine salinity values (Lionard et al., 2005; Nakov et al., 2019) has, in consequence, a negative effect on the presence and abundance of the associated parasitic Chytridiomycota taxa. (III) One of the significant assemble processes for mycoplankton communities in group III was homogenizing dispersal. This may point toward an increased input from upstream regions of the Elbe River, which may include (terrestrial) Ascomycota taxa that are able to proliferate in the marine environment. However, it has to be noted that marine fungal communities can be dominated by zoosporic fungi (Comeau et al., 2016). Studies from the North Sea reported temporary dominance (Priest et al., 2021) and showed, e.g., dependence on host abundance and nutrient availability (Scholz et al., 2016). Despite the different processes acting on the mycoplankton communities, our model showed that the salt gradient was the most important environmental parameter for the structuring of the mycoplankton community. In river estuaries, salt gradients are of general relevance, as they often define structural and functional characteristics of aquatic biota (Telesh and Khlebovich, 2010). In numerous studies, the value around 8 PSU has been described as a threshold value that significantly divides the organism groups under investigation (Sagert et al., 2008; Bleich et al., 2011; Schubert et al., 2011). This was also the case for mycoplankton communities that were studied along a salinity gradient of the Baltic Sea (Rojas-Jimenez et al., 2019). In our study, there was no group splitting at this threshold value. Only when specifically tested for the value of 8 PSU, a significant separation of mycoplankton communities could be observed. The Elbe is under strong tidal influence, and a salt wedge is formed, which is relatively inside the Elbe with a maximum at the level of Stade (samples 11 and 12). Along the salt wedge, there is an increased exchange of fresh and ocean waters. As side effect, marine particulate organic matter can be found in all group II samples (Schöl et al., 2014). Thus, a whole bouquet rather than a single factor impacts on the mycoplankton
community structure, preventing a split of communities at 8 PSU in a multivariate approach. In our study, the most pronounced and significant change in the mycoplankton community structure was registered at a threshold level of 30 PSU, while all brackish water samples with their existing variability formed a significant group. Brackish-water-specific niche formation was already detected, for example, for fluvial bacteria (Selje and Simon, 2003) and fungi in salt marshes (Mohamed and Martiny, 2011). The protistan species-maximum concept developed by Telesh et al. (2013) encapsulates this observation and shows that unicellular eukaryotes are particularly strongly represented in the horohalinicum zone. One reason for this is that biota from two adjacent sites are mixed. Furthermore, for many organisms, the limiting factor is not the organism’s own salinity tolerance but the salinity variability (Attrill, 2002; Telesh et al., 2011). Most eukaryotic unicellular planktonic organisms have a high physiological adaptation to fluctuating salinity, and therefore, the salt content provides subsidy rather than a stressful environment (Stock et al., 2002; Telesh et al., 2011). Thus, they can develop in a system with reduced competitive pressure. Chytridiomycota belong to this group with a small body size of 3–5 μm as zoospores (Gleason and Lilje, 2009). The most abundant OTU, Chytridiomycota SMBZZZZZ9, was found in almost all samples and thus spanned a salt gradient from 0 to 31 PSU. However, this is probably rather an exception. Gleason et al. (2006) tested numerous Chytridiomycota for their salt tolerance. Most of them showed growth success in salt-containing medium mimicking brackish but not ocean water salinity levels. The grouping of the samples along their salinity into the three water types of fresh, brackish, and marine waters revealed no true marine zoosporic fungal clade. Furthermore, each of the identified zoosporic fungal clades was found in at least two of the three water types, mostly in fresh and in brackish waters. The evolutionary history of Chytridiomycota suggests that they originated in a brackish/freshwater-like environment (Berbee et al., 2017) and thus evolved successively in a series of transient brackish waters, where they were able to develop and diversify in parallel with the developing host organisms or existing food (Chang et al., 2015). The dependence of parasitic Chytridiomycota on the adaptation of the host organisms over the salt gradient as well as the reduced adaptability of most Chytridiomycota to high salt concentrations are two important points probably controlling for the presence and abundance of Chytridiomycota in fresh- and brackish water. It remains an open question whether marine Chytridiomycota proliferate due to lower competition in high-salinity water or whether specialization of some taxa has occurred over an evolutionary time scale but underwent so far detection.

