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DISSERTATION / DOCTORAL THESIS

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“Extracellular enzymatic activities of marine pelagic fungi and the influence of temperature and salinity”

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Overview of dissertation

The following thesis is presented as a collective dissertation. This combines a general introduction to the ecology of marine fungi including their biomass and extracellular enzymatic activities (EEAs), followed by four chapters that investigate these ecological parameters, and a general discussion that highlights the principal findings of the mentioned chapters. Chapters I and II are manuscripts published in a peer-reviewed scientific journal, whereas Chapters III and IV are manuscripts in revision also in peer-reviewed scientific journals. Chapter I adapts an extraction method to a low abundance of marine fungi, so that their biomass in the open ocean can be estimated. Chapter II investigates the total EEAs of marine fungi, and the influence of temperature on the kinetic parameters. Similarly, Chapter III investigates the total EEAs of marine fungi, but here, the influence of salinity on the kinetic parameters was analyzed. Finally, Chapter IV presents the contribution of the cell-free fraction to the total EEAs of marine fungi, and how salinity influences the kinetic parameters.

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Summary

Fungi are a eukaryotic kingdom composed of diverse organisms such as mushrooms, molds, and yeasts. Fungi have been widely studied in terrestrial as well as in freshwater ecosystems, but far less in the largest ecosystem on Earth - the global ocean. This inattention has resulted in a lack of knowledge of the basic ecological parameters of marine fungi such as biomass and metabolic activities.

Since ergosterol is almost exclusive to fungi, its quantification has been commonly used as a proxy to estimate fungal biomass in terrestrial and freshwater ecosystems. In Chapter I, a relatively simple lipid extraction method originally developed for fish tissues was adapted to fungi present in low abundance in marine pelagic ecosystems. This adapted extraction method allowed a recovery of ~86% of the ergosterol standards. Furthermore, a combination of high filtration volume, re-extraction, and low resuspension volume of the sample with a Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LC-MS/MS) quantification method allowed the ergosterol quantification from oceanic samples as low as 0.12 pM.

On the other hand, terrestrial and freshwater fungi are well-known decomposers of organic matter. Only recently, some studies have highlighted their potential to degrade carbohydrates, and proteins in marine ecosystems, suggesting that marine fungi might play a key role in the biogeochemical cycles. Extracellular enzymatic activities (EEAs) have been widely used to infer potential functional diversity. In Chapters II and III, the degradation of carbohydrates, proteins, lipids, phosphorus and sulfur moieties by marine fungi was investigated using fluorogenic substrates analogues. Also, the influence of environmental parameters such as temperature and salinity on these activities was assessed. Interestingly, all the marine fungal species studied were capable to break down these substrates, but the majority of enzymatic activities increased with rising temperature and decreasing salinity. However, some potential extremophile enzymes were detected, specially related to sulfatase.

The cell-free fraction of microbial EEAs, mostly attributed to bacteria, has been shown to be relevant in the oceanic environment, but whether pelagic fungi contribute to this pool of dissolved enzymes is unknown. Similar to Chapters II and III, using fluorogenic substrate analogues, the fractions of fungal cell-attached and cell-free enzymes were investigated and compared, as well as the influence of salinity on these activities (Chapter IV). All the marine fungi species studied were capable to release cell-free enzymes. Depending on the enzyme, species, and even salinity, the proportion of cell-free to the total EEAs (i.e., cell-attached plus cell-free) can be up to 85.1%, suggesting fungi as active contributors to the total microbial EEAs pool in the ocean.

Collectively, the widespread presence of fungi in marine ecosystems, as well as their broad enzymatic repertoire both, cell-attached and cell-free, suggests that similar to their terrestrial and freshwater counterparts, marine fungi might be involved in the degradation of organic matter, hence, also in the marine biogeochemical cycles.

Zusamenfassung

Pilze sind ein eukaryotisches Reich, welches sich aus unterschiedlichen Organismen wie etwa Ständer-, Schimmel- und Hefepilzen zusammensetzt. Pilze wurden bereits ausgiebig sowohl in terrestrischen als auch in limnischen Ökosystemen erforscht, aber weit weniger im größten Ökosystem der Erde: dem Weltmeer. Aufgrund der geringen Aufmerksamkeit an Pilzen in marinen Ökosystemen resultierte ein Mangel an Wissen über wesentliche ökologische Parameter, wie deren Biomasse und metabolische Aktivitäten.

Da Ergosterol sehr spezifisch für Pilze ist, wurde es weitgehend als Parameter für die Quantifizierung der Pilzbiomasse in terrestrischen und limnischen Ökosystemen verwendet. In Kapitel I wurde eine relativ einfache, ursprünglich für Fischgewebe entwickelte Lipidextraktionsmethode für die niedrige Menge an Pilzen in pelagischen marinen Ökosystemen angewendet. Diese adaptierte Extraktionsmethode erlaubte eine Rückgewinnung von ~86% an Ergosterol-Standards. Weiters ermöglichte eine Kombination von Filtrationen eines großen Probenvolumens, Re-Extraktion und geringem Resuspensionsvolumen mit einer Liquid Chromatographie-Massenspektometrie/Massenspektometrie (LC-MS/MS) Quantifizierungsmethode die Ermittlung von Ergosterolkonzentrationen mariner Pilze bis zu minimalsten Werten von 0.12 pM.

Andererseits sind terrestrische und limnische Pilze bekannte Zersetzer von organischem Material. Erst kürzlich haben einige Studien deren Abbaupotenzial von Kohlenhydraten und Proteinen in marinen Ökosystem gezeigt, daher könnten diese eine mögliche Schlüsselrolle in biogeochemischen Kreisläufen spielen. Extrazelluläre enzymatische Aktivitäten (EEAs) werden oft herangezogen, um die potenzielle funktionelle Diversität abzuleiten. In Kapitel II und III wurden daher fluorogene analoge Substrate verwendet, um die Zersetzung von Kohlenhydraten, Proteinen, Lipiden, und Phosphor- sowie Schwefelresten, von Meerespilzen zu untersuchen. Weiters wurde auch der Einfluss

von ökologischen Parametern wie Temperatur und Salzgehalt auf deren Aktivitäten berücksichtigt. Interessanterweise waren alle untersuchten marinen Pilzarten fähig diese Substrate abzubauen, wobei jedoch die Mehrheit der enzymatischen Aktivitäten bei höherer Temperatur und niedrigerem Salzgehalt gefunden wurden. Dennoch wurden einige potenziell extremophile Enzyme entdeckt, im Speziellen in Verbindung mit Sulfatase.

Die zellfreie Fraktion von mikrobiellen EEAs, im Allgemeinen hauptsächlich bestehend aus Bakterien, scheinen im marinen Ökosystem relevant zu sein; ob pelagische Pilze zum Pool ungelöster Enzyme beitragen, ist nicht bekannt. Ähnlich zu Kapitel II und III, in denen fluorogene analoge Substrate angewendet wurden, untersuchte und verglich man Fraktionen von zell-assoziierten und zellfreien Pilzenzymen, sowie den Einfluss des Salzgehalts auf deren Aktivitäten (Kapitel IV). Alle untersuchten marinen Pilzarten konnten zellfreie Enzyme freisetzen. Abhängig von Enzym, Spezies und sogar Salzgehalt, konnte der Anteil an zellfreien Enzymen von totalen EEAs (z.B. zell-assoziiert und zellfreie) bis zu 85.1% betragen, was auf einen aktiven Beitrag von Pilzen zum totalen mikrobiellen EEAs Pool in den Ozeanen hindeutet.

Zusammengefasst lässt sowohl die verbreitete Präsenz von Pilzen in marinen Ökosystemen, als auch deren breites enzymatisches Repertoire vermuten, dass sie genauso wie deren Verwandte in terrestrischen und limnischen Ökosystemen an der Zersetzung von organischem Material beteiligt sind und daher auch eine Rolle in marinen biogeochemischen Kreisläufen spielen.

General introduction

In the Linnean taxonomy, fungi were included as part of the "Regnum Vegetabile" (von Linné 1766). However, over centuries, due to its complexity and diversity, fungal taxonomy has undergone major revisions. To deal with these difficulties, Ainsworth (1973), Worrall (1999), and Webster and Weber (2007) proposed that an organism classified as "fungi" should have the following traits: 1) nucleus: present; 2) cell wall: typically of glucan and chitin; 3) nutrition: osmoheterotrophy; 4) reproduction: sexual, parasexual, and/or asexual; 5) propagation: usually through microscopical spores; 6) ecological role: saprotrophs, symbionts, and parasites; 8) habitat: terrestrial, freshwater, and marine; and 7) distribution: ubiquitous. As a result, fungi are nowadays a eukaryotic kingdom classified into nine phyla: Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Glomeromycota, Mucoromycota, Neocallimastigomycota, Opisthosporidia, and Zoopagomycota (Naranjo-Ortiz and Gabaldón 2019). A currently accepted estimate suggests that at least 1.5 to 3 million fungal species exist on Earth (Hawksworth 1991; Hawksworth 2001; Hawksworth 2012), but only 80,000 to 120,000 species have been described (Hawksworth 2001).

1. Marine fungi

A marine fungus is an organism isolated repeatedly from marine environments where it is capable of 1) growing and/or sporulating; 2) being metabolically active; 3) creating a symbiotic relationship with other marine organisms; and/or 4) adapting and genetically evolving (Pang, et al. 2016). In marine environments, fungal species can be obligate or facultative. The obligate species perform the mentioned characteristics exclusively in these environments, whereas the facultative ones are species of terrestrial origin that were introduced to marine environments by river runoff, wind, or even by other organisms (Duarte, et al. 2013; Boddy 2016), but that fulfill the above-mentioned

characteristics (Raghukumar 2017).

Approximately 1,100 fungal species have been isolated from marine environments, but it is estimated that 10,000 fungal species inhabit the oceans (Jones 2000). The failure to isolate, culture, identify, and quantify fungi from these environments might contribute to these low numbers (Blackwell 2011), and also to a lack of genomic libraries (Burgaud, et al. 2022). Consequently, an important kingdom in the largest ecosystem of the Earth's biosphere remains largely unexplored.

1.1. Fungal biomass

Ergosterol is an essential sterol located in the fungal cell membrane (Peacock and Goosey 1989; Ravelet, et al. 2001; Verma, et al. 2002) that protects fungi against mechanical and oxidative stress (Dupont, et al. 2012). Even though it seems to be species-specific (Gessner and Chauvet 1993; Djajakirana, et al. 1996; Montgomery, et al. 2000), ergosterol represents about 80% of the fungal sterols (Gutiérrez, et al. 2020), and 2% of the fungal dry weight (Brennan, et al. 1975). The wide presence, as well as a rare 5,7-double bond, makes ergosterol a promising proxy to estimate fungal biomass (Newell, et al. 1988). As a result, ergosterol has been commonly used to quantify fungal biomass in terrestrial (Seitz and Mohr 1977; Seitz, et al. 1979; Grant and West 1986; Martin, et al. 1990; Gessner and Chauvet 1993; Stahl and Parkin 1996; Montgomery, et al. 2000) and aquatic (Lee, et al. 1980; Newell, et al. 1988; Padgett and Posey 1993; Mille-Lindblom, et al. 2004; Hassett, et al. 2019; Gessner 2020) environments.

Despite the fact that oceans represent the largest ecosystem in the Earth's biosphere (Jannasch and Taylor 1984; Gao, et al. 2010), and that fungi dominated the microbial biomass in bathypelagic marine snow (Bochdansky, et al. 2017), the marine fungal biomass remains largely unquantified. To date, the only available attempt to quantify this via ergosterol was performed by Hassett, et al. (2019) in sea ice and seawater samples from the

productive Arctic, reporting an ergosterol average of 16.5 pM (ranges from 0.12 to 126.9 pM). Using these values as a reference, and also due to the dilute nature of the oceans (McCarthy, et al. 1996; Amon and Benner 2003), highly sensitive methods were needed to extract and quantify the characteristically low fungal biomass in oceanic samples.

