Planktonic marine fungi: A review

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Abstract

Fungi in marine ecosystems play crucial roles as saprotrophs, parasites, and pathogens. The definition of marine fungi has evolved over the past century. Currently, "marine fungi" are defined as any fungi recovered repeatedly from marine habitats that are able to grow and/or sporulate in marine environments, form symbiotic relationships with other marine organisms, adapt and evolve at the genetic level, or are active metabolically in marine environments. While there are a number of recent reviews synthesizing our knowledge derived from over a century of research on marine fungi, this review article focuses on the state of knowledge on planktonic marine fungi from the coastal and open ocean, defined as fungi that are in suspension or attached to particles, substrates or in association with hosts in the pelagic zone of the ocean, and their roles in remineralization of organic matter and major biogeochemical cycles. This review differs from previous ones by focusing on biogeochemical impacts of planktonic marine fungi and methodological considerations for investigating their diversity and ecological functions. Importantly, we point out gaps in our knowledge and the potential methodological biases that might have contributed to these gaps. Finally, we highlight recommendations that will facilitate future studies of marine fungi. This article first provides a brief overview of the diversity of planktonic marine fungi, followed by a discussion of the biogeochemical impacts of planktonic marine fungi, and a wide range of methods that can be used to study marine fungi.

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Key Points:

- Planktonic marine fungi play key roles in the cycling of carbon, nitrogen, phosphorus, sulfur, and metals in the ocean.
- There is a large potential for discovering novel lineages and functions of planktonic marine fungi, particularly in the open ocean.
- The ecological roles of planktonic marine fungi should be studied by combining multi-omics and biochemical tools.

Abstract

Fungi in marine ecosystems play crucial roles as saprotrophs, parasites, and pathogens. The definition of marine fungi has evolved over the past century. Currently, "marine fungi" are defined as any fungi recovered repeatedly from marine habitats that are able to grow and/or sporulate in marine environments, form symbiotic relationships with other marine organisms, adapt and evolve at the genetic level, or are active metabolically in marine environments. While there are a number of recent reviews synthesizing our knowledge derived from over a century of research on marine fungi, this review article focuses on the state of knowledge on planktonic marine fungi from the coastal and open ocean, defined as fungi that are in suspension or attached to particles, substrates or in association with hosts in the pelagic zone of the ocean, and their roles in remineralization of organic matter and major biogeochemical cycles. This review differs from previous ones by focusing on biogeochemical impacts of planktonic marine fungi and methodological considerations for investigating their diversity and ecological functions. Importantly, we point out gaps in our knowledge and the potential methodological biases that might have contributed to these gaps. Finally, we highlight recommendations that will facilitate future studies of marine fungi. This article first provides a brief overview of the diversity of planktonic marine fungi, followed by a discussion of the biogeochemical impacts of planktonic marine fungi, and a wide range of methods that can be used to study marine fungi.

1 Introduction

Fungi in marine ecosystems play crucial roles as saprotrophs, parasites, pathogens, commensals, and symbionts. The definition of marine fungi has evolved over the past century, partially because taxonomic overlap can often be seen with some terrestrial fungi. Currently, "marine fungi" are defined as any fungi recovered repeatedly from marine habitats that are able to grow and/or sporulate in marine environments, form symbiotic relationships with other marine organisms, adapt and evolve at the genetic level, or are active metabolically in marine environments (Pang et al., 2016). Driven by rising interests from multiple disciplines in the past decade, a number of reviews, books, and perspective articles have provided synthesis of our knowledge derived from over a century of research on marine fungi (Amend et al., 2019; Breyer & Baltar, 2023; Burgaud et al., 2022; Cunliffe, 2023; Gladfelter et al., 2019; Gonçalves, Esteves, et al., 2022; Grossart et al., 2019; Hassett et al., 2019; Jones & Pang, 2012; Kempken, 2023; Raghukumar, 2017a; Rédou et al., 2016; Richards et al., 2012; Sen et al., 2022). Planktonic marine fungi refer to those (in the form of spores, yeasts, mycelia, sporangia or other fungal propagules) that are found to be active in suspension or attached to particles, substrates or hosts in the ocean (Wang et al., 2012). This definition includes terrestrial fungi that remain active in the ocean, especially in coastal waters. This review builds upon previous reviews by focusing on the roles planktonic marine fungi play in ocean biogeochemistry and by providing summaries of state-of-the-art methods for investigating the diversity, function, and activity of marine fungi. Finally, we highlight recommendations that will facilitate future studies of marine fungi.

This article first provides a brief overview of the diversity of planktonic marine fungi, followed by a discussion of the ecological roles and biogeochemical impacts of planktonic marine fungi, and a wide range of methods that can be used to study marine fungi.

2 Diversity of planktonic marine fungi

2.1 Diversity of planktonic marine fungi in coastal waters

Coastal regions constitute areas where land masses meet the ocean and vary from ocean and inland systems in terms of precipitation patterns, humidity, food web dynamics, and input of organic matter. Coastal regions are highly dynamic, complex, and productive systems that host about 40% of the human population within a range of 100 km from the shoreline (Baztan et al., 2015). Near-shore marine ecosystems are not as stable as their open ocean counterparts and can

vary in input of terrestrial organic matter, sediment, nutrients, and pollutants, as well as changes in salt concentrations due to evapotranspiration and freshwater mixing (Clipson et al., 2005; Ward et al., 2017).

Coastal planktonic fungi typically constitute a mixture of marine, and terrestrial lineages (Chrismas et al., 2023). There are currently 1,898 described species of marine fungi (https://www.marinefungi.org/, 12/09/2023; Calabon et al., 2023), but their actual diversity is estimated to be much higher, with some studies stating that only around 1% of marine fungi have been identified (Gladfelter et al., 2019; Jones, 2011). In coastal waters, planktonic fungi are commonly filamentous or unicellular types in the form of yeasts, found on aggregates or detritus associated. Moreover, they can be associated with other eukaryotes or phytoplankton as pathogens or symbionts (Grossart et al., 2016; Gutiérrez et al., 2010; Raghukumar, 2017a; Richards et al., 2015; Sen et al., 2022).

Fungi can successfully thrive in aquatic ecosystems due to their unique physiological and biochemical traits (Sridhar, 2020). In coastal areas, planktonic fungal biomass is controlled by organic carbon, nitrogen and phosphorus availability (Jørgensen & Stepanauskas, 2009). In contrast, seasonal changes, in e.g., water temperature, pH, and chlorophyll-a, are mainly responsible for driving community composition and for observed uneven distribution across coastal waters and time (Priest et al., 2021). Highest diversities were found closest to the shoreline, and decreased towards the open ocean (Debeljak & Baltar, 2023; Duan et al., 2021; Z. Gao et al., 2010; Gutiérrez et al., 2010; Sen et al., 2021). Close to shore, proximity and connectivity to estuaries seem to be determining factors in higher observed diversity, probably due to directly linked input of fungi from the inflowing rivers (Debeljak & Baltar, 2023; Taylor & Cunliffe, 2016). Moreover, eutrophication has an impact on the assembly and composition of the most abundant fungal taxa along a eutrophication gradient. After environmental disturbance, however, assembly mechanisms and adaption strategies differed between abundant and rare fungal taxa (H. Zhao et al., 2023). This study also highlighted the essential role of rare taxa in fungal community structure, stability, and diversity in coastal waters. Additionally, the authors emphasized the important biogeochemical role planktonic fungi play in the cycling of carbon, nitrogen, and phosphorus in coastal ecosystems, predominantly via their extracellular enzyme

activities. The fungal communities recovered from coastal waters were mostly dominated by members of Dikarya (Ascomycota and Basidiomycota), including genera such as *Cystobasidium*, *Rhodotorula*, *Aspergillus*, *Penicillium*, and *Cladosporium*, as well as some early diverging lineages including Chytridiomycota (Pham et al., 2021; Sen et al., 2021).

For a worldwide meta-analysis of fungal 18S (V4 region) amplicon data from the water column exclusive to coastal regions, we retrieved information from the publicly available MetaPR2 database (v2.1.1; Vaulot et al., 2022). Sample numbers were significantly different between the southern (N= 99) and the northern hemisphere (N= 730) (Chi-squared test, $p < 0.001$), indicating that coastal habitats south of the equator are still largely understudied. In the southern hemisphere, coastal fungal communities were mainly composed of Basidiomycota (72.3%), Ascomycota (14.1%), and unclassified fungi (10.6%), whereas Ascomycota (36.6%), Chytridiomycota (33.4%), and Basidiomycota (19.3%) dominated the community in the northern hemisphere (**Figure 1a, d**). An analysis of community composition (PERMANOVA on Aitchison distance, 999 permutations) in relation to environmental parameters, however, is rather limited as only coordinates, water temperature, and salinity data were available. It revealed that fungal community composition was mainly driven by latitude ($F = 24.6934$, $p = 0.01$) and water temperature ($F = 10.5938$, $p = 0.01$), but to a smaller, yet still significant extent, by salinity and longitude, with a large residual variance not being explained by any of these variables (**Figure 1c**). Interestingly, relative abundances of chytrids correlated with increasing temperatures, especially in the coastal regions of the North Pacific Ocean (spearman roh = 0.54, $p < 0.01$) and North Atlantic Ocean (spearman roh = 0.17, $p < 0.01$). In the North Atlantic Ocean, Sordariomycetes were more abundant in colder water temperatures (spearman roh = -0.12, $p < 0.05$). In the Southern Ocean, where water temperatures were generally lowest (mean T = 0.8 ± 5.6°C), the relative abundance of fungi was higher in warmer regions. The opposite was true for Cystobasidiomycetes (spearman roh = -0.42, $p < 0.01$) and Tremellomycetes (spearman roh = -0.30, $p < 0.01$), of which the relative abundance was higher at lower temperatures. Fungal alpha diversity was highest in the Baltic Sea (**Figure 1b**), which showed the second lowest mean water temperature after the Arctic Ocean in the northern hemisphere (12.2 ± 2 °C) and lowest mean salinity (9.94 ± 5).

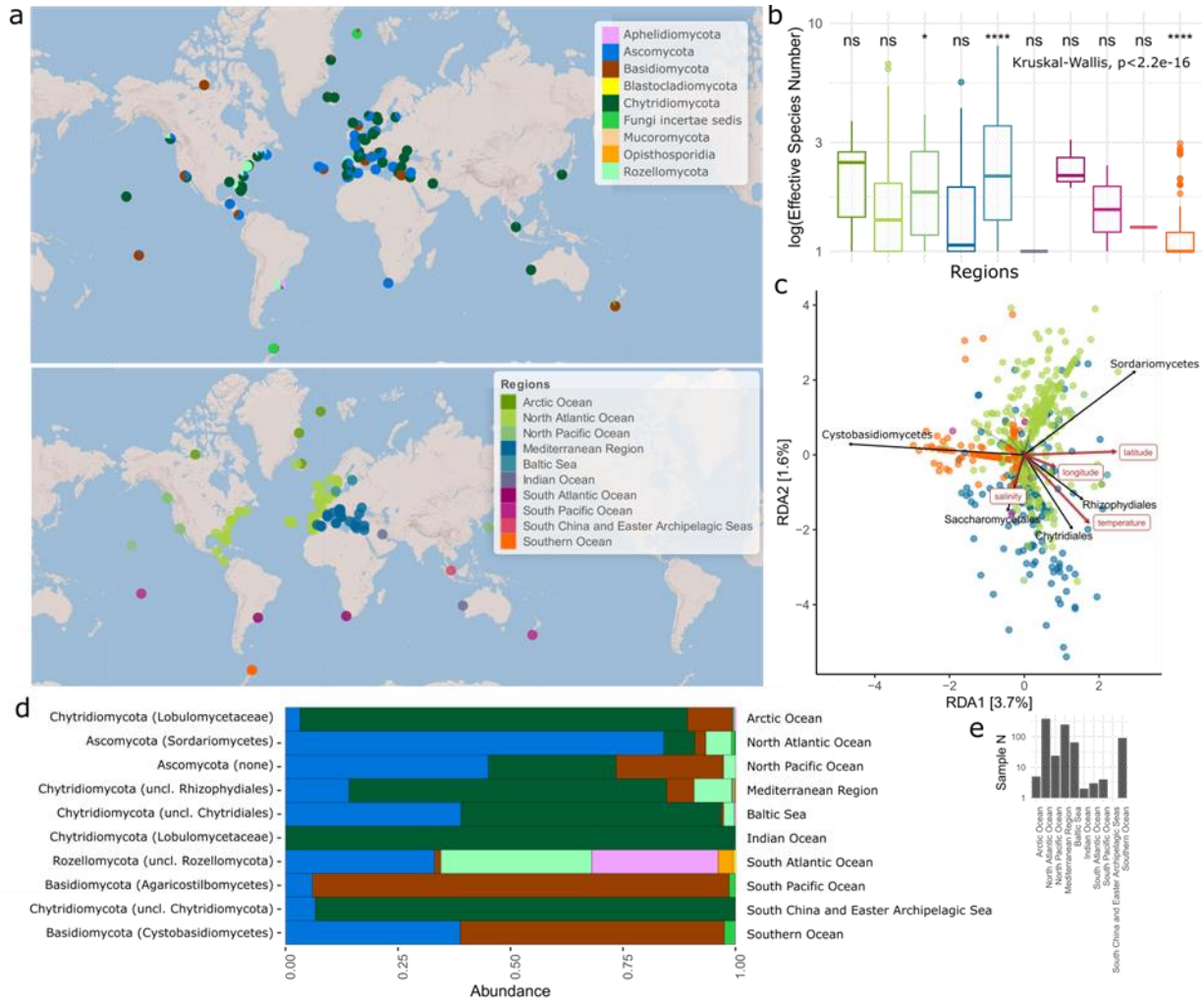

Figure 1. Global distribution of fungi and samples across coastal areas depicting their composition at phylum level, as well as oceanic regions retrieved from the MetaPR2 database (a). Bar plot of differences in alpha diversity given as the logarithm of the effective number of fungal species based on the exponent of the Shannon index (y-axis) between oceanic regions (family-level, Kruskal-Wallis $p < 0.001$) (b). Visualizing patterns (based on a redundancy analysis (RDA) of centred log-ratio transformed abundances with 999 permutations ($p < 0.01$)) between fungal communities, environmental variables (water temperature, salinity, longitude, latitude), and most abundant fungal taxa. Taxa are agglomerated to family level (c). Fungal community composition on phylum level (coloured according to A) between oceanic regions showing the dominant phyla and dominant families in parenthesis (rel. abundance > 0.3) (d). Distribution of the number of samples between oceanic regions indicating potential undersampling of specific coastal regions (e).

In conclusion, members of the phylum Chytridiomycota often are the dominating fungal lineage in coastal regions worldwide, which is consistent with findings in Debeljak & Baltar (2023). Yet, Chytridiomycota are understudied, hence they often contribute to the fungal "dark matter" (Grossart et al., 2016). Partly, this is due to the usage of primer pairs known to have a taxonomic bias towards Dikarya (Tedersoo et al., 2015a), and to the incompleteness of publicly available

databases often failing to classify early diverging fungal lineages to any ecologically meaningful taxonomic level. In addition, coastal waters in the southern hemisphere are predicted to harbor a larger number of undescribed species, suggesting a potentially high and untapped diversity and functionality of fungi in these understudied regions. For example, coastal habitats characterized by extreme environmental conditions, such as very low water temperatures, hold an important potential for structurally novel enzymes produced by fungal lineages with specific adaptions to such hostile environments. Consequently, comparative fungal studies in coastal environments are urgently needed as many questions remain open, particular in a world of rapid and extreme changes.