CONCLUSION

Understanding the relative importance of dispersal and environmental selection in shaping mycoplankton community in water may help predictions of fungal-driven ecological processes, like mycollex, or mycoloop, and conservation of biodiversity in river ecosystems. Our results show that the mycoplankton communities in the Elbe River from the shallow freshwater zone over the estuary and its river plume are subjected to very different assemblage processes and differ significantly from those stations subjected to strong marine influence. Additionally, assemblage processes can change over relatively short distances. Community assembly processes are not static, and the relative importance of one can vary under different conditions and between members of a community. Further work is needed to understand how strongly the assembly processes observed here are related, for example, to the strength of the water runoff and which consequences a shift in the community composition has on the fungal-driven ecological processes.

DATA AVAILABILITY STATEMENT

The generated sequence datasets can be obtained from the European Nucleotide Archive (ENA) with the accession number PRJEB39018. The corresponding environmental data is published in PANGAEA (Reich et al., 2021). The fully annotated OTU table can be accessed over the Supplementary Table 4, representative sequences for each OTU over a Supplementary .fasta-file (Data Sheet 2), and the phylogenetic tree including the inserted generated sequences over a Supplementary .tree-file (Data Sheet 3).

AUTHOR CONTRIBUTIONS

MR, GG, and AW planned and designed the study. YY and SB ran the sequencing pipeline. YY, GG, and MR analyzed the data. YY, SB, and MR wrote the manuscript. All authors have reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.640469/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary Material**

Myoplankton biome structure and assemblage processes differ significantly along a transect from the shallow freshwater area of the Elbe River down to the river plume and the adjacent marine waters

Yanyan Yang, Stefanos Banos, Gunnar Gerdts, Antje Wichels, Marlis Reich

1.1 Supplementary Figures

**Supplementary Figure S1.** Sketch of the fungal phylogenetic tree to explain why environmental clades were classified at different taxonomic levels. Novel diversity clades are colored in blue. Their taxonomic position depends on the branch on which the clade is located and if this branch has a reliable taxonomic assignment.
Supplementary Figure S2. Rarefaction curves calculated on all generated sequences (communities over the total transect) and sample group wise (see PCoA, Fig. 2). Shaded area indicates the 95%-confidence interval.
Supplementary Figure S3. Within the estuary of the Eble River, two different distribution patterns of the abundant OTUs were observed (samples 7-24). OTUs being present (A) over numerous adjacent sampling sites (>4; like OTU SMBZZZ14, Rhizophydiales, saprotroph), or (B) high frequency at single sampling sites if occurring in adjacent sites in a maximum of 3 adjacent sampling stations (like OTU SBMZZZ18, Chytridiomycota clade 01, saprotroph).
Supplementary Figure. S4: PCoA segregating the mycoplankton communities of the end members of the studied transect (marine environment, samples 20-24) with mycoplankton communities of Helgoland Roads described by Banos et al. (2020). PCoA is based on Generalized UniFrac values with the distance parameter of “0” accounting only for the phylogenetic structure and not abundances. Samples 1-24 are samples from the transect. Red, group I; green, group II; blue, group III. Group III are marine samples where the river plume faded off. Samples 45-87 in pink are from Helgoland Roads (Banos et al., 2020, DOI: 10.3389/fmicb.2020.01305).
Supplementary Figure S5: Distance-decay analysis based on linear regression indicating a significant relationship of increasing phylogenetic dissimilarity among communities with increasing distance (km) of sampling sites. Phylogenetic dissimilarity calculated as Generalized UniFrac-value. Distance between sampling sites was calculated as cumulative water channel distance.
Supplementary Figure S6. Definitions of the two spatial submodules used for VPA analysis were based on the scalogram using the Moran’s I coefficient as ordinate. *, significant eigenfunctions (forward selection, \( P < .05 \)).
Supplementary Figure S6: VPA calculated with three different environmental models. Due to high collinearity among some of the environmental variables, three different models were calculated with db-RDA based forward selection. The best model was the one with salinity and PO$_4$. However, all models showed a similar trend indicating that observed variability among mycoplankton communities in the lower reaches of the Elbe River are mainly driven by environmental factors, which are partly under spatial control.
### Supplementary Table S1: Geographic location and grouping of samples

Sample grouping as given by PCoA and confirmed by PERMANOVA analysis \((P < 0.05)\). Additionally, samples were manually grouped into samples of fresh, brackish, and marine water types based on their salinity value. Some samples were spared out from this analysis as they strongly vary in salinity over the course of a year and thus cannot be assigned into one of the groups based on water types (see materials and methods).