1.2. Extracellular enzymatic activities (EEAs)

Osmotrophy is a distinctive feature of the fungal kingdom that has allowed them to conquer diverse habitats (Dix and Webster 1995; Webster and Weber 2007; Richards and Talbot 2013). Fungi, as osmoheterotrophs organisms, produce a wide variety of enzymes that convert large molecules into smaller ones (Worrall 1999; Arnosti 2011; Richards, et al. 2012; Muszewska, et al. 2017). Small molecules can then be absorbed osmotically through the cell membrane, and further internally digested (Worrall 1999).

Enzymes can be constitutive or inductive. The first ones are produced continuously regardless of the cellular and/or substrate conditions, while the inductive enzymes are only released in response to certain conditions, also known as inducers or inducing agents (Priest 1977; Arnosti 2011). Thereby, the production of enzymes might involve important mechanisms such as active chemosensing (Vetter, et al. 1998), and quorum-sensing (Ziervogel and Arnosti 2008).

Enzymes can be located externally or internally. Extracellular enzymes are those located outside the cell membrane, and can be 1) cell-attached, thus linked to the cell surface, either tied or integrated into the membrane (Chróst 1990); and 2) cell-free, which are enzymes released by the cell into the immediate environment (Priest 1977; Chróst 1990; Wetzel 1991). These last ones can be secreted by living cells or released from dead cells (Skujiņš and Burns 1976; Baltar 2018). A commonly used term, total extracellular enzymatic activities (EEAs) refers to a combination of the activities of cell-attached and cell-free enzymes (Wetzel 1991; Baltar 2018).

2. Oceanic biogeochemical cycles

Carbon, hydrogen, nitrogen, oxygen, phosphorus, sulfur, calcium, and iron are the main elements of living organisms, and can represent up to 95% of their biomass (Schlesinger and Bernhardt 2013; Summons 2013). In the biogeochemical cycles, these elements alternate between living and non-living reservoirs (Summons 2013). Microorganisms harbor a large number of genes involved in the conversion of energy and matter into essential compounds, mainly through enzymes (Saunders, et al. 2022). Consequently, in marine environments, microorganisms have been highlighted as "engines" that drive those cycles (Falkowski, et al. 2008).

Due to the renewable nature of organic matter, it is an essential constituent of the biosphere (Summons 2013). In coastal areas, organic matter from terrigenous sources is also available, so the production of autochthonous dissolved organic matter (DOM) is often negligible (Pakulski and Benner 1994; Opsahl and Benner 1997; Benner 2003; Hernes and Benner 2006). In contrast, in the open ocean, as the majority of organic matter from terrigenous sources is degraded (Opsahl and Benner 1997), DOM is mainly produced *in situ* (Druffel, et al. 1992). Here DOM can be composed of simple (amino acids, fatty acids, neutral sugars, vitamins, etc.), complex (lipids, proteins, polysaccharides, etc.), and even not fully characterized molecules (Biersmith and Benner 1998; Repeta 2015).

In marine environments, DOM represents one of the largest reservoirs of carbon (Azam, et al. 1983; Benner, et al. 1992; Amon and Benner 1994; McCarthy, et al. 1996; Hansell, et al. 2009), so its biosynthesis and remineralization are essential processes in the carbon cycle (Benner 2003). As mentioned before, in the open ocean, DOM is mainly produced *in situ* (Druffel, et al. 1992) through photosynthesis, i.e., phytoplankton fixate atmospheric inorganic carbon and transform it into organic matter (Falkowski 1994; Falkowski, et al. 2000). Phytoplankton, and other living and dead cells release DOM into the surrounding environment (Azam, et al. 1983). Normally, organisms tend to use substrates

that are at least one order of magnitude smaller than themselves (Sheldon, et al. 1972). As the majority of marine DOM is composed of molecules smaller than 1000 Da (LMW), but the bioavailable DOM is composed mainly of molecules larger than 1000 Da (HMW) (Wheeler 1976; Benner, et al. 1992; Amon and Benner 1994, 1996; Benner 2002), microorganisms cannot take them up directly through the cell membrane (Weiss, et al. 1991; Arnosti 2004). To overcome this, microorganisms secrete extracellular enzymes capable of cleaving molecules into smaller assimilable ones (Worrall 1999; Arnosti 2011; Richards, et al. 2012; Muszewska, et al. 2017). Consequently, microbial EEAs determine the fate of organic matter in the oceanic water column (Arnosti 2011). Due to this, marine microorganisms and their enzymes are considered the main drivers of the carbon cycle (Vetter, et al. 1998; Alderkamp, et al. 2007), and are also known as "gatekeepers for the carbon cycle" (Arnosti 2011).

3. Ecology of marine fungi

Although fungi have been reported in almost every marine habitat such as coasts (Ueda, et al. 2015; Picard 2017), sediments (Orsi, et al. 2013), and even marine snow (Bochdansky, et al. 2017), their basic ecological parameters are still not determined. Similar to their terrestrial and freshwater counterparts, marine fungi have been flagged as a potential component of the microbial community, which might be involved in the degradation of organic matter (Gutiérrez, et al. 2011). Moreover, as marine fungi contain and express the necessary enzymatic repertoire to degrade an assortment of carbohydrates (Baltar, et al. 2021) and proteins (Breyer, et al. 2022), they might also be drivers of the biogeochemical cycles (Morales, et al. 2019).

Over large time scales, oceans have played a key role in regulating the Earth's climate (Carlton 2000; Reid, et al. 2009). However, over the past two centuries, extensive human activities are leading to changes in its role (McIntyre 1995; Reid, et al. 2009), thus

also in the climate system (Dessler and Theater 1995). Oceans are capable to absorb heat and anthropogenic CO_2 (Reid, et al. 2009; Abraham, et al. 2013). This has led to rising temperatures and retreating sea ice, hence to perturbations in the ocean temperature, and salinity (Trenberth, et al. 2007; Reid, et al. 2009). These two environmental parameters influence the density distribution and stability of the thermohaline circulation (Reid, et al. 2009), and have also been associated with changes in the enzymatic activities of marine microbial communities (Caruso and Zaccone 2000). Taken together, as these "engines" drive the biogeochemical cycles (Falkowski, et al. 2008), the present thesis aims to investigate the ecological role of marine fungi and how their extracellular enzymatic activities might be reshaped under fluctuating environmental conditions due to global warming.

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Chapter I: Adapting an ergosterol extraction method with marine yeasts for the quantification of oceanic fungal biomass

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Abstract

Ergosterol has traditionally been used as a proxy to estimate fungal biomass as it is almost exclusively found in fungal lipid membranes. Ergosterol determination has been mostly used for fungal samples from terrestrial, freshwater, salt marsh- and mangrove-dominated environments or to describe fungal degradation of plant matter. In the open ocean, however, the expected concentrations of ergosterol are orders of magnitude lower than in terrestrial or macrophyte-dominated coastal systems. Consequently, the fungal biomass in the open ocean remains largely unknown. Recent evidence based on microscopy and -omics techniques suggests, however, that fungi contribute substantially to the microbial biomass in the oceanic water column, highlighting the need to accurately determine fungal biomass in the open ocean. We performed ergosterol extractions of an oceanic fungal isolate (*Rhodotorula sphaerocarpa*) with biomass concentrations varying over nine orders of magnitude. While after the initial chloroform-methanol extraction ~87% of the ergosterol was recovered, a second extraction recovered an additional ~10%. Testing this extraction method on samples collected from the open Atlantic Ocean, we successfully determined ergosterol concentrations as low as 0.12 pM. Thus, this highly sensitive method is well suited for measuring fungal biomass from open ocean waters, including deep-sea environments.

1. Introduction

In marine environments, heterotrophic microorganisms are the main drivers of biogeochemical cycles due to their orders of magnitude higher biomass-specific metabolic rates than metazoans. The main heterotrophic organisms in terms of biomass are bacteria, followed by their grazers (Thomson, et al. 2010), the hetero-, and mixotrophic protists (Bar-On and Milo 2019). A recent study on deep-sea marine snow in the North Atlantic showed that although bacteria dominated in terms of abundance, fungi dominated the marine snow-associated microbial community in terms of biomass (Bochdansky, et al. 2017). However, in contrast to terrestrial systems (Grant and West 1986; Stahl and Parkin 1996), freshwater (Gessner and Chauvet 1993; Baldy, et al. 1995; Grattan and Suberkropp 2001), and saltmarsh studies (Newell, et al. 1988), marine pelagic fungi have been poorly studied. It has been assumed that fungi only play a trivial role in the ocean, so their role in the ecology and biogeochemistry of the ocean remains largely enigmatic (Grossart, et al. 2020). A metagenomic study, however, suggests that fungi are potentially involved in many biogeochemical cycles in the ocean (Morales, et al. 2019). Moreover, a recent study supports that fungi contribute to the cycling of carbohydrates in the ocean (Baltar, et al. 2021). Nonetheless, most of the recent discoveries on pelagic fungal ecology are based on molecular methods that inform about the relative abundance and genetic potential and expression; however, the lack of an adequate methodology to reliably determine other basic ecological parameters such as biomass and production precludes a deeper understanding of the ecological role of fungi in the ocean.

For terrestrial and aquatic environments, the concentration of ergosterol has been used as a proxy for fungal biomass (Seitz and Mohr 1977; Grant and West 1986; Stahl and Parkin 1996; Mille-Lindblom, et al. 2004; Hassett, et al. 2019). For the majority of eukaryotic organisms, sterols are essential lipids serving as structural support and precursors for hormones (Dupont, et al. 2012). Ergosterol is an essential component of membranes in

fungi (Ravelet, et al. 2001; Verma, et al. 2002), and its concentration is species-specific and dependent on the physiological state (Gessner and Chauvet 1993). A property that makes ergosterol specially well-suited as a proxy for fungal biomass is that it contains a 5,7-double bond, which is rare among other sterols (Newell, et al. 1988). Additionally, it represents more than 80% of sterols in fungal strains (Gutiérrez, et al. 2020). Not all fungi are capable to synthesize ergosterol such as many fungi belonging to the division Chytridiomycota (Newell 2001). Additionally, some non-fungal organisms such as the green algae *Chlorella vulgaris* and some Protozoa can synthesize ergosterol (Akihisa, et al. 1992; Raghukumar 2017). However, since the division Chytridiomycota is usually either not present or a low contributor to pelagic fungal communities, and only attains relevant biomass sporadically (Taylor and Cunliffe 2016), the determination of fungal biomass via ergosterol quantification appears to be suitable.

The extraction of ergosterol from a sample is the first step in the quantification of fungal biomass. The most commonly used extraction method for ergosterol is the reflux extraction (Seitz and Mohr 1977; Newell, et al. 1988) which has been used for saltmarsh material, and also the chloroform-methanol extraction (Bligh and Dyer 1959) applied to fish tissue samples. It is noteworthy that these methods were developed and applied for environments with considerably higher fungal biomass than in the oligotrophic open ocean. Thus, it is unknown whether the currently applied ergosterol extraction and detection methods are sufficiently sensitive to determine pelagic fungal biomass.

We tested the chloroform-methanol extraction using an oceanic fungal isolate at biomass concentrations varying over nine orders of magnitude. We tested and fine-tuned the method to determine low concentrations of fungal biomass using HPLC-UV and LC MS/MS. Finally, we applied this method to samples collected from the Atlantic Ocean throughout the water column down to bathypelagic waters.