2.2 Diversity of planktonic marine fungi in the open ocean

There are fewer studies focusing on planktonic fungi in the open ocean compared to coastal regions, and even fewer have targeted the mesopelagic and below. This discrepancy can be attributed to logistical challenges associated with reaching the open ocean and the assumption that the open ocean offers fewer substrates for fungi to proliferate. Nearly all cultivation-based studies of marine fungi collected inocula from coastal and estuarine environments (Jones & Pang, 2012). One exception is an early cultivation-based study that collected samples in the Indian Ocean along the 60°E meridian from 12°N to 41°S at depths of 0 - 2,000 m (Fell, 1967). This study found that the density of yeast cells in the Indian Ocean was low (0 to 513 cells L^{-1}), and *Rhodotorula* (Basidiomycota) and *Candida* (Ascomycota) were cosmopolitan yeast genera that were frequently cultivated (Fell, 1967). The tendency to cultivate yeasts from the open ocean is consistent with their apparent dominance (Bass et al., 2007), which is attributed to their large surface-to-volume ratio and flexible physiology (Kutty & Philip, 2008). Currently there are no known early diverging fungi cultivated from the open ocean.

Despite the widespread application of metabarcoding surveys of marine microbial communities, the number of studies that examined open ocean fungal diversity remains very small. A review by Breyer and Baltar (2023) provided an up-to-date and comprehensive summary of metabarcoding surveys of planktonic fungi. Of the 26 studies summarized by Breyer and Baltar (2023), only 9 included samples from the open ocean (Bass et al., 2007; Debeljak & Baltar, 2023; Hassett et al., 2019; Hassett et al., 2017; Li et al., 2019; Morales et al., 2019; Peng & Valentine,

2021; Tisthammer et al., 2016; Wang et al., 2014). Most of these studies focused on one region of the global ocean during one field campaign, while TARA Oceans was the primary source of fungal diversity data providing larger coverage of the global ocean.

While Ascomycota and Basidiomycota consistently account for over two thirds (often > 80%) of the fungal community in the open ocean detected using molecular approaches, there appears to be a systematic difference depending on the primer set used for metabarcoding. The relative abundance of early diverging fungi, particularly Chytridiomycota and Mucoromycota, was usually very low (< 1%) when a primer set targeting the ITS2 region was used (e.g. Li et al. 2019, Peng and Valentine 2021), but when the V4 region of the 18S rRNA gene or the ITS1 region was the target, the relative abundance of early diverging fungi can reach >10% in the open ocean (Debeljak & Baltar, 2023; Jeffries et al., 2016; Orsi et al., 2022). Regardless of the type of primer used, the relative abundance of unclassified fungal taxa, which potentially represent novel lineages, was the highest in the open ocean when compared to coastal and estuarine environments (Debeljak & Baltar, 2023; W. Li et al., 2019; Peng & Valentine, 2021).

While meta-analysis of existing datasets has generated insights into patterns of fungal diversity in the open ocean (e.g. Debeljak and Baltar 2023), most of the large-scale surveys (e.g. TARA Oceans) were not designed to target fungi, so the primers used were typically universal 18S rRNA primers that result in small numbers (e.g. tens to hundreds) of fungal reads per sample due to the low abundance of fungal cells in open ocean microbial communities and potential primer biases. This means if the sequencing depth per sample is low, marine fungal diversity will likely be underestimated. On the other hand, effects of airborne and/or human skin associated fungi contamination may be relatively large but can be minimized if appropriate controls are in place (e.g. abundance filtering before statistical analyses; Marcelino et al., 2019). Regardless of which region of the rRNA gene is targeted (18S, ITS, or 28S), using primers specifically designed to maximize the coverage of fungi (e.g., Banos et al., 2018; Tedersoo et al., 2015b) will be crucial to advance our understanding of fungal diversity in the open ocean.

3 Biogeochemical impacts of planktonic marine fungi

3.1 Carbon cycling

3.1.1 Particle-associated fungi

Introduction and importance of particle-associated fungi

The biological carbon pump (BCP) constitutes the primary natural mechanism for sequestering atmospheric carbon dioxide and facilitating its transport to the deep ocean. It is central to regulating Earth's climate and sustaining marine ecosystems (Boyd et al., 2019). Within the BCP, energy transfer primarily occurs through the gravitational sinking and subsequent decomposition of particulate organic matter (POM) (Guidi et al., 2016). As POM sinks, its molecular composition transforms via heterotrophic degradation, supplying bioavailable nutrients to a broad microbial community (Azam et al., 1983; Worden et al., 2015). Until recently, bacteria and archaea were considered the sole biological decomposers of POM and drivers of the "microbial loop" (Baumas et al., 2021; Duret et al., 2019; Leu et al., 2022; Ollison et al., 2021). However, recent investigations in regions characterized by coastal upwelling demonstrate the prevalence, and at times the dominance, of saprotrophic fungi (and/or fungi-like organisms such as Labyrinthulomycetes) closely associated with POM, herein referred to as "particle-associated fungi" (Bochdansky et al., 2017; Duret et al., 2020; Gutiérrez et al., 2011). Based on recent findings on the active role of pelagic fungi play in carbohydrate and protein degradation (Baltar et al., 2021; Breyer et al., 2022; Chrismas & Cunliffe, 2020), pelagic fungi were recently proposed as significant contributors to the "microbial loop" in the ocean.

Diversity and distribution of particle-associated fungi

Particle-associated fungi are distinct from free-living fungi and are characterized by their attachment to various particles found in the marine environment. These particles encompass a range of substrates such as detritus (i.e. microbial necromass, fecal pellets), and microplastics (Y. Yang et al., 2020). Particle-associated fungal communities can be sampled using a marine snow catcher (Duret et al., 2020) or by using size-fractioned filtration (Peng & Valentine, 2021), but most studies of marine fungi use only a single filter size, which would capture both free-living and particle-associated communities (Morales et al., 2019). Particle-associated fungi are generally dominated by Ascomycota (primarily Dothideomycetes) and Basidiomycota (primarily Microbotryomycetes and Exobasidiomycetes) across the majority of depths and in both coastal

and pelagic regions (Duret et al., 2020; Peng & Valentine, 2021). When fungal data directly obtained from collected POM is unavailable, it is possible to infer a particle-associated lifestyle through functional analysis. In such cases, an enrichment in the production of hydrolytic enzymes relative to cellulose, carbohydrates, lignin, or chitin degradation is likely to be observed (Sen et al., 2022). Additionally, the absence of a positive correlation between fungi and other microbial organisms, such as phytoplankton, can suggest that the fungal group in question is not actively reliant on a biotic host or engaged in a parasitic lifestyle (Taylor & Cunliffe, 2016).

Ecological roles of particle-associated fungi

In marine ecosystems, the decomposition of particulate organic matter is a fundamental process that influences nutrient cycling and overall food web dynamics. Particle-associated fungi likely play a critical role in this process by expediting the breakdown of complex/recalcitrant organic substrates and releasing more readily available nutrients for assimilation by other organisms.

Particle-associated fungi are known to colonize lignocellulosic substrates in marine environments and have been shown in various studies to possess the enzymatic capabilities necessary for their degradation, including laccases, cellulases, amylases, and pectinases (Bonugli-Santos et al., 2010; Kamei et al., 2008; Pointing & Hyde, 2000; Wang et al., 2016). Additionally, marine fungi have demonstrated their ability to process algal derived polysaccharides and POM (Cunliffe et al., 2017; Yanming Wang et al., 2016). Moreover, fungi assimilating POM such as *Cladosporium* have been identified as prey for zooplankton (Hu et al., 2015). During periods of heightened productivity, particularly in coastal upwelling zones characterized by phytoplankton blooms, the abundance of marine fungi correlates positively with the production of hydrolytic enzymes (Gutiérrez et al., 2011). Dikarya fungi effectively assimilate ^{13}C-labeled algal transparent exopolymer particles or substances (TEP or EPS) (Cunliffe et al., 2017; Orsi et al., 2022), providing direct evidence that saprotrophic fungi in marine environments use algal-derived organic matter.

In addition to their role in particulate matter decomposition, particle-associated filamentous fungi are instrumental in the formation and stabilization of particle aggregates in marine sediments (Damare et al., 2008), a role they might also play in the water column. This is achieved through

physical stabilization, facilitated by hyphal networks, and chemical stabilization via the secretion of transparent exopolymer particles (TEP), a polymer that promotes particle aggregation (Bochdansky et al., 2017; Damare et al., 2008). These aggregates, often referred to as "marine snow", are denser than suspended POM, resulting in accelerated sinking and contributing to long-term carbon storage in benthic oceans. Through these mechanisms, particle-associated fungi play critical roles in organic matter fluxes within marine ecosystems, not only by producing bioavailable substances through particle decomposition but also by actively participating in particle aggregation, ultimately facilitating their sinking to the deep-sea environment, and contributing to the biological carbon pump. On the other hand, a recent study has found that chytrid fungi can delay and reduce aggregation of particles in aquatic environments (Klawonn et al., 2021; Klawonn, Van den Wyngaert, et al., 2023), demonstrating that the specific roles fungi play in particle formation/deformation depend on the lineage.

Functional potential of particle-associated fungi

The enzymatic degradation of complex organic matter by fungi is well documented (Baltar et al., 2021; Breyer et al., 2022; El-Gendi et al., 2022), including in freshwater ecosystems, where fungi are primary degraders of terrigenous organic C, and bacteria act as secondary decomposers of more labile compounds following fungal degradation (Fabian et al., 2017; Gessner, 1997; Roberts et al., 2020; Tant et al., 2015). Analogous to their terrestrial and freshwater counterparts, marine fungi possess an arsenal of extracellular enzymes adept at metabolizing recalcitrant biopolymers. Fungal roles in lipid, amino acid, and carbohydrate metabolism, are depth-dependent, as reflected in the distribution of fungal CAZymes spanning surface waters to the seafloor (Baltar et al., 2021; Cunliffe et al., 2017; Orsi, Edgcomb, et al., 2013; Orsi et al., 2022). Glycosyl hydrolases (GHs) involved with chitin degradation have been identified, as fungi are capable of colonizing chitinous substrates including those of zooplankton exoskeletons in freshwater environments (Czeczuga et al., 2000; K. W. Tang et al., 2006). This suggests fungi may have a role in degrading non-phytoplankton-derived POM such as chitin containing peritrophic membrane encased fecal pellets, zooplankton exuviae and carcasses. This has not yet been recorded in marine systems but suggests this is an important vector for carbon flux in the mesopelagic (Bradford-Grieve et al., 2001; Kobari et al., 2008). Fecal pellets quickly export carbon as part of the BCP’s mesopelagic pump into the deep sea, extending the remineralization

scale (Boyd et al., 2019). Degradation by fungi may influence the efficiency of transport of POM via this pump, which has been demonstrated by recent studies (Klawonn et al., 2021; Klawonn, Van den Wyngaert, et al., 2023). Declining abundance of GHs involved with polysaccharide degradation in the mesopelagic corresponds to decreasing polysaccharide concentrations in the water at these depths. This suggests that varied composition and distribution of POM across spatial scales in the water column may regulate fungal communities, resulting in different functionality at different depths and locations (Chrismas & Cunliffe, 2020). Also, there is a shift with depth in the taxonomic affiliation of fungal CAZymes in the global ocean, indicating that the fungal groups performing the degradation of carbohydrates change with depth (Baltar et al., 2021). Fungal function may also vary over much smaller depth changes in response to changing environmental conditions, e.g. oxygen availability (Orsi et al., 2022). For example, assimilation of diatom extracellular polymeric substances (dEPS) by fungi declined from higher to lower oxygen concentrations (Orsi et al., 2022).

As the degradation of algal-derived polysaccharides and chitinous material has already been characterized in some bacterioplankton (Cunliffe et al., 2017; Datta et al., 2016), there are potential interactions between fungi and bacteria that are yet to be considered. Freshwater POM degrading fungi may release degradation products (e.g. monomers of POM polymers) that are available as substrates for other organisms to utilize thereby fueling the microbial loop (Roberts et al., 2020). Similar processes likely take place on marine POM colonized by fungi. Fungi-like organisms such as Labyrinthulomycetes are also found on particles (Bochdansky et al., 2017) which may result in potential interactions. Recent evidence based on the comparison of the transcriptomic profiles of fungal CAZymes (Baltar et al., 2021) and peptidases (Breyer et al., 2022) to the prokaryotic ones (Z. Zhao et al., 2020), indicate that oceanic prokaryotes and fungi might occupy different ecological niches in the degradation of oceanic organic matter (Breyer & Baltar, 2023).

Marine fungi may exert a range of physical influences over the BCP and marine carbon cycle, with varying importance across coastal and open ocean systems. These could include processing POM, impacting particle dynamics, and physical processes related to the function and efficiency of the BCP such as sinking velocity, density, porosity, fragility, aggregation, and disaggregation

of particles. The presence of filamentous fungi in soil environments can result in larger aggregates with hyphae contributing to the stability of soil aggregates (Bearden & Petersen, 2000); marine filamentous fungi may have a similar role in aggregation of marine particles. Lipid-rich fungi may increase the buoyancy of particles, reducing the settling velocity, an effect that may change over time in response to the life stage and type of fungus present (Thomas et al., 2022). Remineralization rates of POM by fungi are currently unknown but it is important to consider when quantifying the role fungi play in the marine cycle.

Although the presence of specific fungal hydrolytic enzymes retrieved from the water column suggests the preference for a particle-associated lifestyle, studies of fungal community composition and metabolic activity on marine POM *directly* are scarce. Moreover, knowledge of marine fungal community succession as POM degradation occurs is nonexistent although critically important to consider when examining efficiency and microbial controls of the BCP.

Conclusions

Particle-associated fungi represent a significant and often overlooked component of marine ecosystems. Their role in POM decomposition, formation of aggregates such as marine snow, and metabolism of complex organic substrates underscores their importance in marine carbon cycling and the BCP. Clearly, further research is needed to fully understand the dynamics of marine fungal communities, their succession during POM degradation, and their contribution to BCP efficiency.

3.1.2 Microalgae-associated fungi

Introduction and importance of microalgae-associated fungi

Microscopic algae and photosynthetic bacteria, more commonly referred to as phytoplankton, are the primary producers that support most global marine ecosystems. Phytoplankton include diatoms, dinoflagellates, and cyanobacteria, and provide ecosystem services including sequestering atmospheric carbon, sustaining fisheries, and maintaining symbioses that enable coral reef formation (Falkowski, 2012). Primary production delivers the organic carbon that underpins most marine food webs either via direct grazing or cycling through the microbial loop,

and is therefore considered an ecosystem service of global significance (Naselli-Flores & Padisák, 2023).

Marine phytoplankton such as diatoms and dinoflagellates, including widespread bloom-forming taxa, may become infected by fungal parasites, particularly by the zoosporic Chytridiomycota ('chytrids') (Gutiérrez et al., 2016; Hassett & Gradinger, 2016; Lepelletier et al., 2014; Scholz et al., 2016; Sparrow, 1969). Interactions between phytoplankton and parasitic fungi influence the health and dynamics of phytoplankton populations directly, through predation, and indirectly by impacting the flow of organic matter within and among microbial communities (Grossart et al., 2019; Sime-Ngando, 2012; Van den Wyngaert et al., 2022). Despite their ecological importance, parasitic, microalgae-associated marine fungi remain understudied in comparison to other microbial groups, especially with regard to implications for ocean biogeochemistry (Ilicic & Grossart, 2022).