<table>
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<th>Sample</th>
<th>Ost</th>
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<th>Temp. (°C)</th>
<th>DOC (µmol/l)</th>
<th>Chl a (µg/l)</th>
<th>SiO4 (µmol/l)</th>
<th>PO4 (µmol/l)</th>
<th>NO2 (µmol/l)</th>
<th>NO3 (µmol/l)</th>
<th>NH4 (µmol/l)</th>
<th>pH</th>
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## Supplementary Table S2: Collinearity of environmental parameters tested by Spearman rank order correlations.

Only significant correlations (FDR adjusted \( P < .05 \)) with high relevance \( (R^2 \geq 0.5) \) are shown. R, \( R^2 \)-values are indicated in the table.

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<th>PO(_4 )</th>
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<th>NO(_3 )</th>
<th>NH(_4 )</th>
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<th>Salinity</th>
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<td>0.83,0.68</td>
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</table>

## Supplementary Table S3: Taxonomic classification and trophic mode of the most abundant OTUs.

Representative sequences of the OTUs were inserted into the phylogenetic reference tree using phylogenetic placement. Taxonomy of the branch where sequences were placed, were transferred on the OTU. Additionally, BLASTn was carried out and best BLAST hits are reported. Based on the classification over BLASTn, literature was screened for information on taxa specific trophic modes.

> See additional .xlsx-file

## Supplementary Table S4: Fully annotated OTU table.

The table provides frequencies of OTUs and detailed taxonomic information from the phylogenetic tree. Representative sequence for each OTU can be found in the attached .fasta-file Supplementary File S1.

> See attached .xlsx-file
Supplementary Table S5: Correlation analyses of the abundant OTUs with environmental parameters using Pearson rank order correlations. Only significant correlations (FDR adjusted $P < .05$) are shown. $R$, $R^2$ values are shown in the table.

<table>
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<th>SiO$_4$</th>
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Table is continued on the next page…
### OTUs Phy lum                  NO\(_3\)          NH\(_4\)        pH          Salinity        Depth

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**Supplementary Table S5: Distance-based Moran’s eigenvector (dbMEM) analysis** identified seven eigenvectors with positive Moran I-values. Out of those, four were identified to have a significant effect on mycoplankton community structure as detected by dbRDA-based forward selection.

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**Forward selection on positive MEM factors**

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Supplementary Table S3: Taxonomic classification and trophic mode of the most abundant OTUs.

https://www.frontiersin.org/articles/file/downloadfile/640469_supplementary-materials_tables_3_xlsx/octet-stream/Table%203.xlsx/1/640469

Supplementary Table S4: Fully annotated OTU table.

https://www.frontiersin.org/articles/file/downloadfile/640469_supplementary-materials_tables_4_xlsx/octet-stream/Table%204.xlsx/1/640469

Supplementary File S1: Representative sequences of all OTUs detected in this study.


Supplementary File 2: Phylogenetic tree as .tree-file.