2. Materials and methods

2.1. Testing a method to allow detecting low concentrations of ergosterol using a cultured fungal strain

2.1.1. Fungal culture and dilutions preparation

Rhodotorula sphaerocarpa (HB 738), a fungus originally isolated from coastal Antarctic waters close to Marguerite Bay, was obtained from the Austrian Center of Biological Resources (ACBR). Ten fungal concentrations were prepared through serial dilution to identify the relationship between the fungal biomass and ergosterol concentration. For this, an initial amount of *Rhodotorula sphaerocarpa* cultured on yeast malt extract Agar (Wickerham 1939; Wickerham 1951) was diluted in 100 mL of artificial seawater (30 g/L sea salts S9883 Sigma-Aldrich) to obtain an $OD_{660} \approx 1$ (i.e., 1×). The optical density (OD) was measured with a UV-1800 Shimadzu spectrophotometer. Thereafter, 100 mL of the initial fungal biomass were added to 100 mL of artificial seawater. This process was repeated sequentially to obtain dilutions of 2×, 4×, 8×, 16×, 32×, 64×, 125×, 250×, 500×, and 1000×, all in triplicate. Thirty mL from each triplicate were gently vacuum-filtered onto combusted (450°C; 6 h) 25 mm diameter Whatman GF/F filters (WHA1825047 Sigma-Aldrich). Finally, filters were wrapped in a combusted aluminum foil and stored at −20°C until further processing.

2.1.2. Ergosterol extraction

The chloroform-methanol method to extract ergosterol was used as described by Bligh and Dyer (1959) with minor modifications. Briefly, one filter was gently shaken in a glass scintillation vial with 3 mL of 2:1 chloroform:methanol (v/v) in the dark at room temperature for 24 h. Thereafter, the extract was transferred to a 15 mL polypropylene tube, and 0.660 mL of Milli-Q water were added, thoroughly mixed, and centrifuged at 3200× g at room temperature for 3 min. To quantify the ergosterol potentially lost in the discarded

extraction phase, the upper phase (usually discarded according to the standard protocol) was transferred to an amber glass vial. Afterwards, the lower phase was transferred to another amber glass vial and evaporated to dryness under a fume hood. Finally, the extracts were resuspended in 0.50 mL of methanol and stored at −20°C until analysis.

The effect of repeated extractions of the same filter using the chloroform-methanol method was also tested. Each filter containing the various dilutions of *R. sphaerocarpa* was extracted three times using the chloroform-methanol approach as described above. The lower phase of each extraction was evaporated separately to dryness under a fume hood and the extracts were resuspended in 0.50 mL of methanol and stored at −20°C until HPLC analysis.

2.1.3. Determining the extraction efficiency of ergosterol

To determine the extraction efficiency of the chloroform-methanol method, 7.5 mL of the biomass of $OD_{660} \approx 1$ were added to 472.5 mL of artificial seawater to achieve the dilution 64×. Thirty mL were gently vacuum-filtered onto combusted (450°C; 6 h) 25 mm diameter Whatman GF/F filters (WHA1825047 Sigma-Aldrich). In total, 12 filters were prepared in this way and each filter was wrapped in a combusted aluminum foil and stored at −20°C until further processing. Before the extraction, 0.5 mL of ergosterol standards in methanol (1400 nM, 700 nM, and 350 nM final concentration; HPLC grade, Sigma-Aldrich) were added to each filter (containing the biomass) in triplicate, and no ergosterol standard was added to three filters. Subsequently, the filters were placed in the oven at 40°C overnight. Finally, they were extracted using chloroform-methanol extraction as described above, and the extracts were analyzed by HPLC analysis.

2.1.4. Ergosterol quantification by High-Performance Liquid Chromatography (HPLC-UV) analysis

Ergosterol concentrations were quantified by applying HPLC according to the protocol of Gulis and Bärlocher (2017). An Agilent 1260 Infinity Bioinert HPLC System equipped with an autosampler was used together with a Zorbax StableBond Aq Analytical Guard Column (4.6 × 12.5 mm; 5 µm particle size; 80 Å pore size, Agilent Technologies, Santa Clara, CA, USA) and a Zorbax Eclipse AAA Rapid resolution column (4.6 × 150 mm; 3.5 µm particle size, 80 Å pore size, Agilent Technologies). For detecting ergosterol, the wavelength of the UV detector was set at 282 nm. The mobile phase consisted of 100% methanol (HPLC grade, Sigma-Aldrich) with a flow rate of 0.8 mL/min. The injection volume was 400 µL and the samples were run for 20 min in total, including 5 min of purging at a column temperature of 25°C. Ergosterol standards (HPLC grade, Sigma-Aldrich) were prepared in a range from 500 µM to 30 nM in methanol and run together in the same sequence batch with the fungal samples. Furthermore, the temperature of the autosampler was kept at 10°C to protect them from potential degradation. Peak areas were used for calculating ergosterol concentrations in samples.

2.1.5. Limit of detection (LOD) and limit of quantitation (LOQ) of ergosterol by HPLC analysis

The lowest ergosterol concentration that can be reliably measured by HPLC was determined according to the protocol of Wenzl, et al. (2016). Six ergosterol standards (HPLC grade, Sigma-Aldrich) were prepared in five replicates ranging from 10 µM to 30 nM in methanol. Linear calibrations were performed and LOD and LOQ concentrations were calculated.

2.2. Measuring ergosterol concentrations in samples collected throughout the water column of the Atlantic Ocean

2.2.1. Sampling

Samples were collected from the water column of the Atlantic Ocean in March-April 2019 during the Poseidon expedition on board the R.V. Sarmiento de Gamboa from Punta Arenas, Chile to Santa Cruz de Tenerife, Spain. Seawater was collected with a CTD rosette sampler containing 12 L Niskin bottles from three different depths corresponding to epipelagic (5 m), mesopelagic (950 m), and bathypelagic (4000 m) waters. Ten liters of seawater were vacuum-filtered onto combusted 47 mm diameter Whatman GF/F filters (WHA1825047 Sigma-Aldrich). Filters were wrapped in combusted aluminum foil and stored at −20°C until analyses.

2.2.2. Ergosterol extraction and concentration

The ergosterol extraction of the samples collected in the Atlantic Ocean was performed with the chloroform-methanol method as mentioned above with minor modifications. Each filter was extracted twice, so the lower phases from extraction 1 and 2 were transferred to the same amber glass vial and evaporated to dryness under a fume hood. Finally, the dried extract was resuspended in 0.1 mL of methanol and stored at −20°C until analysis.

2.2.3. Ergosterol quantification via Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LC-MS/MS) analysis

To ensure the identity of the analyte applying the HPLC-UV method and to compare quantification, LC-MS/MS analysis was also performed. Ergosterol concentrations were quantified with LC-MS/MS similar to the protocol of Ory, et al. (2020). Briefly, an Agilent (Waldbronn, Germany) 1290 UHPLC System was used together with a Sciex

(Framingham, MA, USA) QTrap 6500+ mass spectrometer and a Phenomenex (Aschaffenburg, Germany) Gemini column (150 × 4.6 mm, 5 µm particle size). Isocratic elution at 35°C was performed with a mobile phase consisting of acidified acetonitrile (HPLC grade, Sigma-Aldrich) with 0.1% formic acid at a flow rate of 2.5 mL/min. The ergosterol standards (HPLC grade, Sigma-Aldrich) were prepared in a range from 1 nM to 100 µM in methanol and an injection volume of 20 µL was used. The retention time was 3.78 min, and the eluent was sent to the atmospheric pressure chemical ionization (APCI) source between 3.0 to 4.5 min.

For the ionization of ergosterol in positive ion mode, a heated nebulizer APCI probe was used in the Turbo V source with the following settings: curtain gas 30 psi, nebulizer gas 40 psi, temperature 500°C, nebulizer current 3 µA. The mass spectrometer was operated in selected reaction monitoring mode using both the $[M^+H^-H_2O]^+$ (declustering potential 100 V) and the unusual $[M^+H^-2H_2]^+$ (declustering potential 50 V) ions as precursors. The following six transitions were monitored for 50 ms each (Q1 mass > Q3 mass (collision energy, CE)): 379.3 > 295.2 (20 eV), 379.3 > 159.1 (30 eV), 379.3 > 145.1 (35 eV), 379.3 > 69.0 (55 eV), 393.3 > 268.2 (30 eV), 393.3 > 173.1 (35 eV). Analyst version 1.6.3 was used for data acquisition and evaluation.

2.3. Correlation between HPLC-UV and LC-MS/MS

For the correlation between the two detection methods, we performed a growth experiment. *Sakaguchia dacryoidea* (HB 877), a fungus originally isolated from the Antarctic waters, was obtained from the Austrian Center of Biological Resources (ACBR). An initial amount of *Sakaguchia dacryoidea* cultured on yeast malt extract Agar (Wickerham 1939; Wickerham 1951) was diluted in 10 mL of artificial seawater (30 g/L sea salts S9883 Sigma-Aldrich) to obtain an $OD_{660} \approx 1$. Afterwards, these 10 mL were added to 1000 mL of liquid media containing: 2 g glucose, 2 g peptone, 2 g yeast extract, 2 g malt

extract, 30 g artificial sea salts, and 0.5 g chloramphenicol. The liquid cultures were divided into ten bottles, each containing 100 mL of liquid media + fungi and incubated at 140 rpm (Aggro Lab, Ski 4) and at room temperature. The cultures were sampled in the lag, exponential, and stationary phase as determined by their optical density. Thirty milliliters from each triplicate were gently vacuum-filtered onto combusted (450°C; 6 h) 47 mm diameter Whatman GF/F filters (WHA1825047 Sigma-Aldrich). Filters were wrapped in a combusted aluminum foil and stored at −20°C until further processing. Finally, the filter was extracted using the chloroform-methanol approach as described above and the extracts were analyzed via HPLC-UV and LC-MS/MS analysis.

3. Results and discussion

Ergosterol is the most abundant fungal sterol (Rodrigues 2018) and it is almost exclusively produced by fungi (Mille-Lindblom, et al. 2004). Hence, it has been used to determine fungal biomass in terrestrial and aquatic environments (Gutiérrez, et al. 2020). However, quantifying ergosterol in the open ocean waters and the deep sea requires highly sensitive methods due to the expected low fungal abundance in these environments. Accordingly, we extracted and concentrated ergosterol, and compared two detection methods.

The chloroform-methanol approach allows ergosterol extraction and purification in a single operation. This method is also less time-consuming and no special equipment is required such as a rotavapor as compared to the reflux method (Seitz and Mohr 1977; Newell, et al. 1988). According to Bligh and Dyer (1959), the chloroform-methanol extraction method produces a biphasic system where the chloroform layer contains the lipids and the aqueous layer contains the non-lipids. Nonetheless, we determined the ergosterol concentration in both the lower and upper phase to test whether ergosterol is carried over and discarded with the upper phase. Most of the ergosterol concentration was recovered in

the lower phase (chloroform layer), irrespective of the fungal dilution (Figure I-1). Approximately 98% of the extracted ergosterol was contained in the lower phase, whereas less than 2% was found in the upper phase. Ergosterol has a logP value of 8.86, which reflects the molecule affinity to the organic portion, in this case, chloroform. Moreover, this extraction allowed a recovery of ~86% of the ergosterol standards. For a toluene extraction, a similar value (90%) was obtained by Verma, et al. (2002).

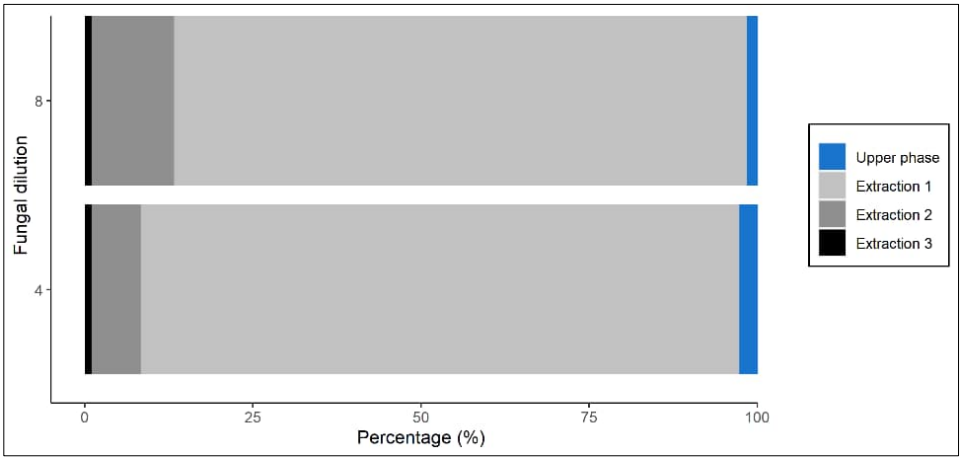

Figure I-1. Percentages of ergosterol extracted with chloroform-methanol approach from two fungal dilutions (4x, and 8x) of the marine fungal isolate *Rhodotorula sphaerocarpa*. Extracts recovered from the upper (one extraction) and lower (three extractions) phases were analyzed.