Diversity and distribution of microalgae-associated fungi

Although parasites have been identified from many early diverging phyla such as Chytridiomycota, Cryptomycota (Rozellomycota) and Aphelida (Corsaro et al., 2014; Kagami, de Bruin, et al., 2007; Moreira et al., 2016), research on fungal parasitism has largely focused on zoosporic fungi from Chytridiomycota (herein referred to as "chytrids"), on which the remainder of this section will focus. Chytrids occur in both coastal and pelagic marine environments, with the highest prevalence contemporaneous with seasonal phytoplankton blooms in coastal upwelling systems and surface waters (Banos et al., 2020; Gleason et al., 2011; Gutiérrez et al., 2016, 2020; Orsi et al., 2013; Taylor & Cunliffe, 2016). In open ocean ecosystems, chytrids are often less dominant in comparison to coastal ecosystems (Breyer & Baltar, 2023).

The diversity and distribution of chytrids is constrained by both biotic and abiotic factors including presence of putative host species, temperature, pH, light intensity, and salinity (Duan et al., 2018; Gutiérrez et al., 2016; Ibelings et al., 2004; Taylor & Cunliffe, 2016). Chytrids demonstrate strong seasonality in coastal environments where variations in water column nutrient composition, oxygen levels, and microbial composition are tightly synchronized with the timing of seasonal phytoplankton blooms (Hassett et al., 2019; Taylor & Cunliffe, 2016).

Chytrid-microalgae interactions are generally considered "host-specific", with several studies utilizing cross-infection experiments to determine generalist vs. specialist tendencies (Kagami et al., 2021; Reñé et al., 2023; Van den Wyngaert et al., 2022). However, there are comparatively few studies where host range is examined empirically, *in situ* within marine systems (Gleason et al., 2011; Kagami, de Bruin, et al., 2007; Kagami, von Elert, et al., 2007). Potential host specificity can be deduced through various means, including analyzing the proportion of attached and detached zoospores in relation to the phytoplankton community composition. For instance, Gutiérrez et al. (2016) demonstrated chytrid fungal seasonality and host preferences, by reporting the highest abundance of attached fungal sporangia coincided with large, colonial diatoms, specifically *Thalassiosira* and *Skeletonema*. Subsequently, a shift in diatom community composition, dominated by the genus *Chaetoceros*, correlated with decreased attached sporangia and increased free-swimming chytrid zoospores (Gutiérrez et al., 2016). Additionally, monitoring changes in fungal community composition throughout the duration of phytoplankton blooms can provide insights into potential fungi-microalgae interactions. Studies conducted off the coast of Plymouth, UK and in Lake Stechlin, Germany demonstrated that chytrid fungi abundance positively correlated with seasonal phytoplankton blooms, implying a likely parasite-host interaction (Taylor & Cunliffe, 2016; Van den Wyngaert et al., 2022).

Interactions between microalgae, fungi, and the environment

During phytoplankton infection, chytrids liberate often inedible biomolecules to heterotrophic grazers via osmotrophic, extracellular degradation as their hosts are generally otherwise inedible to the greater microbial community, a concept incorporated into the "mycoloop" (Kagami et al., 2014). In the mycoloop, chytrid zoospores, rich in essential fatty acids, also serve as a high-quality food source for zooplankton like *Daphnia* in freshwater systems (Kagami, de Bruin, et al., 2007; Kagami, von Elert, et al., 2007). Moreover, chytrid infections may facilitate wider ecosystem functioning upon release of nutrients in surface waters after infection and lysis of host cells (Kagami et al., 2006). Recent work using both the *Synedra–Zygophlyctis* model system and field samples demonstrated that chytrid infections reduced the formation of aggregates, in part by promoting bacterial colonization of parasitized phytoplankton cells (Klawonn, Van den Wyngaert, et al., 2023).

A timely question is if, and how, climate change will impact chytrid-phytoplankton interactions. Ocean warming and ocean acidification directly alter phytoplankton physiology, size structure, and community composition (Basu & Mackey, 2018; Sommer et al., 2012). Indirect impacts of climate change on phytoplankton populations are also possible if these facilitate predation by pathogens such as chytrids. For example, some evidence for these higher-order interactions in freshwater ecosystems is found, where warming increases rates of chytrid infections on phytoplankton (Frenken et al., 2016; Kilias et al., 2020; Velthuis et al., 2017). Other studies propose that increased light penetration resulting from reduced sea ice coverage in Arctic waters may lead to photoinhibition and physiological stress in diatoms, creating favorable conditions for disease proliferation (Hassett & Gradinger, 2016). Kilias et al. (2020) demonstrated that chytrids were positively correlated with the extent of sea ice melting, implying that under periods of prolonged light exposure, due to a reduction in seasonal sea ice coverage, the rate of fungal infection may increase. It is unclear if these patterns extend to other marine systems, but they suggest the possibility that rates of chytrid infection may increase in the future. Given the role of phytoplankton at the base of most marine food chains, this might impact food quality and abundance for higher trophic levels, the composition of photosynthetically derived organic matter released, and even overall global nutrient cycling rates (Rasconi et al., 2020; Sánchez Barranco et al., 2020; Senga et al., 2018).

Conclusions

The ecological roles of microalgae-associated, parasitic fungi, notably chytrids, in marine ecosystems influence marine food webs and global biogeochemical cycles. While our understanding of these fungi in marine ecosystems is expanding, there remains a vast uncharted territory of fungal diversity and ecological roles, especially within pelagic ecosystems, and regarding how these dynamics may change amid environmental stressors like ocean warming and acidification. Recent advancements in culture-independent methods that combine molecular approaches with targeted microscopy are especially promising (Klawonn et al., 2021; Klawonn, Van den Wyngaert, et al., 2023) and will undoubtedly deepen our comprehension of marine fungal diversity, and will ultimately help place marine fungi within the broader context of marine ecosystem functioning as a whole (Seto et al., 2023).

3.1.3 Plastic-associated fungi

Fragments of marine plastic waste that find their way into the oceans swiftly become enveloped by organic matter, giving rise to an “ecocorona”, a distinctive layered structure that modifies the hydrophobic properties of the plastic surfaces and facilitates the adherence and establishment of microbial communities (Latva et al., 2022; Wright et al., 2020; Y. Yang et al., 2020). These communities, referred to as the "plastisphere" (Zettler et al., 2013), promptly establish intricate biofilms, typically within minutes to hours (Latva et al., 2022), reaching a mature state within 15 to 30 days (Cheng et al., 2021; Kirstein et al., 2018; Odobel et al., 2021). Extensive research in the field of prokaryotic communities' colonization dynamics has shown that over large spatial scales environmental factors, rather than the type of polymer, play a pivotal role in shaping the composition of the plastisphere (Kettner et al., 2017; Wright et al., 2021). However, different types of polymers that were locally incubated (Zettler et al., 2013) or harvested from the same field location (Vaksmaa et al., 2021), showed different microbial communities.

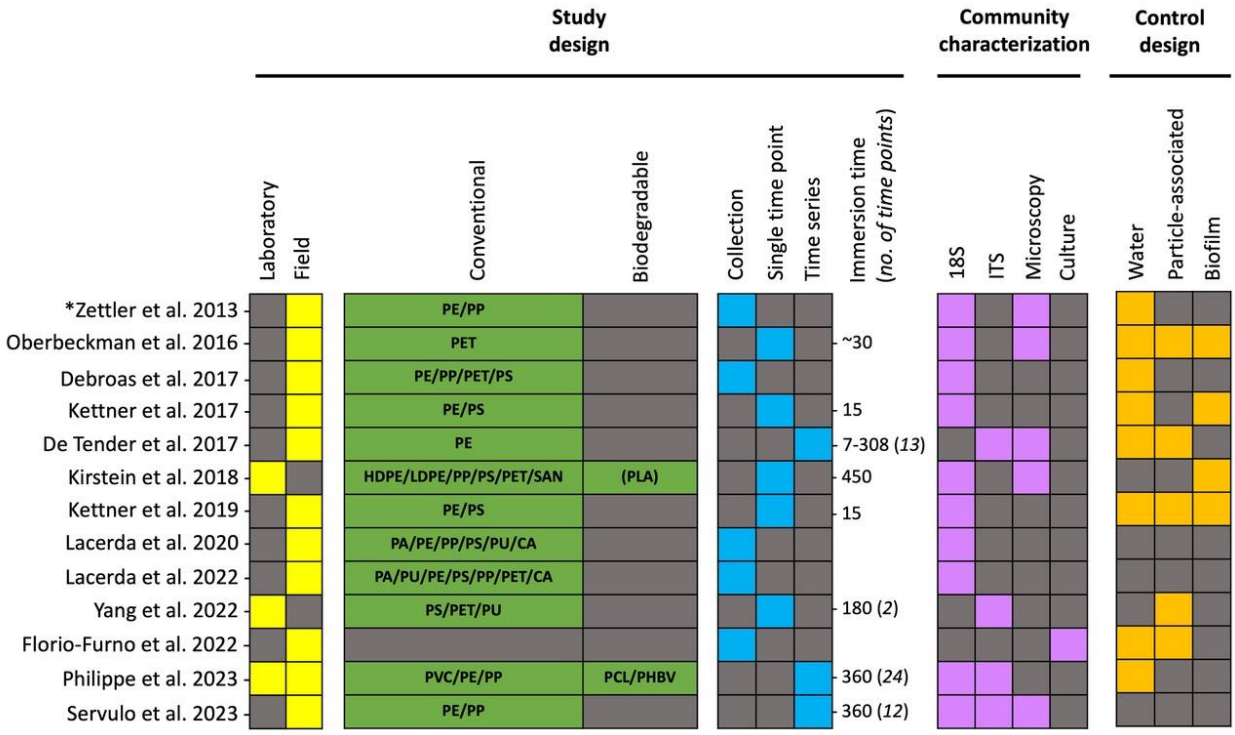

Figure 2. Litterature review of 13 studies analyzing the Plastisphere on marine plastic debris with a focus on fungal communities (updated from Wright et al., 2020). Study design highlights whether the study was conducted in a laboratory or in the field (yellow), the different types of plastics, i.e. conventional or biodegradable (green), and whether the plastics were naturally collected from the sea (collection) or introduced by the researchers and collected either at a single time point or at a series of time points (blue). Numbers indicate the first and last days of incubation, with numbers in brackets indicating the number of points included in the time series. Fungal community characterization (purple) indicates whether the

Plastisphere was analyzed via 18S or ITS rRNA high throughput sequencing or by a culture-based approach, and whether microscopy was used to visualize the biofilm. The controls (orange) highlight those studies that compare the Plastisphere with the microbial community of the surrounding seawater (either bulk or between 0.2 and 3 μm) or particulate organic matter (>3 μm), or biofilms that develop on inert surfaces (e.g., glass, rock, wood, etc.). Meaning of polymer acronyms: PE (Polyethylene), PP (Polypropylene), PET (Polyethylene terephtalate), PS (Polystyrene), HDPE (High-density polyethylene), LDPE (Low-density polyethylene), SAN (Styrene-Acrylonitrile), PA (Polyamide), PU (Polyurethane), CA (Cellulose Acetate), PVC (Polyvinyl chloride), PLA (Polylactic acid), PCL (Polycaprolactone), and PHBV (Poly(3-hydroxybutyrate-co-3-hydroxyvalerate. PLA has been enclosed in brackets due to its biodegradability under specific conditions. * The publication by Zettler et al. 2013 was added, even though it was not focused on fungi, because it represents the first publication indicating the presence of microeukaryotes on marine plastic debris.

Here we have reviewed primary research articles dedicated to elucidating fungal communities associated with marine plastics (**Figure 2**), and we acknowledge another recent review highlighting the potential role marine fungi play in plastic degradation (Zeghal et al., 2021). Planktonic marine fungi associated with plastics are not necessarily degrading plastics, and only a few fungal lineages have been shown to degrade plastics. *Zalerion maritimum* (Paço et al., 2017), *Alternaria alternata FB1* (R. Gao et al., 2022) and *Rhodotorula mucilaginosa* (Vaksmaa et al., 2023) have been identified to degrade polyethylene (PE) and *Cladosporium halotolerans* 6UPA1 was shown to degrade polyurethane (PUR) (K. Zhang et al., 2022). Our investigation revealed that the study of plastic-associated fungi, primarily conducted through a metabarcoding approach, constitutes an emerging field, as evidenced by 6 out of 13 relevant studies published between 2020 and 2023. For those that included a comparable seawater control, plastic-associated fungal communities were significantly distinct from fungal communities in the surrounding bulk seawater. Interestingly, a specific study delved into fungal communities inhabiting marine sediments and associated plastics, employing a culture-based methodology (Florio Furno et al., 2022). This investigation yielded findings consistent with earlier research, demonstrating disparities in species richness between plastic samples and sediment or water samples. Among the selected research articles featuring an inert control material such as wood or glass, three studies (Kettner et al., 2017, 2019; Kirstein et al., 2018) reported notable distinctions between the control and plastic substrates. However, in one study (Oberbeckmann et al., 2016) specifically examining fungal communities colonizing polyethylene terephthalate (PET), no significant differences were observed when compared to those colonizing glass substrates. These findings imply that geographical locations and inherent environmental variables may exert a stronger influence on fungal colonization than the type of plastic/material.

Most published studies indicate that the polymeric composition of plastics does not exert significant influence on OTU/ASV richness or community composition (Kettner et al., 2017, 2019; Lacerda et al., 2020, 2022; Sérvulo et al., 2023). Only one study detected differences in community composition, of fungal communities on polystyrene (PS) and polyethylene terephthalate (PET), which contrasted with those on polyurethane (PU) (Yang et al., 2022). A recent study comparing fungal colonization on conventional vs. biodegradable plastics underscores the significant impact of immersion duration on fungal colonization (Philippe et al., 2023). This is supported by temporal variability observed in fungal communities (De Tender et al., 2017). However, these results diverge from those of Sérvulo et al. (2023), who reported stable communities over a one-year immersion period. In terms of taxonomic diversity, Ascomycota dominate plastic-associated fungal communities, where Basidiomycota, Chytridiomycota, Glomeromycota, Mucoromycota, Zoopagomycota, and Cryptomycota are also present (De Tender et al., 2017; Kettner et al., 2017; Lacerda et al., 2020, 2022; Philippe et al., 2023; Sérvulo et al., 2023; Zeghal et al., 2021), which aligns with the general patterns in pelagic marine fungi diversity (Jones et al., 2019). Moreover, plastic-associated Chytridiomycota exhibit higher abundance during the autumn and winter seasons (Sérvulo et al., 2023) or at higher latitudes (Kettner et al., 2017; Kirstein et al., 2018).

These findings reveal emerging trends and distribution patterns of plastic-associated fungi. However, it is crucial to interpret these preliminary insights with caution due to the diverse array of plastic formats (plates, sheets, pellets, etc.) used in the highlighted studies, putative variations due to the presence of additives in the plastic matrices, distinct environmental conditions, and differences in immersion durations across these investigations. This collective variability raises significant questions about the development of a comprehensive understanding of fungal colonization of plastics. The study of plastic-associated fungi is further complicated by interactions with bacterial and protist communities, as well as the influence of stochastic processes during initial colonization stages. This underscores the need for standardized research to gain additional insights into whether plastics genuinely constitute a unique habitat for fungi, thereby extending the concept of niche partitioning on plastics, highlighted for bacteria (Odobel et al., 2021), to the fungal kingdom.

3.1.4 Fungi at the ocean atmosphere interface

The ocean-atmosphere interface (or sea surface microlayer, SML) covers approximately 71% of the Earth's surface and is the site at which all substances entering or leaving the ocean must pass through (Engel et al., 2017). The combined physical, chemical and biological properties of the SML impact ocean-atmosphere exchange processes, including those that are part of the marine carbon cycle (Cunliffe et al., 2013). In terms of the first stages of the marine carbon cycle, ocean-atmosphere CO_2 flux is globally important, with an estimated uptake of approximately 25% of atmospheric CO_2 causing a net sink and offset of the anthropogenic burden in the atmosphere.