4. Discussion

4.1 Primer toolkit

The primer toolkit exhibited in Chapter I is comprised of an exhaustive list of 18S rRNA gene sequence primers of different characteristics in terms of amplicon length, potential to cover the total fungal community or specific fungal taxon groups, and avoiding co-amplification of non-fungal eukaryotes. This toolkit can facilitate the selection of the appropriate primer pair relevant to the aims of a given research study and simultaneously reduce the potential amplification biases (Chapter I). Moreover, it could function complementary to current existing fungal specific 18S rRNA gene sequence tools, allowing an easy, direct, and user-friendly classification of fungal sequences. Primer pair nu-SSU-1334-5'/nu-SSU-1648-3' holds an essential position in the proposed list as the most suitable Illumina sequencing pair for the amplification of environmental communities. In all three projects, nu-SSU-1334-5'/nu-SSU-1648-3' managed to amplify sequences assigned to nearly all major fungal phyla and subphyla (Chapter I, II, III). In addition, nu-SSU-1334-5'/nu-SSU-1648-3' proved effective in retrieving sequences belonging to the different groups of Basal fungi lineages (Chapters I, II, III), a group of fungal taxa comprising a large proportion of fungi in aquatic environments (Richards et al., 2015; Hassett et al., 2020). Similar performance of the primer pair was also observed in recent studies of aquatic fungi from the Baltic Sea (Rojas-Jimenez et al., 2019) and sediments in the Eastern Tropical Pacific (Rojas-Jimenez et al., 2020). In addition, with the adoption of a phylogeny-based classification strategy (Yarza et al., 2017), novel diversity belonging to Basal fungi lineages was identified with 6 (Chapter II) and 16 (Chapter III) new clades. Phylogenetic analyses of soil fungi have already revealed several clades of previously unclassified taxa belonging to Basal fungal lineages (Tedersoo et al., 2017). The detection of new clades filled the gaps in the fungal tree of life and contributed to the update of the higher classification in the kingdom of Fungi (Tedersoo et al., 2017; Tedersoo et al., 2018a; Wijayawaredene et al., 2020). Similarly, in aquatic ecosystems the discovery of new clades of underscribed taxa, associated with lineages at the base of the fungal tree, could further shed light to the composition, taxonomy, and ecology of the so called “dark matter fungi” (Grossart et al., 2016, 2019).

In Chapters II and III, blocking oligos were implemented in the amplification of the DNA of environmental samples as there was expected a high risk of co-amplification from members of
the SAR group (Chapter I). In aquatic environmental samples, a large portion of the planktonic eukaryotic community still remains unknown (Leray & Knowlton, 2016). When FF390/FR1 is used without a blocking oligo approach, it is likely that a notable fraction of the sequences originates from non-fungal co-amplified eukaryotes (Naim et al., 2017; Rojas-Jimenez et al., 2017, 2019). In Chapter I, the use of blocking oligos in the aquatic samples proved effective in the reduction of the co-amplified sequences. However, the application of the proposed blocking oligos cannot ensure a complete reduction of the co-amplification. The SAR group includes diverse groups of eukaryotic parasites (Pawlowski et al., 2012) common in aquatic systems (de Vargas et al., 2015; Bjorbækmo et al., 2019) with new clades being discovered frequently (Taib, 2013; Massana et al., 2015). Using blocking oligos could improve the sequencing output, but unknown diversity from SAR group members or other aquatic eukaryotes could still pose a co-amplification threat. Similarly, the often reported coamplification of Metazoa lineages can further reduce the fungal sequence output. Nevertheless, the use of nu-SSU-1334-5'/nu-SSU-1648-3’ paired with the blocking oligos in Chapters II and III and in other studies (Priest et al., 2021) produced highly diverse mycoplancton assemblages, allowing the investigation of the structure, dynamics, and role of fungal communities in aquatic environments.

4.2 Pelagic fungal community dynamics in time series samples

My work in Chapter II contributes to the understanding of the temporal dynamics of marine mycoplancton communities within a specific location, and explores the potential biotic and abiotic drivers of these dynamics. Time series observations are an essential tool, being able to push forward our knowledge about the diversity, structure, and functionality of environmental microbial communities (Coenen et al., 2020). Changes in communities through time could be linked to natural or anthropogenic changes in environmental conditions, allowing to address questions about the various ecosystem mechanisms and processes (Buttigieg et al., 2018; Zingone et al., 2019). Despite the importance of time series studies in microbial ecology, little is known about pelagic marine fungal communities, with very few available investigations (Duan et al., 2018) following a tight weekly strategy over a year period as in Chapter II.