The LOD for HPLC-UV was 37.2 nM, while the LOQ was 122.8 nM. As a result, we were able to determine ergosterol concentrations in 7 out of 9 fungal dilutions (4x to 250x) (Figures I-2A and I-3). Due to high biomass, 30 mL of the dilution 2x could not be filtered onto a 25 mm diameter Whatman GF/F filter and therefore, was not measured. The ergosterol concentration was linearly related to fungal biomass using *Rhodotorula sphaerocarpa* (Figure I-4). Therefore, the chloroform-methanol extraction resulted in a relation between ergosterol concentration and fungal biomass (R^2= 0.9715).

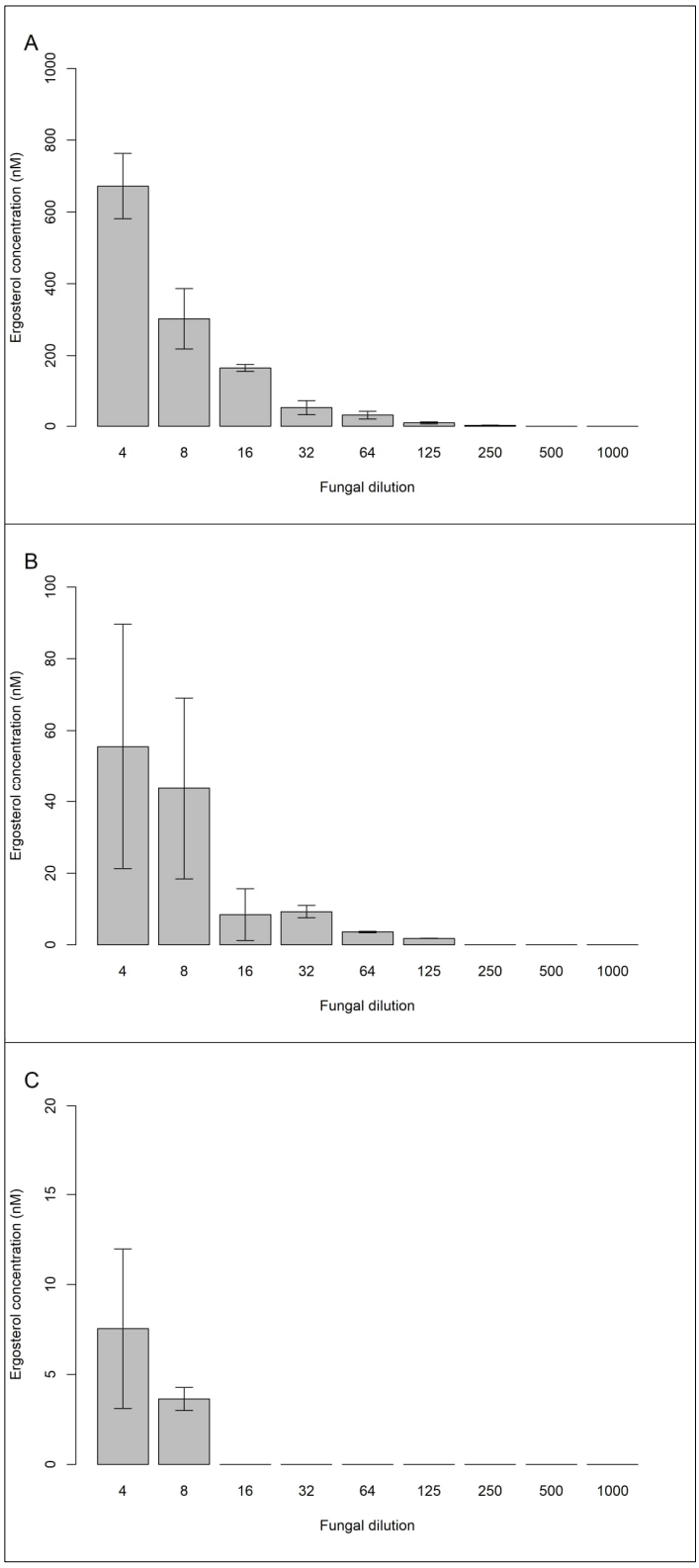

Figure I-2. Ergosterol concentration in nM obtained with chloroform-methanol extraction from the marine fungal isolate *Rhodotorula sphaerocarpa*. Nine fungal dilutions in artificial seawater were used (4×, 8×, 16×, 32×, 64×, 125×, 250×, 500×, and 1000×). Extracts from the first (**A**), second (**B**), and third (**C**) extractions were analyzed.

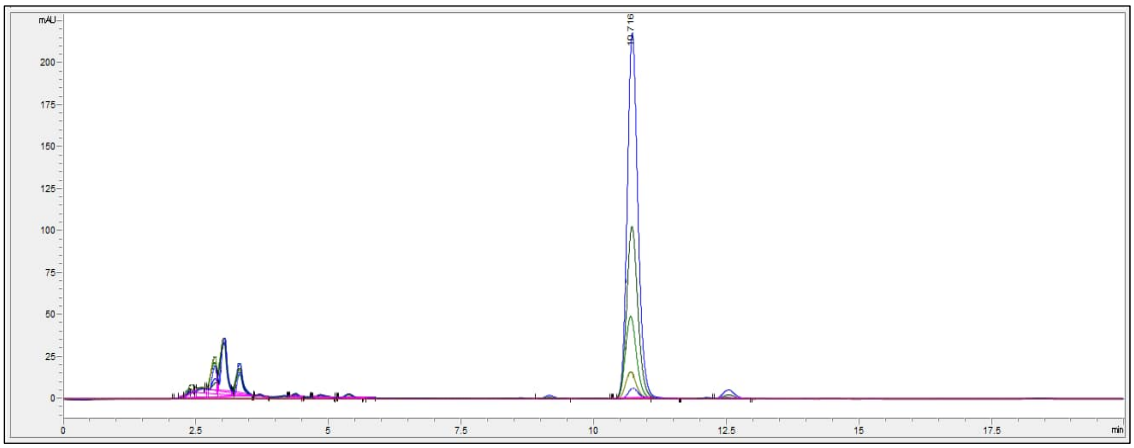

Figure I-3. LC-UV-chromatogram of ergosterol obtained with chloroform-methanol extraction from the marine fungal isolate *Rhodotorula sphaerocarpa*. Five fungal dilutions in artificial seawater are shown (4×, 8×, 16×, 32×, and 64×), and the x-axis shows minutes of the run.

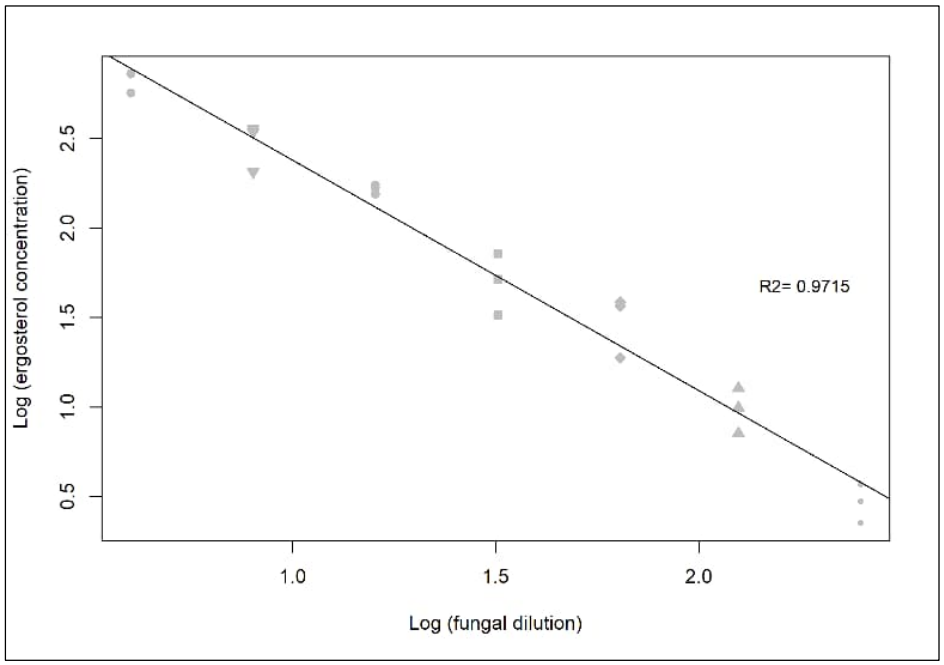

Figure I-4. Relationship between ergosterol concentration and fungal biomass dilution.

We also tested the significance of a second and third extraction (Figures I-2B and I-2C). In the study of Bligh and Dyer (1959) and Roose and Smedes (1996), a second extraction yielded an additional lipid recovery of 6% and 8–10%, respectively. We found

that a second extraction accounts for 5–16% of the total extracted ergosterol in the sample. For fungal dilutions 4×, 8×, 16×, 32×, 64×, and 125×, a second extraction allowed an additional recovery of 7.6%, 12.6%, 4.9%, 15.1%, 10.3%, and 15.5%, respectively. A third extraction led to an additional 1% of the total extracted ergosterol. Therefore, we recommend performing a second extraction, particularly when very low fungal biomass is expected as in oceanic samples.

The two detections, HPLC-UV and LCMS-MS were in good agreement (R^2 = 0.9985) (Figure I-5). Only in the lag phase, where the lowest ergosterol concentrations were detected in the culture, both methods differed remarkably with 380.2 ± 59.0 nM for HPLC-UV and 97.6 ± 57.6 nM LC-MS/MS. In open ocean waters and the deep sea, highly sensitive methods are required to quantify ergosterol as a low fungal abundance is expected in these environments. Thus, for oceanic samples collected from the Atlantic Ocean over three depths, we determined ergosterol with LC-MS/MS.

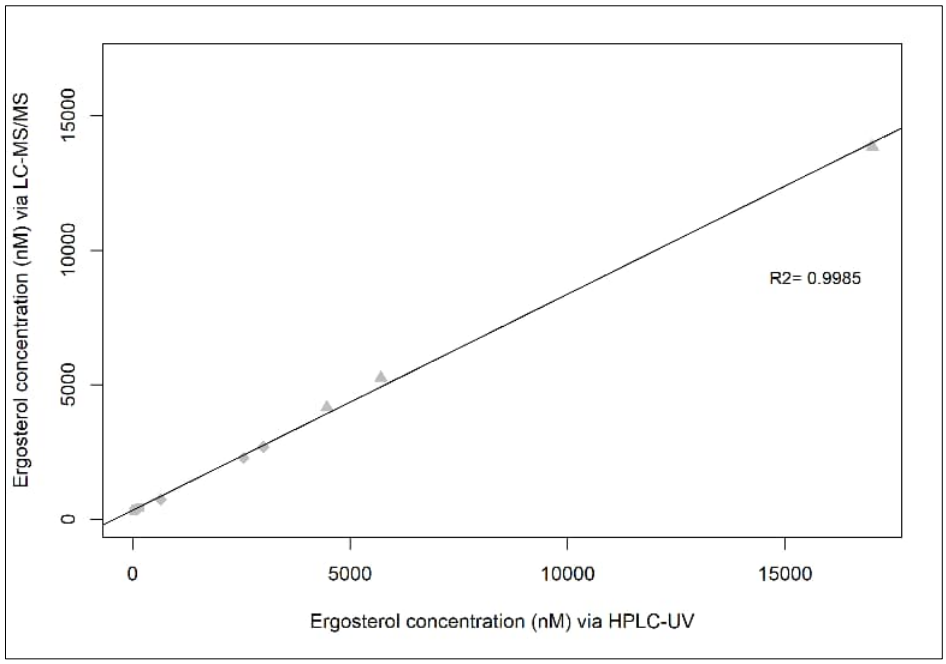

Figure I-5. Ergosterol content in nM detected via HPLC-UV vs. LC-MS/MS from the marine fungal isolate *Sakaguchia dacryoidea* covering three growth phases (lag,

exponential, and stationary).