Microbial life in the SML is often referred to as the neuston, with some groups such as bacteria (i.e. bacterioneuston) relatively well-studied (Cunliffe et al., 2008; Franklin et al., 2005; Wurl & Holmes, 2008). In terms of impact on ocean-atmosphere exchange processes, neuston communities can modify air-sea gas transfer directly, either through the degradation of specific gases (e.g. methane, carbon monoxide) (Conrad & Seiler, 1988; Frost, 1999; Upstill-Goddard et al., 2003) or general microbial activity impacting net CO_2 and O_2 flux (Reinthaler et al., 2008), or indirectly by creating and modifying SML materials that impact the physicochemical properties of the SML, such as surfactants, that impact gas exchange (Kurata et al., 2016). Biological surfactants on the surface of the Atlantic Ocean caused up to a 32% reduction of air-sea CO_2 exchange likely through turbulence repression (Kurata et al., 2016). Enhanced microbial activity in the SML, particularly associated with slicks, can also modify the physicochemical properties of the 'cool ocean surface skin' (Wurl et al., 2018), which also likely impacts CO_2 exchange (Watson et al., 2020).

Fungi in the SML (i.e., myconeuston) are so far very poorly studied. Environmental DNA (eDNA)-based assessment of myconeuston diversity has been conducted in coastal marine waters in the Western English Channel (Taylor & Cunliffe, 2014) and in the open waters of the Mediterranean Sea (Zäncker et al., 2021), with both studies showing that fungal diversity in the SML is distinct from the underlying water column. In the Mediterranean Sea study, particularly for samples collected from the Ionian Sea, fungi were a major eukaryote group in the SML, with the dominant fungal taxon in the study belonging to the genus *Cladosporium*. Other studies have

shown, using stable isotope probing, that marine *Cladosporium* actively degrade phytoplankton-produced high-molecular-weight polysaccharides that form gels (Cunliffe et al., 2017), indicating that myconeuston may have a role in processing SML organic material (Zäncker et al., 2021) and therefore impact the physicochemical composition of the ocean-atmosphere interface. These two studies are based on eDNA assessments of myconeuston diversity and therefore only show the presence of fungi in these locations. Currently, we have no information on fungal general (e.g., respiration) or specific (e.g., polysaccharide processing) activity in the SML, and we are thus still unable to establish the significance of myconeuston on ocean-atmosphere exchange.

3.2 Nitrogen cycling

Nitrogen (N) is the primary limiting nutrient in many parts of the ocean (Gruber, 2008). Many planktonic microorganisms possess extremely high affinity for fixed inorganic N such as ammonium and nitrate (Martens-Habbena et al., 2009; Mulholland & Lomas, 2008). Because fungal cells typically have a smaller surface-to-volume ratio, planktonic fungi may be at disadvantage when competing with prokaryotes for dissolved inorganic nitrogen such as ammonium and nitrate. On the other hand, aquatic fungi demonstrate highly plastic C:N ratios in their cellular biomass (Danger et al., 2016; Danger & Chauvet, 2013), which may allow them to better survive in ocean biomes where nitrogen is the limiting nutrient (Moore et al., 2013). Additionally, fungi can fulfill their N requirements by degrading organic matter (Hodge & Fitter, 2010), which may be an important source of fixed N for oceanic fungi. As marine bacteria also assimilate dissolved organic nitrogen (Bronk & Glibert, 1993; Korth et al., 2012), there is potentially competition between marine fungi and bacteria for dissolved organic nitrogen, but this hypothesis still requires experimental verification. A recent study, assigning fungal peptidase-like sequences in the TARA oceans metagenomes and metatranscriptomes, found that pelagic fungi actively degrade proteins throughout the water column in the world's ocean (Breyer et al., 2022). The relative abundance of fungal proteases increases with depth, suggesting that fungi may be better equipped to degrade refractory organic material than bacteria. Ascomycota and Basidiomycota were mainly responsible for protease production in the ocean (Breyer et al., 2022). In sum, marine fungi play important roles in the recycling and assimilation of fixed organic nitrogen.

Besides their roles in fixed nitrogen assimilation and remineralization, fungi could also play an important role in the dissimilatory cycling of nitrogen in the ocean. Specifically, many fungal species can perform incomplete denitrification, the dissimilatory reduction of nitrate sequentially to nitrite, nitric oxide, and nitrous oxide (Maeda et al., 2015). While fungal denitrification is an important process in soil (Chen et al., 2014; Laughlin & Stevens, 2002), this process has been reported in marine environments (Lazo-Murphy et al., 2022; Su et al., 2021; Wankel et al., 2017), including the eastern tropical North Pacific oxygen minimum zone (Peng & Valentine, 2021). On one hand this suggests fungi make a small (up to 10%) but significant contribution to fixed nitrogen loss from the ocean water columns. On the other hand, because nitrous oxide (N_2O) is a potent ozone-depleting greenhouse gas (Ravishankara et al., 2009) and no fungi have been identified to reduce N_2O to dinitrogen gas (Hirofumi Shoun & Fushinobu, 2016), fungi may make a disproportionately large contribution to N_2O emission from the ocean, especially as fungal denitrification appeared to be less sensitive to oxygen inhibition (Peng & Valentine, 2021; Phillips et al., 2016). The apparent lack of sensitivity to oxygen of fungal N_2O production in a number of tested strains (Phillips et al., 2016; Zuo et al., 2023) suggests that if these strains were present and active in the oxygenated surface ocean, they have the potential to contribute to N_2O efflux from the ocean.

The diagnostic gene for fungal denitrification is an unconventional nitric oxide reducing cytochrome P450 (*P450nor*) likely acquired by fungi via horizontal gene transfer from Actinobacteria (Chen et al., 2014; Shoun & Tanimoto, 1991). Although fungal N_2O production has been demonstrated by many strains (Jirout, 2015; Maeda et al., 2015), a recent study using comparative genomics analyzed >700 fungal genomes, challenged the paradigm that anaerobic respiration is responsible for N_2O production by fungi. By identifying genes associated with secondary metabolisms in the vicinity of *P450nor* in a subset (up to a third) of the analyzed fungal genomes, Higgins et al. (2018) suggested that secondary metabolism might be responsible for fungal N_2O production. However, the fungal genomes analyzed by Higgins and colleagues were nearly exclusively from terrestrial environments. To understand the mechanisms behind fungal denitrification in the ocean, there is an urgent need to conduct studies using fungal strains isolated from the ocean, particularly oxygen minimum zones.

3.3 Other elemental cycling

3.3.1 Sulfur

Marine fungi participate in sulfur (S) cycling primarily via sulfate assimilation to S-bearing amino acids (Morales et al., 2019; Sen et al., 2021). Nonetheless, extracellular mineralization of organic S (e.g., algal sulfate esters) by pelagic fungi (Ascomycota and Basidiomycota) was recently reported in studies of fungal isolates from the Atlantic Ocean and the Antarctic Peninsula (Salazar Alekseyeva et al., 2022). Those authors showed extracellular activity of fungal sulfatases that can hydrolyze sulfate esters present in algal cell walls. The activity (hydrolysis rate and half-saturation constant) of those enzymes was temperature- and species dependent, and agreed with previous studies that described fungal sulfatases as thermo-sensitive enzymes with an optimal temperature of 25^{o}C (Korban et al., 2017). The impact of temperature on S-related enzymes suggests that global ocean warming may alter (in some cases increase) the contribution of pelagic fungi to marine S cycling. Other fungal S-hydrolyzing enzymes that can contribute to mineralization of S-bearing compounds in the ocean include putative metallopeptidases that cleave or modify dimethylsulfoniopropionate (DMSP) to dimethyl sulfide (DMS) (Todd et al., 2009). DMSP is an abundant organosulfur compound in the marine environment (that is produced by marine bacteria and phytoplankton (Yoch, 2002) and plays a major role in the global S cycle and in marine food webs (Mahajan et al., 2015; Teng et al., 2021). Marine Ascomycota taxa are known to hydrolyze DMSP (Bacic & Yoch, 1998) using homologues to the *dddP* gene that modify DMSP to DMS and were likely acquired via horizontal gene transfer from bacteria (Todd et al., 2009).

The role of pelagic fungi in S cycling and S mineralization may extend to the breakdown of polycyclic organosulfur hydrocarbons found in oil spills or anthropogenic S compounds that enter marine waters as detergents, pesticides, or pharmaceuticals. Assimilation of complex organosulfur hydrocarbons by fungi depends on desulfurization (breakdown) of the C–S bond (Linder, 2018). Terrestrial basiodiomycetes and ascomycetes are reported to desulfurize polycyclic organosulfur compounds using aromatic peroxygenases and cytochrome P450 monooxygenases (e.g., Piontek et al., 2013); however, polycyclic organosulfur degradation by pelagic fungi is not yet fully understood. Oil spills are shown to result in marine sediment

692 eukaryotic communities almost exclusively dominated by fungal taxa (Bik et al., 2012). Oil spills
693 also change the structure and function of pelagic fungal communities, increasing the abundance
694 of presently unclassified fungi within the mycoplankton that have an uncharacterized role in
695 degradation of hydrocarbons (Neethu et al., 2019). Nonetheless, the overall ability of pelagic
696 fungi to degrade hydrocarbons is related to the toxicity of hydrocarbons and the ability of
697 specific fungal lineages to tolerate this toxicity. In the case of oil spills, the abundance of
698 *Candida* and *Rhodotorula* decreased (Neethu et al., 2019).
699
700 Pelagic marine fungi may also contribute to S cycling through the production of S-bearing
701 secondary metabolites; however, this is still an area of active research. Production of secondary
702 metabolites can enhance survival and is a strategy utilized by many microorganisms, in particular
703 those with particle-associated lifestyles (e.g., Geller-McGrath et al., 2023). Marine fungi can
704 produce a suite of different S-bearing secondary metabolites with antimicrobial and cytotoxic
705 properties (e.g., (Julianti et al., 2022; Liu et al., 2022). These S-bearing secondary metabolites
706 were isolated primarily from deep sea fungi and taxa that inhabit sediments. Whether these
707 compounds are also produced by pelagic fungi remains to be investigated, however it is likely we
708 will find that they play a role in competition with co-colonizing bacteria on sinking particles.
709 Such S-bearing metabolites could allow fungi to couple sulfur cycling with defense mechanisms
710 against prokaryotes and other fungi that colonize particles.

711 **3.3.2 Phosphorus**

712 Inorganic phosphorus (Pi) is an important macronutrient utilized by marine microorganisms for
713 the synthesis of macromolecules (DNA, RNA, proteins) and under conditions where it is in
714 limited supply, the effect on marine ecosystems can be substantial (F. Zhang et al., 2022). Our
715 knowledge of the role of pelagic fungi in cycling of phosphorus (P) derives to a great extent from
716 Tara Ocean metagenome and metatranscriptome analyses that show the presence and expression
717 of fungal genes encoding proteases and peptidases involved in protein cleavage, tight coupling of
718 protein and carbohydrate degradation, and the likely preference of fungi for a particle-associated
719 lifestyle (Baltar et al., 2021; Breyer et al., 2022). Breyer et al. (2022) found that phosphorus
720 availability was one important controlling factor of fungal peptidase gene expression. Overall,
721 most non-marine fungi regulate the uptake and mobilization of Pi using the phosphate-responsive

signaling pathway (PHO) which is activated upon Pi-deprivation (Vila et al., 2022 and references therein). Pelagic and coastal waters, however, are not Pi-limited (Karl & Björkman, 2015). This implies that the PHO pathway in pelagic fungi (if present) might not be active considering that its activation depends on Pi-limitation. Indeed, Breyer et al., (2022) suggested that pelagic fungi could potentially cover their needs for Pi and participate in P mineralization by recycling proteins using proteases and peptidases. Expression levels of fungal proteases were found to be regulated by P, N and Fe availability while the abundance of fungal peptidases in detected metagenomes was significantly correlated with temperature, O_2, Fe and net primary production (Breyer et al., 2022). To determine whether pelagic fungi can only participate in P mineralization via protein recycling or whether additional mechanisms are involved will require laboratory experiments using additional fungal isolates from pelagic settings.

3.3.3 Iron and manganese

Micronutrients like iron (Fe^{3+}) are important for the growth of microeukaryotes including fungi and phytoplankton because they play a catalytic role in enzymes and because they are critical for production of energy-rich molecules such as NADPH and ATP. Because of its low solubility in oxic waters, Fe^{3+} is found at concentrations < 1 nM in the marine water column (Street & Paytan, 2005). To cope with such low Fe concentrations phytoplankton has efficient mechanisms for its uptake (Sutak et al., 2020). Little is known about the role of pelagic fungi in the cycling of micronutrients like iron (Fe), and the mechanism(s) for how they overcome low Fe^{3+} solubility require further investigation. Metagenome and metatranscriptome surveys from 68 Tara Ocean stations showed that the ability of fungi in the 3-2,000 μm size range to express carbohydrate-active enzymes (CAZymes) was correlated with Fe availability (Baltar et al., 2021). This suggests that at least for pelagic fungi with saprophytic feeding modes (on particles) they need to have mechanisms for Fe^{3+} acquisition. Laboratory studies showed certain ascomycetous isolates from the coast of West India produce siderophores that facilitate solubilization and sequestration of Fe^{3+} from the surrounding environment (Baakza et al., 2004; Vala et al., 2006). Additionally, under aerobic conditions, many strains of marine fungi were reported to produce hydroxamate-type siderophores during iron limitation, suggesting they can compete with bacteria for Fe in the environment (Holinsworth & Martin, 2009). The types and structure of most fungal

siderophores, as well as the mechanisms involved in transportation of siderophore-bound Fe^{3+} into the fungal cell still need to be elucidated (Holinsworth & Martin, 2009).

Manganese is a micronutrient required for the water-splitting complex of photosystem II in photosynthetic organisms, and for many other biological activities (van Hulten et al., 2017 and references therein). Marine fungi are suggested to mineralize dissolved Mn(II) that exists in nM concentrations in the open ocean (e.g., Tebo et al., 2005). Marine fungi enhance the oxidization rates of Mn(II) to Mn oxides in the water column when dissolved Mn from the ocean's surface or from phytoplankton decay is abundant (Sunda & Huntsman, 1994; Sutherland et al., 2018; Tebo et al., 2005). Likewise, experimental studies showed that filamentous Ascomycota can oxidize Mn(II) at their hyphal tips using extracellular superoxide produced during cell differentiation (Hansel et al., 2012; Y. Tang et al., 2013). Aside from hyphal-associated Mn(II) oxidation, analyses of fungal secretomes (the set of biomolecules produced by an organism and secreted into the extracellular environment) documented the capacity of various filamentous Ascomycota to oxidize Mn (II). This capacity is dictated by species-specific Cu-dependent (e.g., tyrosinase) and FAD-dependent (e.g., glucosemethanol-choline oxidoreductases) enzymes (Zeiner et al., 2021). Further, the capacity of these Ascomycota to oxidize Mn(II) varied with secretome age (decreased enzymatic Mn(II) oxidative capacity after 21 days) (Zeiner et al., 2021). These findings are intriguing and require a thorough examination of the role of pelagic fungi in Mn(II) cycling in the water column. Fungal biomineralization of Mn(II) may also support the biogeochemical cycling of other essential micronutrients in the water column (e.g., selenium; Rosenfeld et al., 2020). Finally, this capability may have technological applications where generation of sustainable electrochemical materials produced from fungal Mn biomineralization may have advantages (Q. Li et al., 2016).