Samples of Helgoland Roads revealed a highly diverse and dynamic pelagic mycoplanктон community over the course of the year. In some cases, great differences were
observed in the overall community structure even between samples separated just by one week (Chapter II). On the temporal scale, seasonality was detected on the phylogenetic structure of the fungal assemblages from samples that have been collected mainly in autumn and winter (Chapter II). To explore further the detected dynamics, I looked at the abundant members of the community by investigating their sequence abundance patterns over time. The identification of distinct abundance patterns could be indicative of the OTUs lifestyle and relationships with the environmental parameters. In Chapter II, all abundant OTUs could be categorized into four distinct categories as a result of their similar succession curves. Analogous patterns were identified also for other non-fungal aquatic microbes (Gerphagnon et al., 2013; Needham et al., 2013).

To shed light on the observed patterns and dynamics, I explored the interactions of single OTUs against the various environmental factors and the relations between the different members, forming the whole community co-occurrence network (Chapter II). Co-occurrence networks are a powerful tool with the ability to provide insights into the ecological interactions between the different species of an aquatic microbial community and the environment (Bunse et al., 2016; Milici et al., 2016; Pearman et al., 2016; Mikhailov et al., 2019). The data showed that the observed diversity could be explained by the inter-phyla fungal relations and by the rare OTUs involved in the co-occurrence network (Chapter II). The detected network was formed mainly by rare OTUs, which means that they shaped the backbone of the total fungal community. Recent studies brought to the spotlight the rare fraction of the community as a prominent ecological and evolutionary component (Logares et al., 2014; Coveley et al., 2015; Lynch & Neufeld, 2015; Jousset et al., 2017). Rare taxa can form a pool of diversity, allowing rapid responses to temporal and environmental fluctuations, displaying their potential as a source of functional diversity (Logares et al., 2014; Jiao et al., 2017b; Wang et al., 2017).

Besides, the results demonstrated many OTUs that significantly correlated with phytoplankton and zooplankton groups and with other members of the fungal community. The nature of the potential interactions combined with the specific OTU patterns implied strong antagonistic relationships between fungi and the other planktonic groups (Chapter II). Indeed, aquatic fungi are well recorded as parasites in a variety of phytoplankton species (Rasconi et al., 2009, 2012; Kagami et al., 2011; Lepelletier et al., 2014). On the other side, zooplankton organisms can use fungal zoospores as a nutrient rich food source, improving their survival
chances (Kagami et al., 2011; Schmeller et al., 2014; Agha et al., 2016). From an ecological point of view, the antagonistic relationships could be interpreted as a top-down control of fungi to planktonic organisms or as a transfer of organic material and nutrients from fungi towards zooplankton. Both viewpoints are in line with the proposed conceptual models demonstrating the different processes, like the “Mycoloop”, by which fungi can affect the food web structure and the flow of organic matter in aquatic environments (Gutierrez et al., 2016; Amend et al., 2019; Grossart et al., 2019).

### 4.3 Spatial Dynamics in Elbe River

Chapter III presents one of the first studies focusing on aquatic fungal organisms along a transect line of salinity from freshwater river to marine waters. Riverine systems create across their length a continuum where their water masses are coming under the influence of diverse physiochemical and hydrological gradients. This Chapter explores the influence of the environmental and spatial parameters across these gradients on the fungal communities and provides critical insights on their structure, diversity and assemblage processes.

Samples were clustered in three groups based on their phylogenetic composition: Group I, II and III including freshwater samples upstream from Hamburg, freshwater and brackish samples until the mouth of the Elbe River and marine samples until the island of Helgoland in the North sea, respectively. Group samples I and II were dominated mainly by Chytridiomycota whereas Group samples III were dominated mainly by Ascomycota OTUs. Although the first two groups presented a similar community composition in higher taxonomic levels, they were still separated due to the higher phylogenetic diversity of Group I and the differences in abundance of commonly shared OTUs. Group III marine samples originated from the same or nearby sea region in the North Sea as the time series samples of Chapter II, and shared with them a similar overall taxonomic composition. When all samples from both projects were compared phylogenetically, Group III clustered together with the time series project and not with Group I and II samples. Chapters II and III were consistent as far as it concerns the structure of the marine pelagic mycoplankton communities of the Helgoland region. The consistency in the structure of the marine pelagic mycoplankton communities between Chapters II and III, combined with the
overall phylogenetic differences with the brackish and freshwater samples, points out salinity as a potential driving force behind the observed dissimilarities.