For the developed LC-APCI-MS/MS method, the observed ionization was in agreement with results obtained from Ory, et al. (2020), with the $[M+H-H_2O]^+$ and the $[M+H-2H_2]^+$ ions being the most intense. An LOD of 1.5 nM (S/N = 3/1) and an LOQ of 5.0 nM (2 ng/mL) at S/N = 10/1 could be obtained, thus being about 25 times more sensitive than with the HPLC-UV method. A chromatogram of an ergosterol standard (1 µM) with all six SRM transitions is shown in Figure I-6. We were able to successfully concentrate ergosterol and detect it in the pM range (Table I-1). Marine samples were concentrated 10,000-fold (10 L water filtered and finally taken up in 0.1 mL); thus, the LOQ in seawater was about 0.05 pM (0.02 pg/mL). The highest concentration of ergosterol was 0.31 pM corresponding to epipelagic (5 m), whereas the lowest concentration was below the limit of detection corresponding to bathypelagic (4000 m). In the mesopelagic (950 m), the concentration was 0.12 pM. These numbers correspond to those of Hassett, et al. (2019), reporting an ergosterol concentration of 0.12 pM at a 246 m depth in the Arctic Ocean.

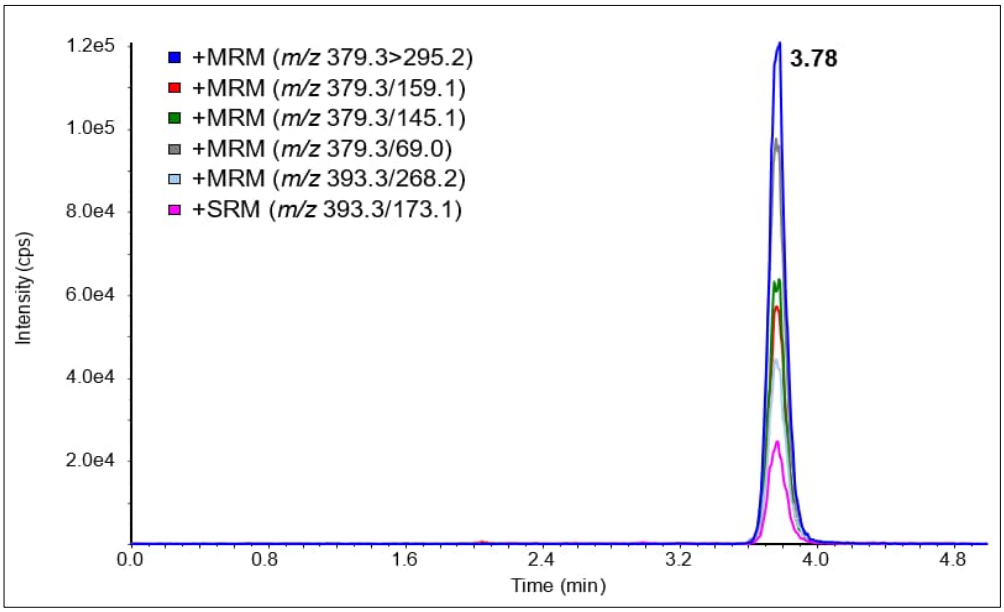

Figure I-6. LC-APCI-MS/MS chromatogram of a 1000 nM ergosterol standard.

Table I-1. Ergosterol content in the seawater of the Atlantic Ocean obtained with the chloroform-methanol approach (Bligh and Dyer 1959).

Coordinates		Pelagic zone	Depth (m)	Ergosterol concentration (pM)
Latitude	Longitude			
13°35.748	-29°42.830	Epipelagic	5	0.306
		Mesopelagic	950	0.120
		Bathypelagic	4000	0.000

Overall, we successfully adapted a method originally developed for ergosterol extraction from fish tissue (Bligh and Dyer 1959) to pelagic fungi. Based on our findings, we propose an ergosterol extraction method (detailed in Figure I-7) and quantification method specially adapted for low fungal concentrations. We found three crucial steps in the determination of ergosterol which are (1) a re-extraction of the filter, (2) the volume of the extract, and (3) the filtration volume of the water sample. A second extraction of the filter accounts for an additional ~10% of the ergosterol recovered. Additionally, the final volume of the extraction was adjusted to 0.5 mL for culture samples and 0.1 mL for oceanic samples, which might also be essential for the ergosterol quantification. Nonetheless, as mentioned before, it is important to consider that the ergosterol concentration can vary between species, and also that it can be influenced by different environmental parameters (Gessner and Chauvet 1993; Baldy, et al. 1995; Grattan and Suberkropp 2001). Thus, it is now relevant to test this method with other fungal species from oceanic origin (including hyphae like), and to consider the importance of environmental conditions on the ergosterol concentration when estimating the biomass of oceanic fungi. In spite of these few limitations, the method characterized here represents an important step for future research on the ecological role of

fungi in the ocean and an adequately sensitive method for environments with low fungal biomass.

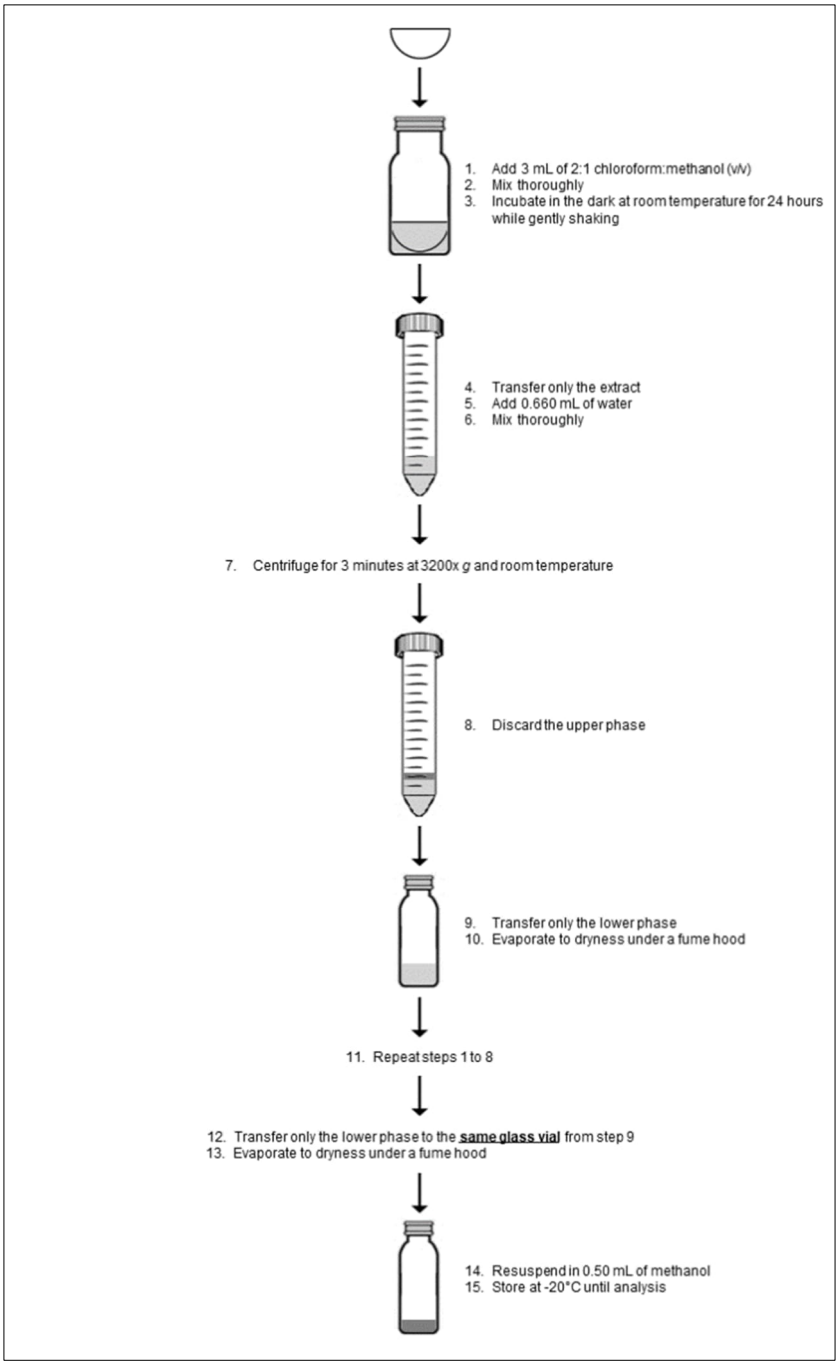

Figure I-7. Ergosterol extraction method modified and adapted for pelagic fungi.

Acknowledgments

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Chapter II: Extracellular enzymatic activities of oceanic pelagic fungal strains and the influence of temperature

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Abstract

Although terrestrial and aquatic fungi are well-known decomposers of organic matter, the role of marine fungi remains largely unknown. Recent studies based on omics suggest that marine fungi potentially play a major role in elemental cycles. However, there is very limited information on the diversity of extracellular enzymatic activities performed by pelagic fungi in the ocean and how these might be affected by community composition and/or critical environmental parameters such as temperature. In order to obtain information on the potential metabolic activity of marine fungi, extracellular enzymatic activities (EEA) were investigated. Five marine fungal species belonging to the most abundant pelagic phyla (Ascomycota and Basidiomycota) were grown at 5°C and 20°C, and fluorogenic enzymatic assays were performed using six substrate analogues for the hydrolysis of carbohydrates (β-glucosidase, β-xylosidase, and *N*-acetyl-β-D-glucosaminidase), amino acids (leucine aminopeptidase), and of organic phosphorus (alkaline phosphatase) and sulfur compounds (sulfatase). Remarkably, all fungal strains were capable of hydrolyzing all the offered substrates. However, the hydrolysis rate (V_{max}) and half-saturation constant (K_m) varied among the fungal strains depending on the enzyme type. Temperature had a strong impact on the EEAs, resulting in Q_{10} values of up to 6.1 and was species and substrate dependent. The observed impact of temperature on fungal EEA suggests that warming of the global ocean might alter the contribution of pelagic fungi in marine biogeochemical cycles.

1. Introduction

Fungi are eukaryotic and osmoheterotrophic organisms depending on organic matter to grow and obtain energy (Nevalainen, et al. 2014). Osmotrophy involves the secretion of different enzymes to break down complex biological polymers into smaller monomers that can then be taken up through the cell wall (Richards, et al. 2012). Due to this conversion of organic matter, osmoheterotrophs such as fungi should be major players in the recycling of organic matter. In the marine environment, most of the research on extracellular enzymatic activity (EEA) has been focused on prokaryotes (Arrieta and Herndl 2002; Baltar, et al. 2009). Only a few studies have reported on fungal EEA related to phytoplankton blooms (Gutiérrez, et al. 2011) or the degradation of plant-derived matter (Cunliffe, et al. 2017). As a result, the ecological role of fungi in the biogeochemistry of the oceans, which represents the largest habitat in the Earth's biosphere, remains poorly known (Gao, et al. 2010). This contrasts with recent evidence of fungal biomass dominating the bathypelagic marine snow (Bochdansky, et al. 2017). Additionally, recent studies based on omics suggest that pelagic fungi harbor genes indicative of an active role in marine biogeochemical cycles (Morales, et al. 2019). It has also been shown that a large variety of carbohydrate active enzymes (CAZymes) are expressed in marine pelagic fungi (Baltar, et al. 2021). Still, there is very limited information on the diversity of EEA in pelagic fungi in the ocean, and how it might be affected by community composition and/or environmental parameters such as temperature.