4 Methods for studying planktonic marine fungi

4.1 Cultivation-based methods

Recent culture-independent studies of planktonic marine fungal diversity have revealed the dominance of Ascomycota, Basidiomycota and Chytridiomycota (Hassett et al., 2019; Ilicic & Grossart, 2022; Morales et al., 2019), although sequence signatures of other groups of fungi (e.g. Cryptomycota (Rozellomycota), Glomeromycota, Mucoromycota, Neocallimastigomycota) were also observed (Debeljak & Baltar, 2023; Duan et al., 2018; Orsi et al., 2022; Sen et al., 2022). On

the other hand, culture-based techniques resulted in the isolation of fast-growing ascomycetous and basidiomycetous yeasts (e.g. *Aureobasidium*, *Rhodotorula*, *Rhodosporidium*) or filamentous Ascomycota (e.g. *Aspergillus*, *Penicillium* and *Cladosporium*) in seawater (Sen et al., 2022).

Recent advancements in sequencing techniques have improved our comprehension of the overall diversity of planktonic marine fungi, and isolation of fungi provides an avenue for investigating their ecophysiology, yielding key insights into their functional roles and adaptations within the marine environment (Pang et al., 2020; Velez et al., 2015). The isolation and preservation of marine fungi in global culture collections not only helps to maintain the representative biodiversity of an ecosystem at a particular moment but also carries significant potential for long-term research, particularly in the context of climate change's effects on organisms. It is conceivable that in the coming decades, we will be able to unveil profound genetic and ecophysiological adaptations through the study of isolates preserved in culture collections (V. Kumar et al., 2021). The labor-intensive and time-consuming process of isolating marine fungi, involving collection, isolation, identification, and preservation, must persist. We seize here the opportunity to recognize Jan Kohlmeyer and Brigitte Volkmann-Kohlmeyer's substantial contributions as leading collectors and identifiers of marine fungi in the 20th and early 21st centuries, with an herbarium housing over 25,000 specimens (Cunliffe, 2023).

Among cultivated marine fungi, members of Ascomycota clearly dominate, representing 77% of cultured marine fungal species, followed by Basidiomycota (~11%), Microsporidia (~7%), and Chytridiomycota (~2%), the remaining species represented by other basal-fungal lineages. However, it's worth noting that this documented count falls far short of the estimated 10,000 marine fungal species (Jones, 2011), a discrepancy underscoring the vast fungal diversity residing in the oceans, yet to be comprehensively isolated and cataloged. This section provides a brief overview of culture methods for planktonic marine fungi, and the approaches well-suited for studying their physiology.

For sampling of seawater, small sterile bottles, tubes, bags or buckets (H. Zhao et al., 2023) are common containers that have been used for near shore locations. Van Dorn bottles can also be used for sampling after proper washing before use (Kimura et al., 1999). Niskin bottles fitted in a

rosette arrangement with a CTD (conductivity, temperature and depth) device are suitable at offshore sites and samples can be taken at different depths from the top 1 meter (Duan et al., 2018) to hundreds of meters (Z. Gao et al., 2010; Peng & Valentine, 2021) and even exceeding 1,000 meters (Breyer et al., 2023). A disadvantage of Niskin bottles however, is contamination from overlying seawater as the rosette descends with the bottles open to the environment. To collect particle-associated communities, particles can be separated using devices such as the Marine Snow Catcher (Riley et al., 2012) or by using size-fractioned filtration (Peng & Valentine, 2021). Neuston samples (air-sea interface samples) can be collected by a mesh screen sampler (Cunliffe et al., 2011). It is essential to ensure the sterility of sampling equipment and consumables throughout the collection process, with constant vigilance for potential contamination risks. Managing these risks necessitates conducting checks at different stages of the process, such as deploying sealed bottles/tubes/bags/buckets containing sterile seawater on-site to detect any potential contamination. Aerosol samples at sea can be taken at the time of seawater sampling, using common air samplers such as quartz fiber or glass fiber filter-based devices for culture-independent analysis (Fröhlich-Nowoisky et al., 2012), and six-stage Andersen impactor (Yu et al., 2013) or Burkard sampler (Mescioglu et al., 2021) for culture-based examination. This allows for the assessment of culturable fungal diversity in the sampled air, providing a basis for comparison with the fungal diversity in the seawater at the same time of sampling.

Samples should be handled immediately either on board or transported to the laboratory at a low temperature (4 °C) for culture as soon as possible. Seawater samples can be serially diluted (Pham et al., 2021) if there is a high sediment load in coastal waters, or membrane filtered (G. Wang et al., 2012) and the residue resuspended in a smaller volume of sterile seawater for oceanic water samples with the relatively lower number of fungal propagules (Vrijmoed, 2000). The diluent/suspension can be spread plated onto agar plates or inoculated into liquid media in flasks or microplates. Ideally, sinking particles can be separated from the seawater using particle interceptor traps (Fontanez et al., 2015) or filtered (Bochdansky et al., 2017) in order to compare fungal isolates that are in suspension to those attached to particles. Some common media suitable for the isolation of planktonic marine fungi include Sabouraud dextrose agar (SDA), malt extract agar (MEA), potato dextrose agar (PDA), Czapek Dox agar (CDA), cornmeal agar (CMA) (L. Li

et al., 2014; Pham et al., 2021), glucose-yeast extract agar (Vera et al., 2017) and a medium containing glucose, yeast extract peptone and starch (Breyer et al., 2023) to list a few (all supplemented with sea salts matching the salinity of the sampling site). Original culture media can also be designed in order to mimic the *in situ* conditions as much as possible to select for marine fungi (e.g. Panno et al., 2013; Álvarez-Barragán et al., 2023). Some studies used 1/5 strength of the media so as to imitate environmental concentrations (Pang et al., 2020). Antibiotics such as chloramphenicol, streptomycin sulfate and penicillin may be added to the media to inhibit bacterial growth. However, some studies may opt not to use antibiotics in order to promote fungi-bacteria interactions, ultimately enhancing culturability, as already demonstrated on deep-sea samples (Rédou et al., 2015). The inoculated media can be incubated at different temperatures and/or light regimes, always mimicking the natural conditions as much as possible. The plates should be checked daily and colonies of different types (e.g., color, pigment on agar, mycelial density, branching) be subcultured onto fresh agar plates (same media) as pure cultures for further identification based on morphology or molecular analysis. For liquid media inoculated with seawater samples, aliquots can be spread plated onto agar media to isolate colonies of different morphologies for identification as mentioned above. Rose Bengal may also be added to the media to slow down fast-growing species for the isolation of slow-growing species (Ottow, 1972). Few Mucoromycota and related taxa have been reported from the marine environment (Calabon et al., 2023), and might be sensitive to salinity (Johnson & Sparrow, 1961) and so a low salinity medium may be required to isolate these groups of fungi.

Dilution to extinction technique can also be used to culture slow-growing marine fungi (Overy et al., 2019). Seawater samples, either undiluted or filtered and resuspended in sterile seawater, are inoculated into wells of a microplate. A series of dilutions (e.g., 2-fold) is made with a liquid medium (mentioned above), preferably with a reduced strength, along the rows/columns of the microplates. Dilutions with the highest frequency of single colonies are plated out (Collado et al., 2007).

Direct plating (spread plating, streaking) of samples and baiting of pine pollen, snake skin, other keratinous substrates, or live hosts are common methods used to isolate saprobic zoosporic true fungi (e.g., Van den Wyngaert et al., 2022). Seawater samples can be directly plated/streaked

onto Emerson's 1/4 YpSs agar medium (S.-F. Chen & Chien, 1998). For the baiting method, seawater samples are seeded with sterilized pine pollen, and/or snake skin fragments supplemented with antibiotics (Guo et al., 2023). Infected pollens or snake skin fragments are picked by a loop and streaked onto Emerson's 1/4 YpSs agar medium or the seawater with zoospores is spread plated onto the medium. Colonies with large sporangia on the agar medium after incubation (both plating or baiting methods) are picked up under a stereomicroscope, subcultured by streaking, purified and maintained on the same medium. The colony morphology of zoosporic true fungi and Labyrinthulomycetes is very similar rendering isolation of the former group very difficult.

Parasitic species of zoosporic true fungi require co-culturing with the hosts. Seawater samples can be directly (or serially diluted) inoculated into the culture medium (e.g. Guillard's medium (f/2-Si), F/2 medium, Jaworski's medium) in multi-well plates with the enrichment of monocultures of diatoms (Scholz et al., 2017) or dinoflagellates (Fernández-Valero et al., 2022). Cultures are obtained after a few subcultures into axenic hosts (Fernández-Valero et al., 2022).

Recent mid/high-throughput culturing approaches (M/HTC) may also be implemented to enhance culturability and the number of marine fungal isolates. M/HTC methods enhance the recovery and isolation of the broadest possible representation of *in situ* fungi based on the fact that a large number of parameters can be modified at once (temperatures, salinities, pH, oxygen concentrations, different substrates and different concentrations of each). Possible combinations can be constrained based on knowledge of the environment (e.g., concentrations and co-distribution of nutrients and substrates such as nitrate, ammonium, phosphate) to select a reasonable number of conditions to test for isolation of environmentally relevant fungi. Laser nephelometry (BMC Labtech) has already been used as a M/HTC approach. Laser nephelometry measures light scattered by particles (unicellular and/or filamentous cells) in 96 to 384-wells and was recently used to generate >150 isolates from lower oceanic crust samples (Quemener et al., 2021). Mini/micro-satellite primed-PCR amplification can be used as a strategy to select unique fungal isolates from such a large collection generated by the M/HTC approach (Rédou et al., 2015).

Long term preservation of fungi without genetic change allows further studies in basic and applied research (Nakagiri, 2012). Planktonic marine fungi can be preserved through culture transfer, drying and freezing (Nakagiri & Jones, 2000). Freeze- or liquid-drying are suitable methods for planktonic marine fungi which produce abundant spores. Freeze-drying (in the presence of a lyoprotectant) was found feasible for long term preservation of filamentous fungi (Tan et al., 2007) and yeasts (Bond, 2007). Filamentous fungi and yeasts can be cryopreserved for years to decades at -80 °C while zoosporic true fungi can be kept in liquid nitrogen (-196 °C). Examples of cryoprotectants prepared in seawater include 10% glycerol, 10% dimethysulfoxide (DMSO), and 10% glycerol + 5% trehalose (Nakagiri & Jones, 2000). Cryopreservation in 10 % glycerol or DMSO as cryoprotectants was found to be suitable for chytrids (Gleason et al., 2007). For parasitic species, diatoms/dinoflagellates infected with chytrids can be submerged in a mixture of 10% glycerol and 5% trehalose (Nakagiri, 2012). As part of this process, when new species are obtained and described, isolates must be deposited within at least two internationally recognized culture collections, usually CBS (Westerdijk Fungal Biodiversity Institute, formerly known as Centraal Bureau voor Schimmelcultures) and DSMZ (German Collection of Microorganisms and Cell Cultures, also known as Deutsche Sammlung von Mikroorganismen und Zellkulturen) for fungi.

4.2 Methods to study the physiology of planktonic marine fungi

Physiology of planktonic marine fungi refers to the responses (metabolism, growth, reproduction, spore germination, death) under their immediate biological (symbiosis), chemical (salinity, pH, nutrition, interference competition, pollution) and physical (temperature, ultraviolet irradiation) surroundings (adapted from Walker and White, 2017). Little is known about the physiology of planktonic marine fungi, but they were found to be affected by temperature, pH, chlorophyll a, insolation, salinity, and dissolved inorganic carbon (DIC) (Breyer et al., 2023; Duan et al., 2018; Heitger & Baltar, 2023; Salazar Alekseyeva et al., 2022; Sen et al., 2022) and also the chemical defense by planktonic macroalgae (Lam et al., 2008). Fungal growth has been the main attribute to assess in response to different environmental factors.

Plate assay based on colony diameter or liquid assay based on biomass or absorbance are common methods for assessing growth of filamentous fungi and yeasts. For a plate assay,

actively growing mycelia (on an agar plug/block) are inoculated onto the surface of the assay agar (i.e., with supplementation of different organic or inorganic nutrients, salinities, pHs or pollutants and/or incubated under different physical conditions such as temperature). Colony diameter (average of two perpendicular diameters) represents growth of the fungi. To study substrate degradation, substrates are incorporated in the agar media; clearance zone diameter (average of two perpendicular diameters) of the colored agar (e.g. Poly R-478/Remazol Brilliant Blue R/Toluidine Blue agar for peroxidases) or dyeing zone diameter of the colorless agar (e.g. 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) agar for laccases) represents degradation (Pointing et al., 1998; Rojas-Jimenez et al., 2017). For some enzyme assays such as cellulose degradation (e.g. carboxy-methyl cellulose), dyes are used to stain the agar after incubation (Masigol et al., 2021; Pointing et al., 1998). For detection of chitinase activity, a basal medium composed of colloidal chitin and the dye Bromocresol Purple containing crab shell flakes can be used (Masigol et al., 2021), and the appearance of purple to black area around the mycelia represents a positive reaction. However, some fungi produce abundant aerial mycelia and so this method does not reflect the true intensity of growth. This method is also not suitable for fungi with yeast-like growth.

The growth response of three planktonic fungi (*Scheffersomyces spartinae*, *Rhodotorula sphaerocarpa*, *Sarocladium kiliense*) under different temperatures and salinities was investigated using a liquid culture method (Breyer et al., 2023). Spores, yeasts (in suspension) and fragmented mycelia in suspension or as an agar plug/block are inoculated into a liquid assay medium in flasks. For yeast species, optical density of subsamples is measured periodically at 660 nm, which reflects growth (Heitger & Baltar, 2023; Salazar Alekseyeva et al., 2022). Filamentous fungi do not produce homogenous growth in liquid media, and so total mycelial biomass in flasks after incubation is collected by filtration, dried and weighed to represent growth.

Different environmental factors exert an interactive effect on fungal growth (Pang et al., 2020). It is often impractical to examine the effect of all possible combinations of physicochemical conditions (with replication) on fungal growth. Pang et al. (2020) studied the physiological growth of filamentous fungi isolated from substrates collected at a marine shallow-water

hydrothermal vent site in Taiwan under the combined effect of temperature, salinity and pH using microtitre plates. Spores/fragmented mycelia in suspension are inoculated into wells of a 96-well Costar 3595 (Corning, Maine, USA) microtitre plate prefilled with the liquid assay medium. Due to the much smaller volume of medium being used (200 μL) in each well, the combined effects of multiple factors with replications on fungal growth can be assessed by optical density at 630 nm (Langvad, 1999). This method is also applicable to zoosporic true fungi (Guo et al., 2023).

Sporulation can be used to study reproduction success of planktonic marine fungi under environmental pressure, especially those attached to a substrate. In this approach, fungi (mainly hyphomycetes) are either plug- or point-inoculated onto an assay agar medium (e.g., malt extract agar; Damare et al., 2008). After incubation, agar plugs are retrieved from the colony, and spores are dislodged from each of the plugs by shaking with a solution made of 0.02% Tween 80 and glass beads in a sterile tube and counted by a haemocytometer (Byrne and Jones, 1975). The concentrations of spores from each agar plug are expressed as number of spores per cm square of colony. For saprobic zoosporic true fungi, isolates are spread-plated or streaked onto an Emerson's 1/4 YpSs agar medium. After incubation, seawater is flooded onto the agar plate, which is then incubated for up to an hour (Guo et al., 2023). Samples are taken from the overlaying seawater and stained/fixed with lactophenol cotton blue. The number of zoospores is counted, and the result is expressed as number of zoospores per number of colonies produced on the agar plate. This method can apply to parasitic species as number of zoospores per number of cells of diatoms/dinoflagellates.