Indeed, salinity gradient was the most substantial environmental factor in the formation of the aquatic fungal communities across the transect sampling locations (Chapter III). A salinity value around 8 PSU has been reported in multiple studies, as a critical threshold value driving changes and fluctuations in the community composition of the various aquatic organism groups (Telesh & Khlebovich, 2010; Herlemann et al., 2011; Schubert et al., 2011). A similar threshold was described by Rojas-Jimenez et al. (2019) for the mycoplankton assemblages along a salinity gradient in the Baltic Sea. Besides, other studies showed differences between the fungal communities across broad salt gradients in Arctic and Antarctic regions (Zhang et al., 2016; Rojas-Jimenez et al., 2017), marshes (Mohamed & Martiny, 2011), intertidal wetlands and shallow marine sediments (Picard, 2017) and estuarine, coastal and pelagic waters (Jeffries et al., 2016). Our community in Chapter III did not display the most pronounced changes at 8 PSU, but at 30 PSU, where the brackish samples were clearly separated from the marine ones. This is directly reflected in the dominance of Chytridiomycota and other zoosporic fungi in the freshwater and brackish samples but not in the ones close to the Helgoland area, with salinity values over 30 PSU. Chytridiomycota species have been shown that are able to grow successfully in media with brackish salt content, but their tolerance to ocean salt levels is lower (Gleason et al., 2006). Aquatic environments with lower salinities could be more favorable for Chytrids because their zoospores do not possess cell walls, making them more vulnerable to osmotic stresses (Gleason et al., 2008; Gleason & Lilje, 2009). Indeed, NGS studies in non-marine environments demonstrated Chytridiomycota as the most abundant phylum in river (Bai et al., 2018; Chen et al., 2020), lake (Wurzbacher et al., 2016; Zhang et al., 2018), and estuarine regions (Rojas-Jimenez et al., 2019). However, there are also available studies reporting that Chytridiomycota could be prevalent in samples from pelagic and coastal waters (Comeau et al., 2016; Picard et al., 2017). Chytrid species are able to parasitize various groups of phytoplankton in marine environment, hinting that these organisms could have developed other mechanisms to deal with salinity (Gleason et al., 2011, Hassett et al., 2017).

A possible reason for the separation of samples at 30 and not at 8 PSU could be the presence of a salt wedge along the Elbe River until the level of the city of Stade. This salt wedge allows the exchange of fresh and marine waters and extents the influence of the marine organic
matter to all brackish and freshwater samples of Group II. Thus, multiple parameters and not only salinity could be the drivers shaping the fungal community in Chapter III. Besides, it was revealed a broad environmental heterogeneity across the sampling sites with the samples clustering into eight different groups (Chapter III). Spatial factors also cannot be excluded as a potential driver of the observed variation, since multiple assembly processes can simultaneously control the community composition (Vellend, 2010; Stegen & Hurlbert, 2011; Leibold & Chase, 2017). Aiming to discover the forces influencing the assemblage process at the different sampling points, we applied the ecological framework of Stegen et al. (2015). Our data revealed a difference between the dominant assemblage processes between the three Group samples, with variable selection for Group I and undominated processes for Group II and III being the principal ecological drivers.