Approximately 70% of the Earth's biosphere is composed of persistently cold environments, from the deep sea to polar regions (D'Amico, et al. 2006; Yusof, et al. 2021). Depending on the optimal growth temperature, organisms living there can be psychrophilic or psychrotrophic (Morita 1975). These organisms need to be well adapted to low temperatures, low nutrient availability, and light seasonality (Feller and Gerday 2003; Vonnahme, et al. 2020). Moreover, as low temperatures influence the biochemical reaction

rates, organisms must be prepared to overcome those challenges (D'Amico, et al. 2006).

Here, we investigated the kinetic parameters (V_{max} and K_m) of the EEA of five oceanic fungal isolates using six different fluorogenic substrate analogues. β-glucosidase and β-xylosidase were used as a proxy for the degradation of plant-derived matter; *N*-acetyl-β-D-glucosaminidase were used for the utilization of animal and fungal chitinous compounds; alkaline phosphatase and leucine aminopeptidase were used for the cleavage of phosphate moieties and peptides, respectively; and sulfatase was used for the degradation of sulfate esters in macromolecules. We used five fungal strains isolated from the oceanic water column, with two strains belonging to the phylum Ascomycota and three to Basidiomycota. We selected these strains because Ascomycota and Basidiomycota are the most abundant pelagic fungal phyla (Taylor and Cunliffe 2016; Amend, et al. 2019; Morales, et al. 2019). Furthermore, the influence of temperature on fungal EEAs was determined.

2. Materials and methods

2.1. Culture of fungi species

The fungal species *Blastobotrys parvus* (HA 1620), *Metschnikowia australis* (HA 635), *Rhodotorula sphaerocarpa* (HB 738), and *Sakaguchia dacryoidea* (HB 877) were obtained from the Austrian Center of Biological Resources (ACBR). All these species were isolated from Antarctic Ocean waters at temperatures ranging from -1.24°C to 5.60°C (Fell and Hunter 1968; Newell and Fell 1970; Fell and Statzell 1971; Fell, et al. 1973). *M. australis* and *R. sphaerocarpa* were isolated close to the South Shetland Islands and Marguerite Bay, respectively. The fungus *Rhodotorula mucilaginosa* was isolated from the Atlantic Ocean at 21.03°C during the Poseidon cruise on board of RV Sarmiento de Gamboa in March 2019. The maximum temperature growth reported is 25°C for *B. parvus* (Fell and Statzell 1971) and *M. australis* (Fell and Hunter 1968), and 30°C for *R. mucilaginosa* (Sampaio 2011), *R. sphaerocarpa* (Newell and Fell 1970), and *S. dacryoidea* (Fell, et al. 1973). In order to have

fresh cultures, the pure isolates were cultured on yeast malt extract agar (Wickerham 1939; Wickerham 1951) for one week. Afterwards, an initial amount of each fungus was diluted in artificial seawater (30 g/L sea salts S9883 Sigma-Aldrich, Vienna, Austria) to obtain an $OD_{660} \approx 1$ (Salazar Alekseyeva, et al. 2021). The optical density (OD) was measured with a UV-1800 Shimadzu spectrophotometer. Then, 10 mL of this fungal culture was inoculated into an autoclaved growth medium containing 2 g/L of glucose, malt extract, peptone, and yeast extract; 35 g/L of artificial sea salts (S9883 Sigma-Aldrich); and 0.50 g/L of chloramphenicol. Afterwards, 150 mL of this medium containing fungi was filled in Schott bottles and to compare the effect of temperature, all strains were grown in triplicate at 5°C and 20°C on a rotary shaker (Jeio Tech ISS-7100 Incubated Shaker, Daejeon, South Chungcheong, Republic of Korea). The culture growth was tracked daily by OD. Once the exponential phase was reached, bottles with similar OD values were chosen in triplicate for further analysis (EEA and biomass).

2.2. Determining extracellular enzymatic activity and fungal biomass

Fluorogenic substrate analogues such as 4-methylumbelliferyl β-D-glucopyranoside (M3633 Sigma-Aldrich), 4-methylumbelliferyl β-D-xylopyranoside (M7008 Sigma-Aldrich), and 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (M2133 Sigma-Aldrich) were used to estimate the potential activity of the enzymes β-glucosidase (BGL), β-xylosidase (BXY), and *N*-acetyl-β-D-glucosaminidase (NAG), respectively (Table II-1). These enzymes can hydrolyze cellulose (Norkrans 1963; Pointing, et al. 1998), chitin, and xylan (Collins, et al. 2005; dos Santos, et al. 2016), respectively, thus mediating carbohydrate degradation by marine fungi. The hydrolysis of 4-methylumbelliferyl phosphate (M8883 Sigma-Aldrich) and N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (L2145 Sigma-Aldrich) was used to estimate the potential enzymatic activity of alkaline phosphatase (APA) and leucine aminopeptidase (LAP), respectively.

APA is indicative of the capability of microbes to acquire inorganic phosphorus from organic molecules, and LAP is involved in the hydrolysis of proteins and peptides (Hoppe 1983). Finally, 4-methylumbelliferyl sulfate potassium salt (M7133 Sigma-Aldrich) was used to determine the activity of sulfatase (SUL) degrading sulfate esters in macromolecules. The hydrolysis of these fluorogenic substrates analogues were standardized to the corresponding fluorophores. The fluorophores methylcoumaryl amide (MCA) (A9891 Sigma-Aldrich) and methylumbelliferone (MUF) (M1381 Sigma-Aldrich) were dissolved in 2-methoxyethanol to obtain a final concentration of 100, 50, 10, and 1 μM, and 2000, 1000, 100, and 50 μM.

Table II-1. Targeted enzymes with an analogue fluorogenic substrate and their respective standards, methylumbelliferyl (MUF) and methylcoumaryl (MCA) amide.

Type	Code	Name	Standard
Carbohydrates	BGL	β-glucosidase	MUF
	BXY	β-xylosidase	MUF
	NAG	*N*-acetyl-β-D-glucosaminidase	MUF
Proteins, peptides	LAP	Leucine aminopeptidase	MCA
Phosphorus	APA	Alkaline phosphatase	MUF
Sulfur	SUL	Sulfatase	MUF

Sterile microplates of 96 wells with an F bottom and low protein binding (XT64.1, Carl Roth, Karlsruhe, Baden-Wurtemberg, Germany) were used. The standards were distributed to each biological triplicate to establish a standard calibration curve, and each biological triplicate without any addition was used as a blank to determine the background fluorescence of the medium. Serial dilutions of the fluorogenic substrate were established

resulting in 12 final concentrations ranging from 100 to 0.05 µM. The fluorescence was measured with a Tecan Infinite 200 PRO at an excitation wavelength of 365 nm and an emission wavelength of 445 nm. An initial measurement was performed, and then, every hour, a measurement was made over a total period of 3 h. Between measurements, the microplates were incubated in the dark at their respective temperature.

For fungal biomass determination, combusted (450°C; for 6 h) Whatman GF/F filters (WHA1825047 Sigma-Aldrich, 47 mm filter diameter) were individually wrapped in aluminum foil and weighed. Then, 40 mL of each fungal culture triplicate was gently filtered onto a combusted and weighed filter, and was dried at 80°C for 3 days. Thereafter, the sample was weighed again to determine the fungal biomass as dry weight.

2.3. Determination of the kinetic parameters of the extracellular enzymatic activity (EEA)

The increase in fluorescence over time (3 h) was transformed into hydrolysis rate [µmol L^{-1} h^{-1}] using the equation obtained from the standard calibration lines of MCA and MUF. The resulting hydrolysis rates were fitted directly with the Michaelis–Menten equation using nonlinear least-squares regression analysis with R software (Huitema and Horsman 2018). The enzymatic kinetic parameters maximum velocity (V_{max}) and half-saturation constant (K_m) were calculated. To obtain the biomass-specific activity, the V_{max} was normalized to the fungal biomass obtained from the dry weight. Additionally, the Q_{10} value was calculated to identify the dependence of enzyme activity on temperature (Sterratt 2015).

2.4. Statistical analyses

For the kinetic parameters V_{max} and K_m, a one-way analysis of variance (ANOVA) was performed to determine differences among species and temperature. Tukey's Honestly

Significant Difference (Tukey's HSD) was used for a multiple and simultaneous comparison between species and to identify significance at the species level. A Student's T-test was used to test the normal distribution of the data. A principal component analysis (PCA) was performed to analyze the fungal enzymatic activity by species and substrate. For this purpose, the prcomp command of the R software was used. To maximize the sum of the variance of the squared loadings, Varimax rotation was performed.

3. Results

Remarkably, all the fungal strains were hydrolyzing all the fluorogenic substrates offered (Figures II-1 and II-2). The kinetic parameters V_{max} and K_m of the EEA varied, however, among the different fungal strains and substrates (Figures II-3 and II-4). Additionally, the response of EEA to temperature was species and substrate dependent (Figures II-5 and II-6).

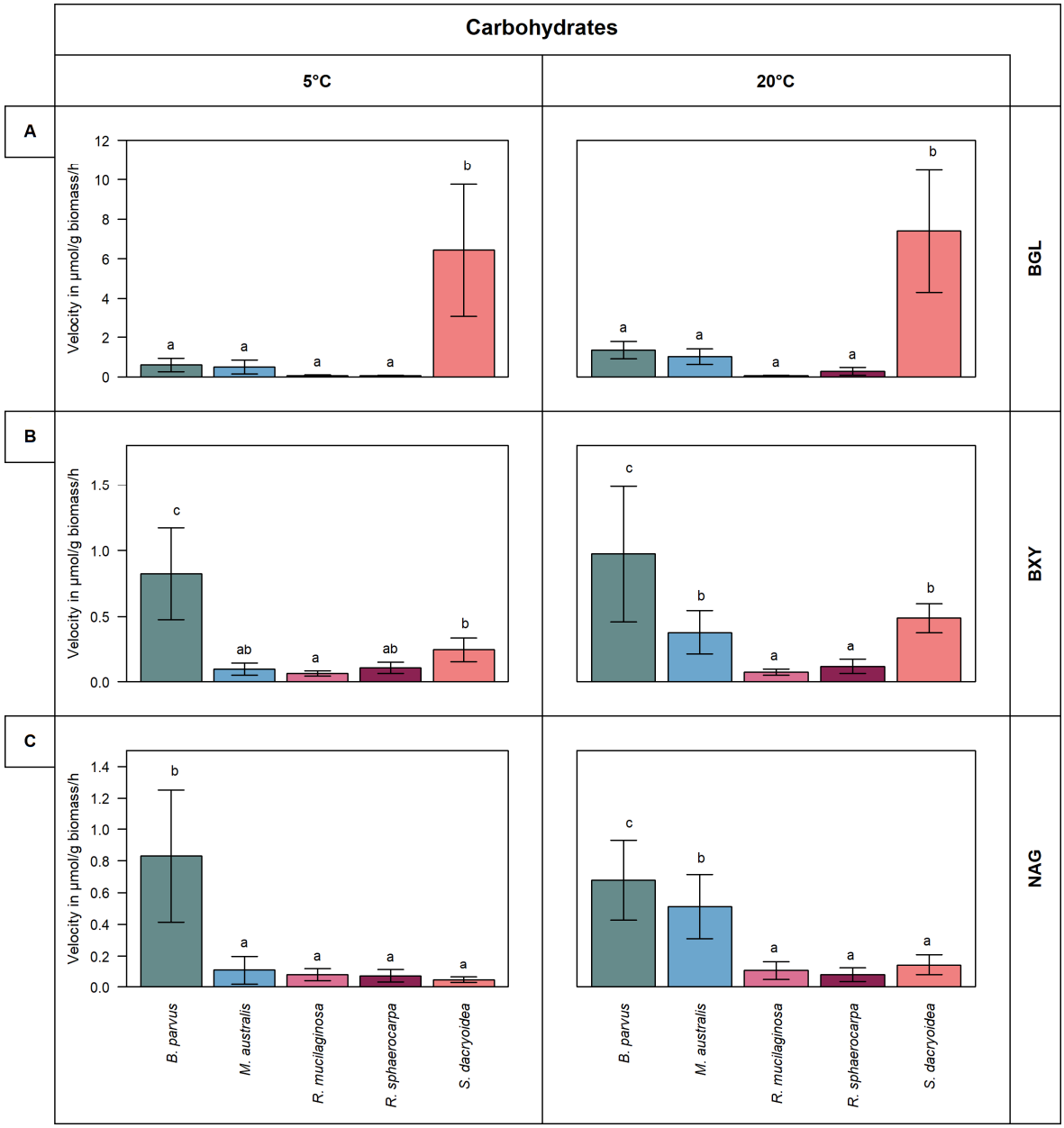

Figure II-1. V_{max} in µmol/g biomass/h obtained from the total enzymatic activity for the substrates representing carbohydrates such as (**A**) β-glucosidase (BGL), (**B**) β-xylosidase (BXY), and (**C**) *N*-acetyl-β-D-glucosaminidase (NAG) of five marine fungal isolates *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* at 5°C and 20°C. According to Tukey's HSD, bars denoted by a different letter (a, b, and c) are significantly different ($p < 0.05$), whereas bars denoted by a common letter (ab) are not significantly different.