Spore germination is the key life cycle stage determining successful colonization of substrates in the pelagic zone of the ocean. A spore suspension is prepared by flooding the top of the colony of the fungi (mostly hyphomycetes) with a solution of 0.02% Tween 80 and shaking to dislodge the spores. This spore suspension can be inoculated into the assay medium in wells of a microtiter plate. Samples are taken from the wells after incubation and the percentage of germinated spores are counted (Damare et al., 2008). Fungal spores/conidia are considered germinated when the length of the germ tube equaled or exceed the largest dimension of the original spores/conidia (Van Long et al., 2017). Following this, germination kinetics, expressed as the % of germination

as a function of time, can then be determined.

The BIOLOG platform not only provides rapid identification and characterization of filamentous fungi and yeasts, it can also be applied to substrate utilization and metabolic profiling of planktonic marine fungi under the influence of physical and chemical variables (Breyer et al., 2023; Chou et al., 2022). A suspension with spores or mycelial fragments is inoculated into the 96 wells of the microplate, each with a different substrate (e.g., BIOLOG FF MicroPlate™). Optical density is measured daily at 490 nm for substrate utilization (reduction of iodonitrotetrazolium by NADH from colorless to purple) and 750 nm for mycelial growth (turbidity). On a similar basis, Mid/High-Throughput Devices like Laser Nephelometry or oCelloScope (Harirchi et al., 2023) can also be employed to evaluate fungal growth parameters such as lag time and maximal growth rate using e.g. 96-wells microplates. The combination of Laser Nephelometry and OcelloScope provides robust data comprising both quantitative information (number of fungal particles in each well) and qualitative data (microscopic images of each well), thus allowing in-depth analysis of the growth potential of numerous fungal isolates simultaneously.

Transcriptomics, proteomics and metabolomics are modern techniques that can be used to examine physiological changes of planktonic marine fungi under environmental stresses, either biological, chemical and/or physical. Transcriptomic analysis examines transcriptional changes of proteins and involves isolation of RNA, reverse transcription PCR and sequencing (Pang et al., 2020; Velez et al., 2015). Proteomic analysis, on the other hand, studies translational response of fungi. Proteins are extracted from mycelia, analyzed by 2D gel electrophoresis and identified by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) analysis (Velmurugan et al., 2017). For metabolomics analysis, fungi are grown in a liquid medium in fermentation tanks/shake flasks or a solid agar medium, and samples (biomass, spent culture liquid medium, agar) are taken for quenching, extraction and chemical analyses such as NMR, GC-MS and LC-MS, data processing and analyses (Bayona et al., 2022; Gonçalves, Esteves, et al., 2022; G. Li et al., 2022; Oppong-Danquah et al., 2018). All of these methods can be used to examine fungal molecular responses to environmental stress, interactions with other organisms (Durham et al., 2022; G. Li et al., 2022; Pang et al., 2020) and degradation of substrates or

pollutants (Pilgaard et al., 2019; Velmurugan et al., 2017).

4.3 Microscopy

During the 20th century, microscopy observations of marine fungi were limited, and when conducted, protocols were based on phase-contrast optics or non-specific stains (Kohlmeyer & Kohlmeyer, 1979), which presented a challenge when attempting to quantify natural populations. Planktonic yeast cells are typically round shaped with a diameter as small as ~1 μm, so it is difficult to distinguish them from many bacterial cells (**Figure 3a**). Filamentous fungi have distinct morphology compared to most other planktonic microbes (**Figure 3b**), but they are typically expected to be attached to particles, which makes it challenging to distinguish fungal filaments (**Figure 3c**). Later in the 20th century, one of the most widely used methods in mycology and in the medical community was based on the affinity of wheat germ agglutinin (WGA) for chitin. WGA is a lectin with a high affinity for N-acetylglucosamine residues, which, in turn, constitute the monomers of the polymer chitin. This approach has then been utilized independently to detect fungi in both light and electron microscopy (Meyberg, 1988 and references therein). The addition of fluorescein isothiocyanate (FITC) as a conjugate to the WGA (WGA-FITC), enabled the enhancement of fungi detection and quantification by utilizing the fluorescence of the conjugate (Meyberg, 1988). Originally developed for plant-fungal interactions, this method was quickly integrated into the study of aquatic fungi (Montgomery et al., 1990).

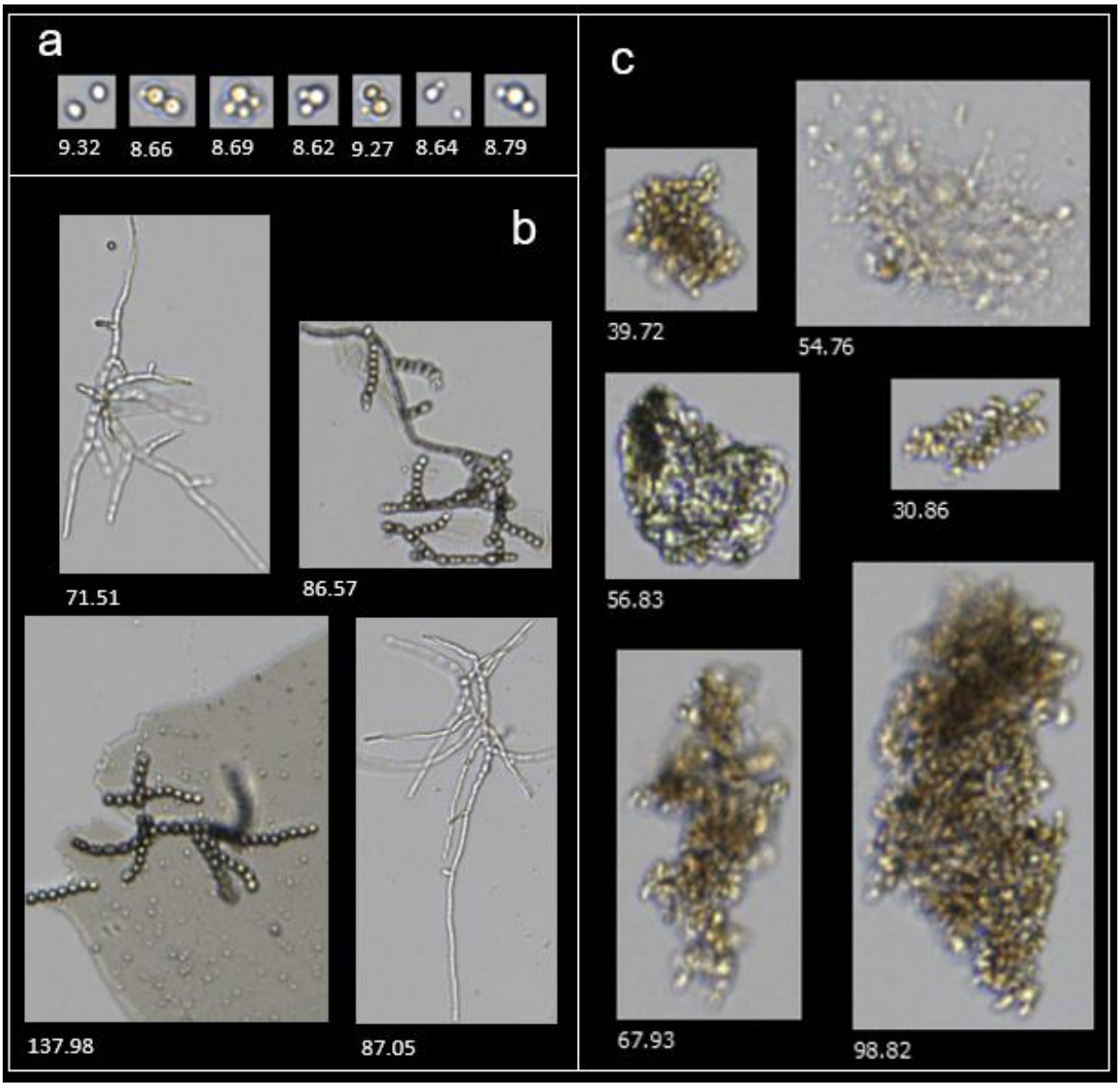

Figure 3. Micrographs of *Rhodotorula sphaeroacarpa* ETNP2018 (a), *Exophiala sp.* ETNP 2018 (b), and particles from seawater collected from the coast of South Carolina (c). Images were generated by FlowCam 8000. Numbers below each image represent area-based diameters in μm (Kydd et al., 2018).

Despite the high specificity of the previous method, the most popular procedure, however, has been another staining method, used in the clinical field to detect fungal infections (Harrington and Hageage, 2003). This method involves the staining of chitin using Calcofluor White (CFW) (Damare & Raghukumar, 2008; Rasconi et al., 2009). CFW has a high affinity for the β1-3 and β1-4 polysaccharides present in chitin, thus effectively staining the fungal wall. Additionally, CFW can be excited under UV light, which is one of the commonly used wavelengths in aquatic

microbiology microscopy, resulting in a deep, bright blue fluorescence (**Figure 4**). While some adjustments may be necessary depending on the samples, such as optimizing staining intensity and adjusting pH to minimize background noise, CFW has become one of the most commonly used methods in aquatic mycology. Its popularity might be then due to its cost-effectiveness and relatively straightforward application (e.g., Frenken et al., 2016; Gutiérrez et al., 2011; Rasconi et al., 2012; Vera et al., 2017). However, CFW is not entirely free of some undesired side effects. One of the main limitations is its lack of specificity for chitin, as it also stains other common organic compounds present in the aquatic environment, such as cellulose, which is widespread in both planktonic and benthic realms. As a result, while it successfully stains fungi, it may also stain other organisms like dinoflagellates, diatoms (these also contain chitin; Durkin et al., 2009), ecdysozoans, or plant and algal debris, among others. This necessitates careful examination to confirm that the stained bodies are indeed fungi, whether they are yeasts or hyphae, by observing fungi-specific characteristics. Another drawback of using CFW is that its excitation and emission spectra overlap with those of a common microbiological stain, DAPI, which specifically binds to DNA and is frequently used for characterizing and counting bacteria and protists.

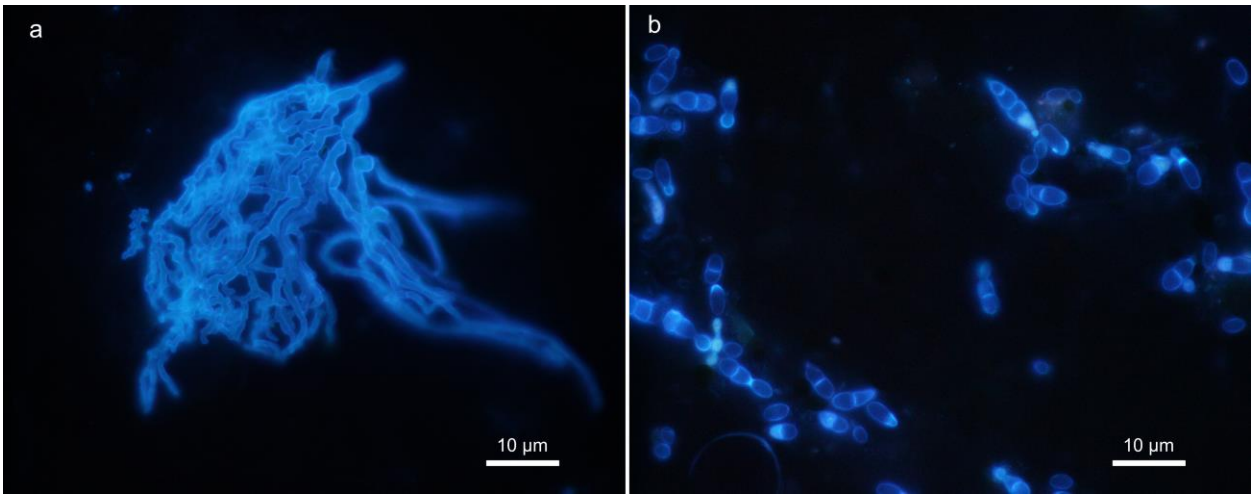

Figure 4. Epifluorescence microscopy photographs of planktonic fungi (1000x; Olympus IX-83; UV Wide filter cube) stained with calcofluor white.

To address these undesired consequences, alternative approaches have been proposed and developed. Researchers have sought new staining methods or modifications to improve specificity and reduce overlap with other stains, thus enhancing the accuracy of fungal

identification and quantification in aquatic environments. One protocol involves the use of fluorescence in situ hybridization (FISH) (Baschien et al., 2008) or catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) (Jobard et al., 2010). These methods utilize labeled probes that specifically bind to certain DNA regions of the target community. The probes are conjugated with fluorescent dyes that can accommodate different wavelengths, allowing for the avoidance of overlap with DAPI or any other stain. While many FISH probes are non-specific and contain mismatches, recent efforts have been made to design FISH probes at a phylum and OTU-level (Priest et al., 2021). Other challenges using FISH/CARD-FISH include the effective permeabilization of the fungal and cell walls, which is necessary to enable the probes to reach their targets. In aquatic samples, the presence of transparent exopolymer particles (TEP) in the filters can obstruct the path of the probes, or even interfere with the binding of common dyes like DAPI (Bochdansky et al., 2017). Consequently, appropriate pre-treatments are required to achieve the desired results and ensure the accurate staining of the fungal community using these methods. Despite these challenges, FISH and CARD-FISH represent valuable alternatives for enhancing the specificity and reliability of fungal detection and quantification in aquatic environments.

Chitin-binding domains (CBD) in bacterial chitinases have been used in conjugation with fluorescein isothiocyanate (CBD-FITC) as an improved staining technique for aquatic fungi (Wurzbacher & Grossart, 2012). This method offers several advantages over previous approaches. CBD-FITC is highly specific for chitin, making it superior to Calcofluor White (CFW) or WGA-FITC in terms of specificity. Unlike CFW, CBD-FITC does not stain other compounds containing sialic acid or N-acetylglucosamine residues, ensuring accurate detection of chitin-containing structures in fungi. CBD-FITC also eliminates the need for pre-treatments to create pores in cell walls, as required in fluorescence in situ hybridization (FISH) and catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH). This simplifies the staining process and reduces the potential for sample alteration during preparation. Another characteristic of CBD-FITC (shared with WGA-FITC) is its compatibility with other stains like DAPI and FISH. Researchers can combine the chitin-specific CBD-FITC staining with other staining techniques, allowing for a comprehensive understanding of the aquatic fungal community, and facilitating better quantification and characterization of the organisms present. Unfortunately,

CBD-FITC is no longer available commercially (Klawonn, Dunker, et al., 2023), but laboratories equipped for protein purification could perform FITC labeling in-house with commercially available conjugation kits.

The approach of using commercial conjugates can be further expanded to include various fluorescent probes with different emission wavelengths. By utilizing a wide range of fluorescent probes, researchers can combine multiple staining procedures to avoid overlapping emission wavelengths and achieve a more comprehensive analysis of the aquatic fungal community. However, it is essential to acknowledge that incorporating multiple methods does increase the complexity of the staining procedures. The order in which staining or probing is performed becomes critical to avoid interference and to obtain accurate results. Careful optimization and testing are required to ensure compatibility and successful accommodation of various staining techniques, such as WGA-FITC, CARD-FISH, and DAPI (Biancalana et al., 2017). Finally, it is essential to note that marine fungi, like many other fungi, exhibit autofluorescence when excited under different wavelengths (Breyer et al., 2021). Moreover, this fluorescence can vary depending on the growth state, species, and glucose availability. Therefore, researchers should carefully consider this property when interpreting fluorescence-based fungal image analyses (Breyer et al., 2021).