Group samples I are located in a saturated eutrophic area of the river with a strong growth and high diversity of phytoplankton. The highly diverse Chytridiomycota taxa dominating these samples include OTUs with both saprotrophic and parasitic nutrition modes (Chapter III). As it was also presented in Chapter II, Chytrids are able to interact with diverse phytoplankton species influencing the various food web dynamics in aquatic environments (Gleason et al., 2014; Kagami et al., 2014). These interactions could be one of the reasons explaining why variable selection was the major assemblage process observed for Group I. Downstream from the city of Hamburg samples Group II fungal communities were formed by undominated processes where neither selection nor dispersal processes dominated. This part of the river is characterized by a large increase in depth because of dredging activities, leading to light limitation and lower oxygen content compared to the Group I region. These conditions cause a negative net phytoplankton growth, changing the nutritional system mode from autotrophic to heterotrophic (Amann et al., 2012; Schöl et al., 2014). Besides, the estuarine region of Elbe is heavily affected by terrestrial, marine, riverine, and anthropogenic sources of organic matter (Middelburg and Herman, 2007). Species in stream systems are expected to be influenced, mainly by environmental factors and by various degrees of dispersal depending on the studied organism and the river characteristics (Heino et al., 2015). The different environmental conditions between the upstream and downstream regions from the city of Hamburg were reflected in the different dominate assemblage processes in Group I and Group II samples. Between these group samples there was a transition from a dominant variable selection in Group I to a mixture of moderate
levels of selection and dispersal where neither one of them was the main assemblage. Selection and dispersal limitation may operate more strongly in geographic areas considered ecological boundaries, for example, due to strong physicochemical change in the seawater, leading to abrupt changes in microbiome composition.

Moving towards to Group III samples, the assemblage processes shift even more towards undominated processes. Group III samples stretch in a relatively small marine region where dispersal limitation is expected to be limited (Heino et al., 2015). In these samples, the influence of the Elbe River fades away and salinity becomes typical marine and the general environmental conditions change to oceanic. Different to Chapter III marine samples, other studies targeting oceanic microbial eukaryotes showed dispersal limitation (Logares et al., 2018b, 2020) or variable selection (Wu et al., 2018) as the main assemblage processes. The reasons behind this discrepancy could be our small number of marine samples, the different climatic conditions, and that the studies aimed at the complete fraction of the eukaryotic microbial communities and not only specific fungi. Different groups of organisms could possible exhibit different assemblage processes when they are studied in parallel (Powell et al., 2015a; Logares et al., 2018a, 2018b; Wu et al., 2018). The community assemblage processes are not fixed, and the level of influence of selection, dispersal and drift may change under different environmental conditions, across relatively short distances and among the various part of a community.

4.4 Future perspectives

The development of third-generation sequencing (TGS) technologies enabled the production of significant longer reads than the NGS methods (Goodwin et al., 2016) in a real-time sequencing process (van Dijk et al., 2018). Pacific Biosciences (PacBio) with single-molecule real-time sequencing (Eid et al., 2009) and Oxford Nanopore Technologies (ONT) with nanopore sequencing (Jain et al., 2015) are the most widely used TGS platforms. Initially, TGS high error rates, relatively low throughput, and limited bioinformatics tools for the processing of produced sequences (van Dijk et al., 2018) made them less attractive for many researchers. Improvements in the error rate by increasing the number of generated subreads (Goodwin et al., 2016) and the introduction of the more affordable with higher throughput new Sequel PacBio platforms, offered an alternative approach to study environmental fungal communities (Tedersoo
et al., 2018b; Purahong et al., 2019). Recently, with the application of PacBio Sequel technology, Tedersoo et al. (2020) used the total ITS together with a small partial region of the 18S rRNA gene sequence to explore the regional-scale diversity of soil environmental samples in the Northern Baltic region. TGS techniques could be proven very promising in the barcoding of fungi offering the possibility to sequence the whole rRNA operon, allowing to combine information from 18S and 28S rRNA genes and ITS spacer regions (Heeger et al., 2018). However, TGS technologies have not been widely applied in the metabarcoding of aquatic fungi. In the following years, TGS techniques are going to replace gradually the NGS techniques. The proposed primer toolkit (Chapter I) is able to meet the challenge of the newer sequencing technologies, proposing primer pairs which can not only amplify longer stretches than the length limitations of Illumina but also cover the whole 18S rRNA gene region.

New fungal studies will provide a higher amount of fungal sequences enriching the public sequencing databases. High quality sequences belonging to Basal fungi will increase their representation in databases, improving potential primer biases towards Dikarya. Improved databases will offer the opportunity to re-check and re-evaluate all primers in our primer list and if it is possible to re-design even more optimal pairs covering either the whole fungal kingdom or specific phyla. Similarly, more eukaryotic non-fungal sequences available in databases, will offer the opportunity to validate even further the co-amplification performance of primer pairs and to develop new blocking oligos.