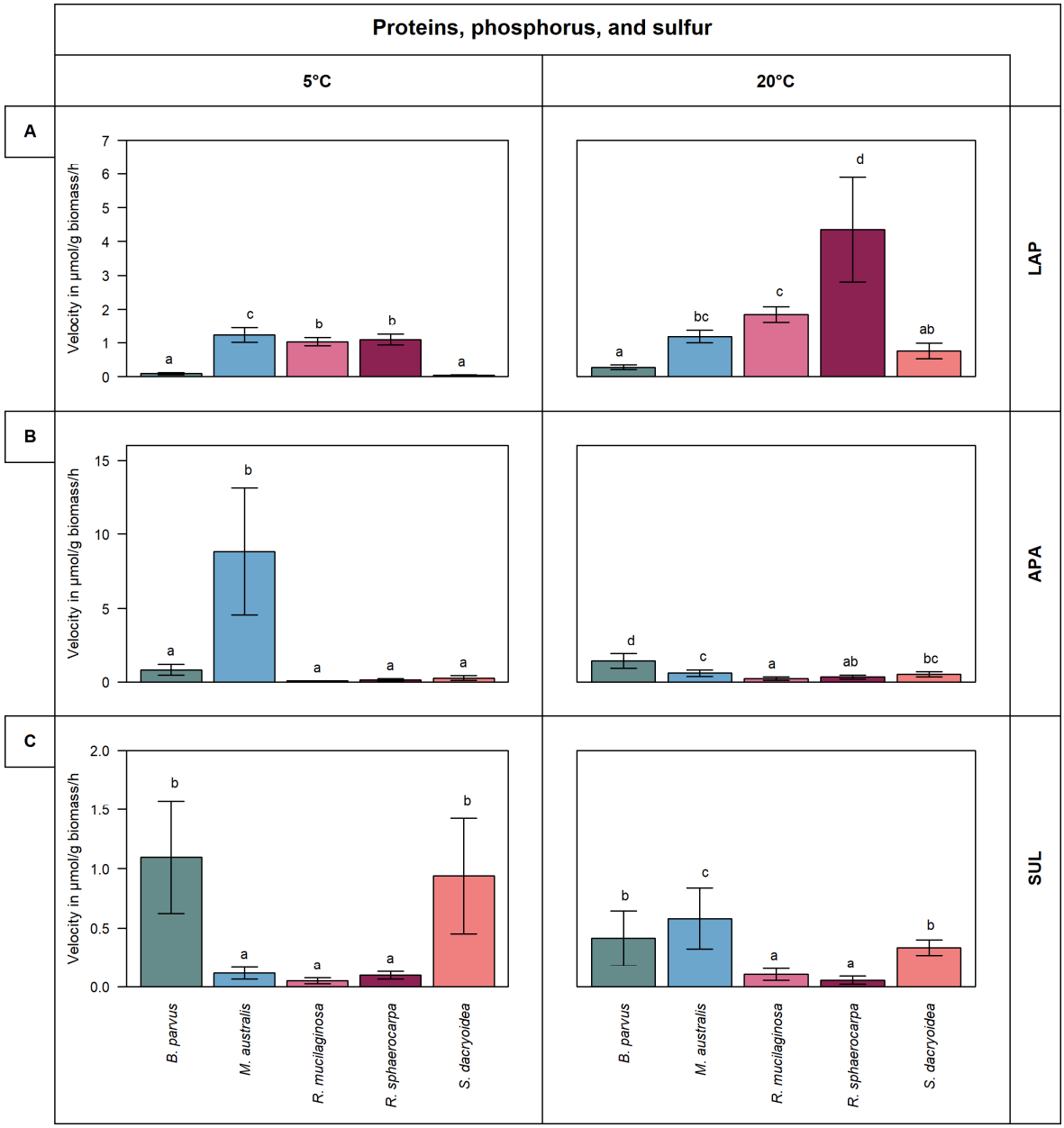

Figure II-2. V_{max} in µmol/g biomass/h obtained from the total enzymatic activity for the substrates representing proteins, phosphorus, and sulfur, such as (**A**) leucine aminopeptidase (LAP), (**B**) alkaline phosphatase (APA), and (**C**) sulfatase (SUL) of the five marine fungal isolates *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* at 5°C and 20°C. According to Tukey's HSD, bars denoted by a different letter (a, b, c, and d) are significantly different ($p < 0.05$), whereas bars denoted by a common letter (ab and bc) are not significantly different.

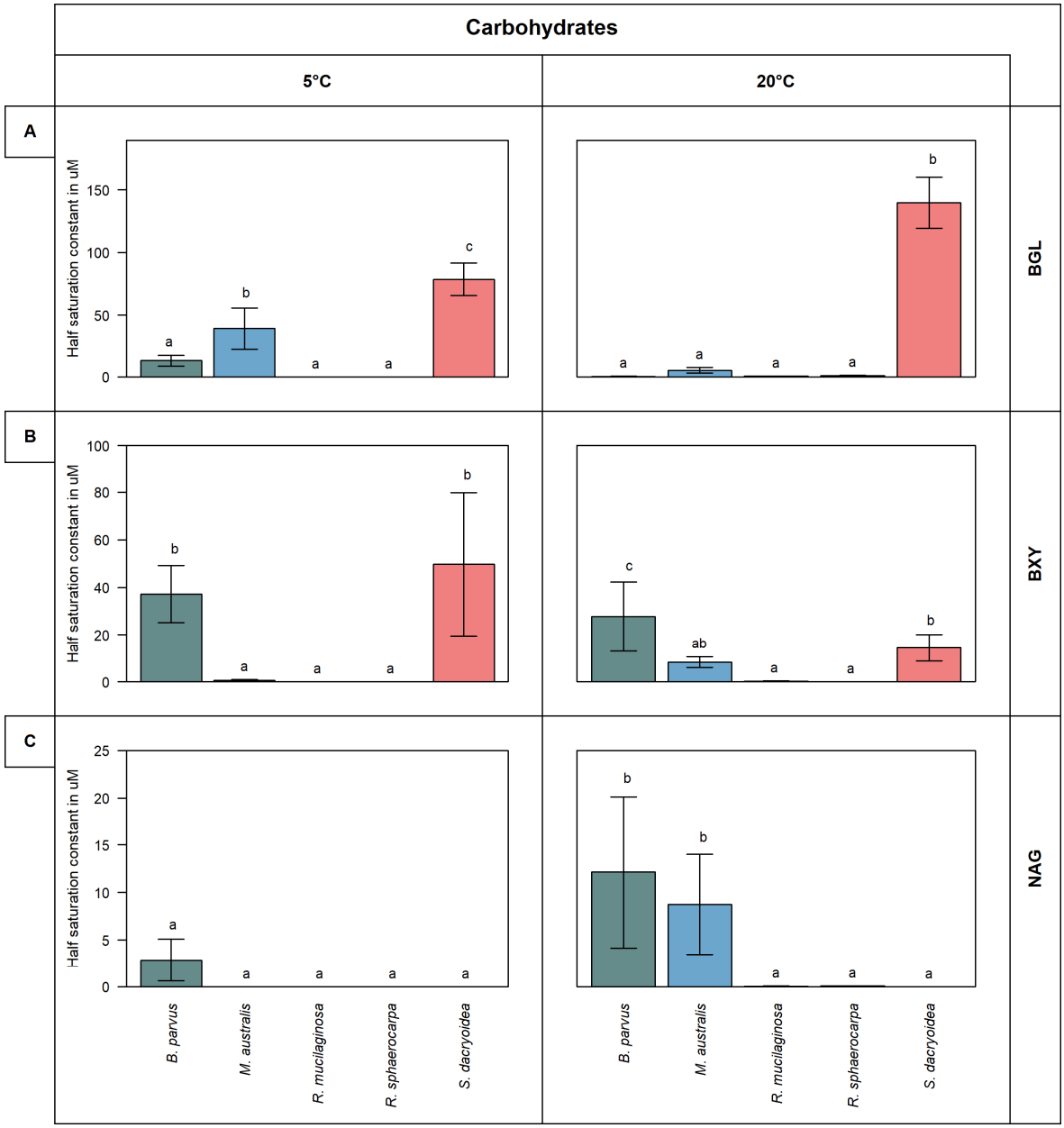

Figure II-3. K_m in µM obtained from the total enzymatic activity for the substrates representing carbohydrates, such as (**A**) β-glucosidase (BGL), (**B**) β-xylosidase (BXY), and (**C**) *N*-acetyl-β-D-glucosaminidase (NAG) of the five marine fungal isolates (*B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*). Measurements were performed at 5°C and 20°C in the exponential phase. According to Tukey's HSD, bars denoted by a different letter (a, b, and c) are significantly different ($p < 0.05$), whereas bars denoted by a common letter (ab) are not significantly different.