4.4 Quantification of marine fungal biomass and abundance

The quantification of biomass is a fundamental prerequisite to quantify microbial contributions to marine biogeochemical cycles. While marine fungi are commonly encountered in DNA-based studies spanning global oceans (Amend et al., 2019; Breyer & Baltar, 2023; Morales et al., 2019) there remains a significant lack of comprehensive information concerning their abundances and biomass. Nonetheless, this enigmatic kingdom can exhibit biomass levels that surpass prokaryotes during phytoplankton blooms (Gutiérrez et al., 2011) and fungi are known to be major contributors to microbial biomass on bathypelagic marine snow (Bochdansky et al., 2017).

Common techniques to quantify the contribution of fungi to marine microbial biomass include the use of fluorescence microscopy (fluorescence in-situ hybridization (FISH); Calcofluor or Wheatgerm Agglutinin staining) or the use of biomarkers (e.g., fatty acids) as indicator for

fungal biomass. Traditionally, qPCR (quantitative polymerase chain reaction) has also been used to estimate fungal abundances in environmental samples based on gene copy numbers (Taylor & Cunliffe, 2016; X. Wang et al., 2014; Yaqiong Wang et al., 2018, 2019) but the results are not as accurate when compared to other methods (Smith & Osborn, 2009). Instead, fluorescent probes, which specifically bind to group-specific fungal rRNA genes, have been used to enumerate fungal cells and quantify biomass in the North Sea (Priest et al., 2021), which has been discussed in section 4.3. Fluorescent staining of marine fungi can be combined with other instruments such as a flow cytometer to achieve high-throughput enumeration of natural populations (e.g., Klawonn, Dunker, et al., 2023). Another approach involves measuring the concentration of ergosterol as a proxy for fungal biomass (Gessner, 2020), with a focus on Ascomycota and Basidiomycota (Dikarya). The ergosterol extraction method was recently adapted to marine fungi to allow for the quantification of fungal biomass in the oligotrophic (low productive) regions, which account for the vast majority of the open ocean (Salazar Alekseyeva et al., 2022). However, the ergosterol-based method falls short when dealing with Chytridiomycota taxa (Gutiérrez et al., 2020), which is important to consider during phytoplankton blooms.

Given the limitations associated with different methods, future studies aiming to comprehensively assess mycoplankton across major ocean basins would benefit from employing a combination of the aforementioned methods. Such a holistic approach has been performed in a recent study estimating the biomass of fungi in the open ocean water column across a wide range of latitudes and productivity regimes (Breyer et al., submitted). This study revealed an overall good agreement among these different techniques to estimate fungal biomass, while also indicating fungi as relevant contributors to open ocean microbial biomass. More comparative studies in similar and contrasting environments will be crucial in unraveling the mysteries of mycoplankton and their significance within the intricate complexity of marine ecosystems.

4.5 Metabarcoding

There are many ways of targeting fungi in a marine sample. Culturing, albeit providing key insights into their functions and physiology, is inherently challenged by the culturability of presumed fastidious organisms, making this approach laborious and resource intensive. Direct observations of fungal structures using optical microscopy and/or FISH or other fluorescence-

based methods enables the unambiguous identification of fungal structures and morphological characteristics within an environment. However, these microscopy-based methods are also limited by their time-consuming and labor-intensive nature. Molecular techniques such as metabarcoding offer an alternative approach for comprehensively evaluating marine fungal communities. The present era of 'omics' has introduced the capability to produce large-scale evaluations of marine fungal diversity and richness by focusing on nucleotide sequences, primarily through rDNA metabarcoding of taxonomically informative regions.

A comprehensive literature review was conducted to assess various studies regarding their objectives and metabarcoding methodologies employed for characterizing planktonic marine fungal communities, as illustrated in **Figure 5**. Our analysis identified that, out of the 18 studies reviewed, 16 employed a single genetic marker (18S, ITS (ITS1 and/or ITS2), or 28S rRNA genes), with 12 focusing on the ITS1 and/or ITS2 genetic marker, 3 on the 18S, and 1 on the 28S. Additionally, some studies adopted dual genetic markers, namely, the 18S and 28S (Hassett et al., 2017) or the 18S and ITS (Sen et al., 2021). Based on this literature search, the primers utilized for the amplification of genetic markers appear highly conserved within studies. Primers ITS1F/ITS2 and ITS3/ITS4 are often employed for the amplification of ITS1 and ITS2, respectively. In the case of the 18S region, primers FF390/FR1, also referred to as nu-SSU-1333/nu-SSU-1647, are used to target the V7/V8 region, while the primers Euk1391f/EukBr are employed to amplify the highly variable V9 region of small-subunit ribosomal RNA genes.

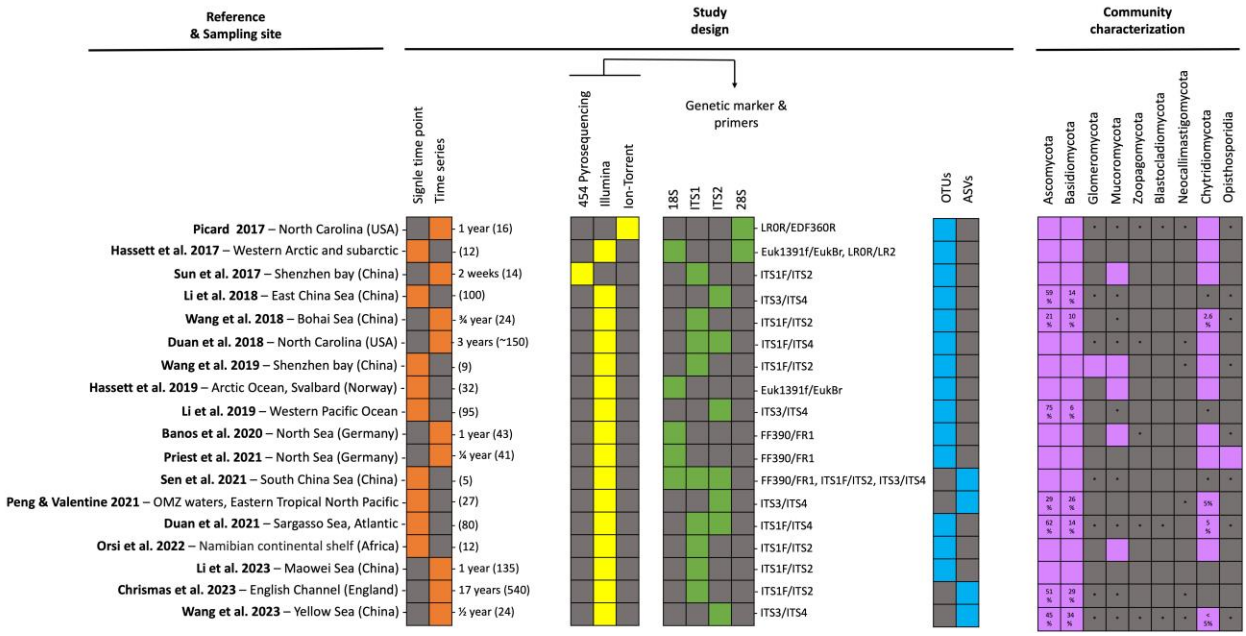

Figure 5. Literature review of 18 studies analyzing planktonic fungal communities using a metabarcoding approach within the period spanning from 2017 to 2023. The reference and sampling site section highlights details regarding the reference source and the locations of seawater sampling. The study design section specifies whether samples were collected at a single time point or at a series of time points (orange), including information about the duration of the time series and the number of samples analyzed (enclosed in brackets), the sequencing methodology employed (yellow), the targeted genetic marker (green), including the primers used, and whether the bioinformatic analysis was based on OTUs or ASVs (blue). Finally, the community characterization section presents the different fungal phyla detected, based on the phylogeny proposed by Naranjo-Ortiz & Gabaldon, 2019, (purple), along with the percentage representation of fungal OTUs when available. The asterisks (*) indicate low-level detections.

Primer pairs are acknowledged for their selective amplification within a target community (Stoeck et al., 2006). For instance, the primers ITS1F/ITS2 exhibit a bias toward specific fungal groups, notably Mucoromycota, Chytridiomycota (Orsi et al., 2022) and also completely miss Microsporidia (Tedersoo & Lindahl, 2016) as part of the Opisthosporidia phylum (based on the phylogeny proposed by Naranjo-Ortiz & Gabaldón, 2019). Furthermore, both ITS1F/ITS2 and ITS3/ITS4 primers have been demonstrated to be less efficient compared to alternative primers, both in terms of amplification efficiency and the identification of OTUs from diverse phyla (Beeck et al., 2014). Despite the possibility of targeting the entire ITS region in metabarcoding studies using ITS1F/ITS4 primers (Duan et al., 2018, 2021), bias may still arise due to discrimination against longer PCR products (Ihrmark et al., 2012). 18S rRNA primers designed for fungal specificity also differ highly in their fungal coverage rate of higher and basal fungal

lineages, as well as in their tendency for co-amplifying non-fungal eukaryotic sequences. For instance, the commonly utilized primer pair FF390/FR1 (nu-SSU-1333/nu-SSU-1647) has recently been demonstrated to co-amplify non-fungal eukaryotic groups such as Stramenopiles, Alveolata, Rhizaria, and Telonema (Banos et al., 2020; Prévost-Bouré et al., 2011). These non-fungal eukaryotes are both abundant and diverse in marine environments, raising concerns regarding the suitability of these primers for aquatic samples although blocking primers were designed to reduce co-amplification (Banos et al., 2020).

Every primer pair employed carries its own set of advantages and disadvantages. However, the results obtained remain valuable, provided that the authors openly acknowledge the limitations of the approach employed and weigh the results with scientific integrity. The diversity described in these studies represents only a glimpse of the whole fungal diversity existing in the coastal sites surveyed. The Internal Transcribed Spacer (ITS) region was recommended by the Fungal Barcoding Consortium as a universal barcode for fungi due to its capacity for species-level resolution across a wide spectrum of fungal taxa (Schoch et al., 2012). However, this region does have the drawback of lacking a discernible phylogenetic signal, resulting in the identification of many OTUs only at the kingdom or phylum level due to the scarcity of reference sequences or inadequately annotated sequences (Nilsson et al., 2016). As discussed in the section above on the diversity of planktonic marine fungi, the current ITS databases (e.g. UNITE) include few sequences sampled from the ocean, rendering many marine (both coastal and open ocean) fungal ITS OTUs unclassified even at the phylum level (Peng & Valentine, 2021). To address this challenge, Banos et al. (2018) proposed a solution by incorporating a segment of the 18S region as a phylogenetic marker during ITS amplification. This approach enables phylogeny-based classification of fungal sequences and subsequently enhances the assignment to lower taxonomic levels, particularly for unknown or poorly annotated taxa. Alternatively, the 28S region can achieve higher taxonomic resolution than the 18S region and allow phylogenetic reconstruction (Xu et al., 2016). Rising interests in the 28S region have led to efforts such as the Fungal 28S Ribosomal RNA (LSU) RefSeq Targeted Loci Project that will likely facilitate taxonomic classifications in future studies (Robbertse, 2023). The entire rRNA region can be sequenced to provide maximum phylogenetic resolution and accuracy (Heeger et al., 2018; Runnel et al., 2022; Tedersoo et al., 2018), but this approach has not been adopted broadly due to cost and

technical challenges such as obtaining high-molecular-weight DNA from environmental samples.

This comprehensive literature review underscores the prevalence of Ascomycota, Basidiomycota, and Chytridiomycota, although other fungal taxonomic phyla are also detected, albeit at lower abundances (Mucoromycota, Glomeromycota, Opisthosporidia, etc.). This trend is consistently observed across studies, whether based on OTUs or ASVs, and in both single time point and time series investigations (**Figure 5**). Notably, in polar marine environments, there is an intriguing departure from this pattern, with Chytridiomycota appearing to dominate, suggesting a unique ecological niche for these fungi (Burgaud et al., 2022).

Metabarcoding also provides the potential to categorize fungal OTUs/ASVs into ecological roles (e.g., animal pathogens, plant pathogens, wood saprotrophs) within three trophic modes (pathotroph, symbiotroph, and saprotroph) (Nguyen et al., 2016). Recent application of this tool to coastal water samples in the Western Pacific Ocean suggests that planktonic fungi significantly influence biogeochemical cycles and food webs through their multi-trophic nutrition (W. Li et al., 2019). Despite some limitations, such as underrepresentation of specific taxa, both higher (e.g., *Aspergillus*, *Cladosporium*) and lower basal fungal lineages (e.g., Chytridiomycota, Opisthosporidia), leading to biases in trophic mode estimation, this approach offers insights into ecological roles. Furthermore, network analyses based on weighted topological overlaps (Banos et al., 2020) can reveal co-occurrence patterns among fungal OTUs/ASVs, allowing the inference of specific lifestyles like saprotrophy, antagonism, parasitism, etc., thus providing an additional layer of information.

A recent investigation into planktonic marine fungi in Chinese coastal waters, utilizing metabarcoding and metatranscriptomics, has revealed a significant disparity. ITS2 metabarcoding failed to detect early diverging fungal lineages that were abundant in the metatranscriptomic dataset (M. Wang et al., 2023). This observation aligns with findings from a prior investigation that focused on oceanic oxygen minimum zones and employed metabarcoding and metagenomics. In this earlier study, a comparable pattern emerged, wherein the metagenomic dataset revealed approximately one-third of early diverging fungal phyla that were

not detected by metabarcoding (Peng & Valentine, 2021). This underscores the imperative need for employing integrated approaches, such as metabarcoding in conjunction with metagenomics and/or metatranscriptomics, to provide an accurate depiction of the community structure of planktonic marine fungi.

4.6 Omics methods

The application and integration of genomics, transcriptomics, proteomics, and metabolomics in marine microbiology in the past two decades have brought about significant advances (Kim, 2016). However, the application of these modern approaches to study marine fungi has been limited. For example, the 1000 fungal genome project has brought the entire mycological community into the genomics age (Spatafora et al., 2017), but fewer than ten of the sequenced genomes were of marine origin. The number of individual genomics studies on marine fungi is very small, and most published studies using genomics to study marine fungi are motivated by potential biotechnological applications of marine fungi (Ameen et al., 2021; Kempken, 2023; A. Kumar et al., 2018; Xue et al., 2022). Given that nearly 1,900 marine fungal species have been described (Calabon et al., 2023), there needs to be a community effort to increase the number of genomic studies of marine fungi.

Phylogenomic investigation of marine fungi can help elucidate evolutionary relationships between marine fungi and terrestrial fungi, as there is still a large uncertainty whether fungi originated in the sea or on land (Raghukumar, 2017b). A recent comparative genomics study of 15 red yeast *Rhodotorula* species isolated from different environments showed that the oceanic strain hosts the smallest of the 15 genomes and yet fully conserves core metabolic pathways, suggesting the adaptation of *Rhodotorula* to the oligotrophic ocean (Lane et al., 2023). A genomic characterization of *Emericellopsis atlantica*, wood-associated *Amylocarpus encephaloides* and algae-associated *Calycina marina* showed that these marine fungi have a generalist lifestyle and can degrade multiple types of marine biomass while possessing a large repertoire of CAZymes (Hagestad et al., 2021).