The described correlations and interactions between fungi as well as the biotic and abiotic factors in Chapters II and III could be further investigated with a tighter sampling regime and a further collection of environmental parameters. Specifically, in Chapter II a more intensive sampling strategy, close and during the phytoplankton period could improve our understanding on the behavior and dynamics of specific fungal OTUs. Future transect line projects on the fungal communities in the Elbe River should include seasonality, different degrees of water runoff and more samples between the three different Group Samples. The application of these sampling strategies in follow-up studies will shed light on the observed assembly process of Chapter III, and the influence of the strength of the water runoff, of the various ecological boundaries across the river and of the anthropogenic activities on them. In addition the inclusion of data about the phyto- and zooplankton communities in future Elbe riverine studies could reveal the potential ecological drivers behind the various assemblage processes, as it was exhibited in Chapter II.
Bacterial cell count numbers were missing for both projects. Bacterial data should be also incorporated in future experimental designs as their interactions with the mycoplancton community need to be investigated. Finally, the knowledge of the various potential interaction patterns of fungi and the nature of relations with them could be advantageous for future co-cultivation experiments.

Statistical data analysis tools such as Network analysis are very important in getting further insights into the potential biotic interactions of aquatic fungi, and they should be widely implemented in future studies. Similarly, the ecological framework of Stegen et al. (2015) could be applied in more studies. This framework allows a better understanding of the dispersal patterns of mycoplankton communities in aquatic habitats, and a deeper investigation of the ecological processes in which fungal organisms are involved.

The primer toolkit is also important for real-time Q-PCR studies, as it indicates potential biases, such as low coverage or co-amplification threats of the fungal 18S rRNA primers. To overcome these problems, it is critical to test and choose the most suitable primer for the fungal biomass assessment, something that cannot be facilitated by the extensive list presented in Chapter I. The limitations of techniques like fluorescence in situ hybridization (FISH) on aquatic fungi could be overwhelmed by better probes designed at a taxon, OTU-level, and if it is possible for the whole kingdom of fungi. In this direction, a follow-up study on Chapter II already applied catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) aiming to explore the dynamics of unicellular mycoplankton during a spring phytoplankton bloom (Priest et al., 2021).

4.5 Concluding remarks

This dissertation aimed to develop a set of missing tools for the analysis of fungal high-throughput 18S rRNA gene sequences and then apply them to shed light on the spatial and temporal dynamics of aquatic mycoplankton communities. The studied regions revealed diverse fungal communities with several new clades of unclassified taxa belonging to Basal Fungi. Marine samples were dominated primarily by Ascomycota and Basidiomycota whereas brackish and freshwater samples by Chytridiomycota and Rozellomycota OTUs.
The type of the biotic and abiotic interactions in Chapter II appeared to be connected to the OTUs abundance and seasonal frequency patterns. In chapter II, the majority of the detected interactions between fungi and plactonic organisms were negative. From an ecological standpoint, this can be explained either as a top-down control of fungi on phyto- and zooplankton or as a predation of fungi from zooplankton. Mainly rare OTUs formed the backbone of the community, exhibiting the ecological importance of the rare marine fungal biosphere, as functional guild and probable stabilizing factor against environmental changes. In addition, the number of the detected negative inter-fungal interactions could be indicative of antagonistic relationships. The aquatic fungal communities in Chapter III were under the influence of high dynamics of assembly processes, different for the three distinct formed sample groups. Freshwater samples upstream the city of Hamburg were governed by variable selection while downstream regions together with marine samples were governed by undominated processes like ecological drift.

In conclusion this dissertation advances our understanding about the diversity, structure and dynamics of aquatic mycoplankton communities. In addition, it presents data that could be the basis for follow up studies exploring the position of fungi on the aquatic trophic webs and their functional role on the various biogeochemical cycles.

4.6 References

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Erklärungen zur elektronischen Version und zur Überprüfung einer Dissertation


Ich bin mit der Überprüfung meiner Dissertation gemäß §6 Abs. 2, Punkt 5 mit qualifizierter Software im Rahmen der Untersuchung von Plagiatsvorwürfen einverstanden.

Stefanos Banos

Unterschrift
Ort, Datum: Bremen, 18.10.2021

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