A challenge for studying microbial eukaryotes stems from the added complexity of eukaryotic genome assembly and annotation compared to relatively straightforward prokaryotic genomes

that can typically be performed using an unsupervised, self-trained bioinformatic pipeline (Hyatt et al., 2010). Eukaryotic genome annotation usually requires supervision, and given the presence of variable number of repeated regions that require masking, introns, and exons, is best complemented with transcriptomic and proteomic evidence to improve model accuracies (Stanke & Waack, 2003; Yandell & Ence, 2012). Facing these challenges, transcriptome sequencing has been adopted as a complementary approach to investigate the functional diversity of marine microbial eukaryotes (Marine Microbial Eukaryotic Transcriptome Sequencing Project, "MMETSP") (Keeling et al., 2014). Like the 1000 fungal genome project, marine fungi are poorly represented by the MMETSP despite the large number of cultivated fungi from marine environments. Future studies using transcriptomics can be designed to study physiological adaptation of marine fungi to environmental changes. However, transcriptomic profiling is ideally performed with reference genomes, which are still rare for marine fungi. To avoid this "chicken-and-egg" problem, both genomics and transcriptomics should be incorporated in experimental designs.

When applied to natural assemblages of microorganisms, genomics and transcriptomic sequencing takes the "meta" form and can reveal the diversity, function, and activity of marine fungi. However, the low relative abundance of fungal DNA (as low as less than 0.02%, e.g. Peng and Valentine 2021) and RNA (~1% in seawater) (Kolody et al., 2019) poses a major challenge. Theoretically, increasing the metagenome sequencing depth to at least ten billion reads (up to 250 bp per read) per sample could recover millions of fungal reads, but even at today's reduced cost of high-throughput sequencing, the cost of performing such an experiment would be prohibitive. Most up-to-date studies use a sequencing depth up to hundreds of millions of reads per sample (Lan et al., 2022), making it infeasible to reconstruct fungal metagenome-assembled genomes (MAGs), which have been achieved in environmental samples with relatively low diversity and high fungal abundance (Peng et al., 2021; West et al., 2018). Despite these limitations, metagenomics have been used to assess fungal diversity in the ocean (Hassett et al., 2020; Morales et al., 2019; Peng & Valentine, 2021), as it has the advantage of avoiding biases associated with PCR, a required step of metabarcoding methods. The analysis of a metagenome dataset from the eastern tropical North Pacific oxygen minimum zone revealed that early diverging fungi accounted for about one third of the fungal community at locations where

metabarcoding of the ITS2 region detected no more than 5% of early diverging fungi (Peng & Valentine, 2021). The accuracy of classifying metagenomic reads depends upon the quality of the reference database, so it is critical to increase the number of marine fungal genomes which will serve as references.

Although current metagenome datasets typically do not have sufficient sequencing depth to enable the recovery of fungal MAGs, many fungal contigs are included in the general metagenomes assemblies and can be separated using tools such as EukRep (West et al., 2018). Moreover, eukaryotic metatranscriptome libraries constructed by targeting the polyadenylated tails of mature transcripts selecting for mainly protein-coding messenger RNA are another important method to investigate the function and activity of marine fungi. This approach has enabled the discovery of fungal transcripts coding for cell division and hydrolases involved in lipid, carbohydrate, and protein degradation in the deep biosphere (Orsi, Edgcomb, et al., 2013; Quemener et al., 2020), as well as the ocean water columns (Orsi et al., 2022; M. Wang et al., 2023). Additionally, there is a large potential for discovery in publicly available marine metagenomes and metatranscriptomes, most of which were primarily explored targeting bacteria and archaea. For example, analyses of the TARA Oceans datasets revealed a widespread utilization of different types of enzymes that hydrolyze carbohydrates and proteins by pelagic fungi, which potentially occupy a deeper niche than marine bacteria (Baltar et al., 2021; Breyer et al., 2022; Chrismas & Cunliffe, 2020).

Proteomic analysis has been used to study the enzymatic profile of marine fungi, especially the secreted proteins (secretome) (Pilgaard et al., 2019). Proteomics are typically performed to complement genomic and/or transcriptomic analysis of isolated fungal strains, and provide an additional line of evidence for fungal metabolisms such as polysaccharide depolymerization (Pilgaard et al., 2019) and secondary metabolite production (Kramer et al., 2015). In contrast, untargeted metaproteomics remains highly challenging not only because of the lack of reference genomes and transcriptomes of planktonic marine fungi, but the low concentration of proteins in the water column makes sample acquisition highly demanding, which requires filtration of at least tens to hundreds of liters of seawater (Saito et al., 2019).

Metabolomics measure a group metabolites from organisms and has been a staple tool for systems biology for decades (Kell, 2004). Application of metabolomics in fungal research, including the subfield of marine fungi, has largely focused on the measurements of secondary metabolites and usually driven by the interests in natural product discovery (G. Li et al., 2022; Stuart et al., 2020). Metabolic profiling of marine fungi captures snapshots of fungal metabolites under different growth conditions. Dissolved metabolites in aqueous phase are the most common type of metabolites analyzed using moder instruments such as high-performance liquid chromatography-mass spectrometry (G. Li et al., 2022). Very few metabolomic studies have been performed using planktonic marine fungi as subjects. Metabolic profiling of the marine fungus *Emericellopsis cladophorae* demonstrated its ability to produce antimicrobial and anti-inflammatory compounds (Gonçalves, Hilário, et al., 2022).

4.7 Stable isotope-based methods

Stable isotopes have been applied broadly in biogeochemistry, microbial ecology, and oceanography to study the microbial transformation of elements from molecular to ecosystem levels. The tracing of added stable isotopes in incubations is often combined with other techniques to provide novel insights. In this section we highlight three stable isotope-based methods that are based on and complement the abovementioned methods: secondary ion mass spectrometry (SIMS), stable isotope probing, and biogeochemical rate measurements.

The incorporation of substrates labeled with stable isotopes (e.g. ^{13}C and ^{15}N) by individual cells in mono- or co-cultures or in a natural assemblage of microbial community can be visualized using secondary ion mass spectrometry (SIMS), or nanoSIMS that can focus the primary ion beam down to 50 nm, enabling the quantification of stable isotopes with extremely high spatial resolution (Mayali, 2020). This technique quantitatively determines the number of both the rare (e.g., ^{15}N) and abundant isotopes (e.g., ^{14}N), as well as their spatial distribution, within the cell. Very few studies have applied nanoSIMS to the study of marine or aquatic fungi, partially due to limited instrument availability and the high cost associated with the analysis. A recent study incubated ^{13}C-labeled polyethylene with mono-cultures of *Rhodotorula mucilaginosa* isolated from seawater and used nanoSIMS to trace the incorporation of plastic-derived carbon into individual cells in addition to polyethylene degradation rates (Vaksmaa et al., 2023). They found

that *R. mucilaginosa* incorporated UV-treated ^{13}C-polyethylene, which suggests the red yeast could contribute to plastic degradation in marine environments. NanoSIMS has also been used to demonstrate that in a model system of freshwater diatom and chytrid fungi, fungi derived ~100% of their carbon content from the diatom (Klawonn et al., 2021). Klawonn et al. (2021) also showed that in a complex microbial community, unidentified taxa can be identified using FISH at the phylum or class level. Given the versatility of stable isotope labeled substrates, future experiments including nanoSIMS as a method can reveal novel biochemical pathways of planktonic marine fungi, as well as the roles fungi play in marine food webs.

While nanoSIMS offers a visual approach to quantify the incorporation of stable isotope-labeled substrates by cells, DNA stable isotope probing (DNA-SIP) reveals the identity and functional potential of the microorganisms responsible for the metabolism of added substrates, which is particularly useful in a complex microbial community of which most members are uncultivated. In marine microbial communities, saprotrophic fungi and bacteria play similar roles, such as in biomass degradation. DNA-SIP allows the identification of both fungal and bacterial lineages responsible for biomass degradation. The DNA of microorganisms that incorporated labeled substrate (e.g. ^{13}C-labeled polysaccharide) can be separated from the DNA without stable isotope labels by ultracentrifugation (Neufeld et al., 2007). Separated ^{13}C-labeled DNA can be used for metabarcoding and metagenomic analysis as discussed above, but they represent the microbial community that incorporated the added substrate. Seawater incubations with ^{13}C-labeled diatom biomass have demonstrated that *Malassezia* and *Cladosporium* assimilate microalgae-derived biomass in the ocean water column (Cunliffe et al., 2017; Orsi et al., 2022). The design of DNA-SIP experiments should consider ways to minimize potential cross feeding, which refers to the incorporation of stable isotope labels by cells that did not directly metabolize stable isotope-labeled substrate (e.g., by feeding on other cells that directly metabolize the labeled substrate).

Incubation with stable isotopes is a common method used to determine the transformation rates of chemical elements in any environment on earth, including in the ocean (Glibert et al., 2019). When stable isotope incubations are applied to a natural assemblage of microorganisms, the overall rates of elemental transformation mediated by microbial enzymes and abiotic processes are measured (e.g. nitrification by both archaea and bacteria in the ocean; Peng et al., 2015). The

contribution of fungi to a biogeochemical process, such as denitrification, can be constrained by applying antimicrobial compounds that inhibit specific microbial groups in stable isotope incubations (Peng & Valentine, 2021). For example, chloramphenicol, a broad-spectrum antibiotic commonly used to isolate fungi from the environment (see section 4.1), inhibits both bacteria and archaea (Yunis, 1988). On the other hand, antifungal agents such as cycloheximide could be used to inhibit fungal activities. In theory, if the antimicrobial compound achieved 100% specific and effective inhibition of the targeted groups (e.g. bacteria and archaea), the remainder rates measured represent the contribution of the non-targeted groups (e.g. fungi and other microbial eukaryotes). Peng & Valentine (2021) combined seawater incubations with ^{15}N-labeled nitrate with chloramphenicol treatments and found that fungi in the eastern tropical North Pacific Ocean can contribute up to 10% of the production of N_2O, a potent ozone-depleting greenhouse gas. However, one of the major limitations of this approach using antimicrobial compounds is that the inhibition of targeted groups may not be 100% (Salkin & Hurd, 1972), sometimes due to antibiotic-resistance (Larsson & Flach, 2022), and the antimicrobial compound may affect non-targeted organisms (Castaldi & Smith, 1998; Rousk et al., 2009). Therefore, the results from incubations with antimicrobial compounds must be interpreted with caution, and ideally the optimal concentration of antimicrobial compounds that maximizes their effectiveness and specificity for each sample type (e.g., open ocean vs. coastal seawater) should be determined with pilot experiments.

5 Outlook

This review of the study of planktonic marine fungi shows the large potential for new discoveries of fungal diversity and functions in marine environments, especially in the open ocean. To reconcile some of the inconsistent reports of fungal diversity based on metabarcoding surveys using different primers, we recommend using primer pairs targeting the large subunit of the rRNA gene because they are less biased against early diverging fungi compared to ITS primers and they typically allow taxonomic classification at lower levels than the small subunit of the rRNA gene. Once long-read sequencing becomes more affordable and tractable, it will be another promising alternative as nearly the entire rRNA region can be sequenced. Metagenomics and metatranscriptomics are becoming increasingly useful tools for assessing the diversity, function, and activity of planktonic marine fungi.

In addition to assessing planktonic fungal diversity with metabarcoding surveys, it is essential to determine the biomass of marine fungi to elucidate the flow of energy and nutrients in marine food webs. Future cultivation efforts could learn from the development of marine bacteria studies, in which it took decades to isolate the most abundant and prevalent heterotrophic bacteria in the ocean. Specially, enrichment and isolation media would ideally be designed to mimic in situ nutrient and oxygen concentrations, temperature, light level, and pressure. Analysis of marine fungal genomes and transcriptomic profiling will generate valuable insights into how planktonic fungi would adapt to a changing ocean and interact with other members of the microbial communities.

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Open Research

Data used in this manuscript were deposited at https://app.metapr2.org/metapr2/. The micrograph images will be deposited at https://marinemicrobe.github.io/.

Figure Captions

Figure 2. Global distribution of fungi and samples across coastal areas depicting their composition at phylum level, as well as oceanic regions retrieved from the MetaPR2 database (a). Bar plot of differences in alpha diversity given as the logarithm of the effective number of fungal species based on the exponent of the Shannon index (y-axis) between oceanic regions (family-level, Kruskal-Wallis $p < 0.001$) (b). Visualizing patterns (based on a redundancy analysis (RDA) of centred log-ratio transformed abundances with 999 permutations ($p < 0.01$)) between fungal communities, environmental variables (water temperature, salinity, longitude, latitude), and most abundant fungal taxa. Taxa are agglomerated to family level (c). Fungal community composition on phylum level (coloured according to A) between oceanic regions showing the dominant phyla and dominant families in parenthesis (rel. abundance > 0.3) (d). Distribution of the number of samples between oceanic regions indicating potential undersampling of specific coastal regions (e).

Figure 2. Litterature review of 13 studies analyzing the Plastisphere on marine plastic debris with a focus on fungal communities (updated from Wright et al., 2020). Study design highlights whether the study was conducted in a laboratory or in the field (yellow), the different types of plastics, i.e. conventional or biodegradable (green), and whether the plastics were naturally collected from the sea (collection) or introduced by the researchers and collected either at a single time point or at a series of time points (blue). Numbers indicate the first and last days of incubation, with numbers in brackets indicating the number of points included in the time series. Fungal community characterization (purple) indicates whether the Plastisphere was analyzed via 18S or ITS rRNA high throughput sequencing or by a culture-based approach, and whether microscopy was used to visualize the biofilm. The controls (orange) highlight those studies that compare the Plastisphere with the microbial community of the surrounding seawater (either bulk or between 0.2 and 3 μm) or particulate organic matter (>3 μm), or biofilms that develop on inert surfaces (e.g., glass, rock, wood, etc.). Meaning of polymer acronyms: PE (Polyethylene), PP (Polypropylene), PET (Polyethylene terephtalate), PS (Polystyrene), HDPE (High-density polyethylene), LDPE (Low-density polyethylene), SAN (Styrene-Acrylonitrile), PA (Polyamide), PU (Polyurethane), CA (Cellulose Acetate), PVC (Polyvinyl chloride), PLA (Polylactic acid), PCL (Polycaprolactone), and PHBV (Poly(3-hydroxybutyrate-co-3-hydroxyvalerate. PLA has been enclosed in brackets due to its biodegradability under specific conditions. * The publication by Zettler et al. 2013 was added, even though it was not focused on fungi, because it represents the first publication indicating the presence of microeukaryotes on marine plastic debris.

Figure 3. Micrographs of *Rhodotorula sphaeroacarpa* ETNP2018 (a), *Exophiala sp.* ETNP 2018 (b), and particles from seawater collected from the coast of South Carolina (c). Images were generated by FlowCam 8000. Numbers below each image represent area-based diameters in μm (Kydd et al., 2018).

Figure 4. Epifluorescence microscopy photographs of planktonic fungi (1000x; Olympus IX-83; UV Wide filter cube) stained with calcofluor white.

Figure 5. Literature review of 18 studies analyzing planktonic fungal communities using a metabarcoding approach within the period spanning from 2017 to 2023. The reference and sampling site section highlights details regarding the reference source and the locations of seawater sampling. The study design section specifies whether samples were collected at a single time point or at a series of time points (orange), including information about the duration of the time series and the number of samples analyzed (enclosed in brackets), the sequencing methodology employed (yellow), the targeted genetic marker (green), including the primers used, and whether the bioinformatic analysis was based on OTUs or ASVs (blue). Finally, the community characterization section presents the different fungal phyla detected, based on the phylogeny proposed by Naranjo-Ortiz & Gabaldon, 2019, (purple), along with the percentage representation of fungal OTUs when available. The asterisks (*) indicate low-level detections.

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