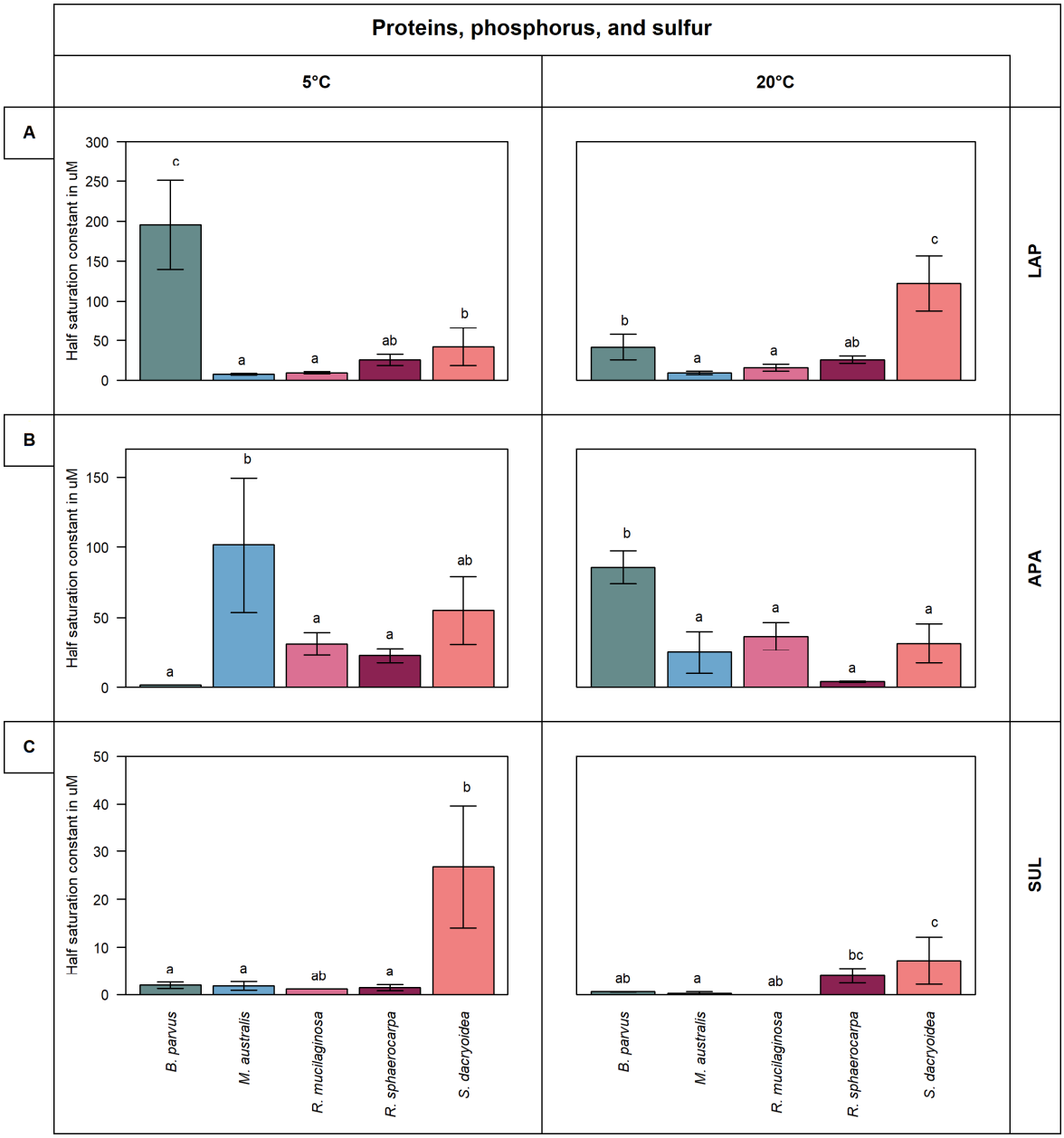

Figure II-4. K_m in µM obtained from the total enzymatic activity for the substrates representing proteins, phosphorus, and sulfur, such as (**A**) leucine aminopeptidase (LAP), (**B**) alkaline phosphatase (APA), and (**C**) sulfatase (SUL) of the five marine fungal isolates (*B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*). Measurements were performed at 5°C and 20°C in the exponential phase. According to Tukey's HSD, bars denoted by a different letter (a, b, and c) are significantly different ($p < 0.05$), whereas bars denoted by a common letter (ab and bc) are not significantly different.

Figure II-5. Q_{10} of the normalized total enzymatic activity (V_{max}) with the biomass (dry weight) for the substrates β-glucosidase (BGL), β-xylosidase (BXY), *N*-acetyl-β-D-glucosaminidase (NAG), leucine aminopeptidase (LAP), alkaline phosphatase (APA), and sulfatase (SUL) and the species *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*.

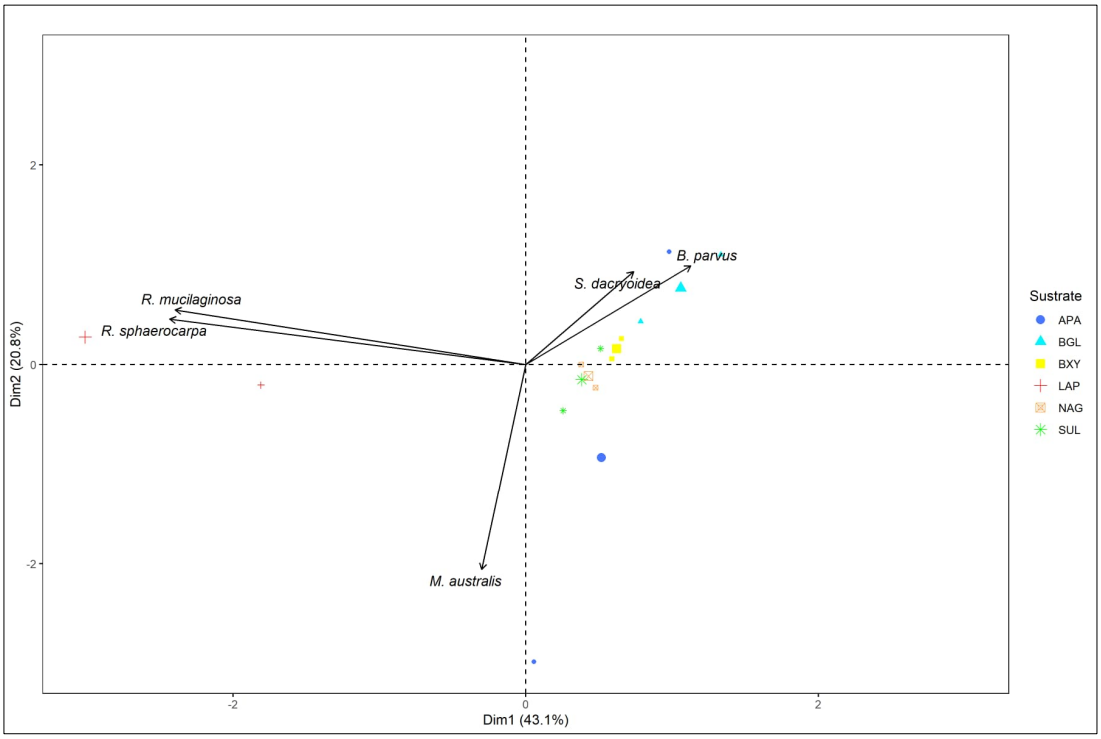

Figure II-6. PCA plot from the normalization of the total enzymatic activity (V_{max}) with the

biomass (dry weight) at 5°C and 20°C for all the species. This was compared for each substrate, which corresponds to alkaline phosphatase (APA), β-glucosidase (BGL), β-xylosidase (BXY), leucine aminopeptidase (LAP), *N*-acetyl-β-D-glucosaminidase (NAG), and sulfatase (SUL).

3.1. Carbohydrate active enzymes

3.1.1. β-Glucosidase (BGL)

S. dacryoidea exhibited a significantly higher V_{max} for BGL than the other fungal strains *(t-test*; $p < 0.001$) at 5°C (6.4 ± 3.2 µmol/g biomass/h) and 20°C (7.4 ± 3.0 µmol/g biomass/h) (Figure II-1A). The other fungal strains exhibited generally low BGL activity, particularly at 5°C. At 20°C, the BGL activity was slightly higher than at 5°C, except for *R. mucilaginosa*, which maintained a low BGL activity at both temperatures. Consequently, V_{max} was a species- and temperature-specific value, with Q_{10} values varying between 1.4 and 2.4, except for *R. mucilaginosa* (Figure II-5).

The K_m was significantly higher in *S. dacryoidea* than in the other fungal strains *(t-test*; $p < 0.001$) and ranged between 139.6 ± 19.1 µM and 78.6 ± 11.9 µM (Figure II-3A). Moreover, the K_m of *S. dacryoidea* was significantly higher at 20°C than at 5°C (*t-test*; $p < 0.001$). Nonetheless, the other two species of the phylum Basidiomycota, *R. sphaerocarpa* and *R. mucilaginosa*, showed low K_m values, specially the latter. Finally, for the Ascomycota species, the K_m was highest at 5°C (*t-test*; $p = 0.01$) with 12.9 ± 3.4 µM for *B. parvus* and 38.7 ± 14.6 for *M. australis*.

3.1.2. β-Xylosidase (BXY)

All fungal strains tested were capable to cleave xylose, however, at low rates (Figure II-1B). *B. parvus* exhibited a significantly higher V_{max} for BXY than the other strains (*t-test*; $p < 0.001$). In *B. parvus*, the V_{max} was 0.8 ± 0.3 µmol/g biomass/h at 5°C and 1.0 ±

0.5 µmol/g biomass/h at 20°C. In the other strains, the hydrolysis rates were higher at 20°C than at 5°C, amounting to 0.5 and 0.1 µmol/g biomass/h, respectively. When all the fungal species were compared, the temperature had a greater effect in *M. australis* and *S. dacryoidea* with Q_{10} values of 2.1 and 1.8, respectively, than in the other fungal strains (Figure II-5). For *B. parvus* and both species of the genus *Rhodotorula*, Q_{10} values close to 1 suggested that V_{max} was independent of the temperature.

The K_m varied significantly between both temperatures (*t-test*; p= 0.90) (Figure II-3B). At 5°C, *S. dacryoidea* showed a high K_m (49.7 ± 27.6 µM) followed by *B. parvus* with a K_m value of 37.2 ± 8.5 µM. In contrast, at 20°C, the K_m decreased in both *B. parvus* and *S. dacryoidea* but increased in *M. australis*. The other two species, *R. sphaerocarpa* and *R. mucilaginosa*, showed low K_m values corresponding to their low V_{max}.

3.1.3. *N*-acetyl-β-D-glucosaminidase (NAG)

B. parvus exhibited similar NAG hydrolysis rates at 5°C and 20°C (*t-test*; p= 0.39) (Figure II-1C). At 5°C, the V_{max} was 0.8 ± 0.4 µmol/g biomass/h and at 20°C 0.7 ± 0.2 µmol/g biomass/h. Although the other strains exhibited low NAG enzymatic activity, it increased from 5°C to 20°C, particularly in *M. australis* and *S. dacryoidea*. In *M. australis* the V_{max} increased from 0.1 ± 0.08 µmol/g biomass/h to 0.5 ± 0.2 µmol/g biomass/h and in *S. dacryoidea* from 0.04 ± 0.02 µmol/g biomass/h to 0.1 ± 0.06 µmol/g biomass/h. The Q_{10} values for NAG in *M. australis* and *S. dacryoidea* were 2.1 and 1.1, respectively (Figure II-5).

The high NAG enzymatic activity detected in *B. parvus* coincided with a high K_m (Figure II-3C). At 5°C, the K_m was 2.9 ± 1.8 µM, while at 20°C it was 12.1 ± 5.6 µM. At 20°C, the K_m of *M. australis* increased to 8.7 ± 4.6 µM. Thus, the K_m values of these two Ascomycota species increased with temperature, but they remained low in the three Basidiomycota species.

3.2. Extracellular enzymes targeting proteins, phosphorus, and sulfur compounds

3.2.1. Leucine aminopeptidase (LAP)

Generally, the LAP activity was higher at 20°C than at 5°C in all the fungal strains (*t-test*; $p < 0.001$), except in *M. australis* (*t-test*; $p = 0.46$) (Figure II-2A). The LAP activity in *R. sphaerocarpa* at 20°C was 4.3 ± 1.5 µmol/g biomass/h; however, it was only 1.1 ± 0.2 µmol/g biomass/h (*t-test*; $p < 0.001$) at 5°C resulting in a Q_{10} of 6.1 (Figure II-5). All the other fungal strains had Q_{10} values ranging from 1.9 to 1.1. Thus, the LAP activity was the only extracellular enzymatic activity tested that showed a temperature dependency in all the fungal strains (Figure II-5).

B. parvus and *S. dacryoidea* exhibited significantly higher K_m values than the other fungal strains (*t-test*; $p < 0.001$) (Figure II-4A). The K_m values varied between 195.4 µM and 41.6 µM in *B. parvus* and between 122.3 µM and 42.2 µM in *S. dacryoidea*. While in *B. parvus*, the K_m decreased with increasing temperature, and in *S. dacryoidea*, the K_m value incremented with increasing temperature.

3.2.2. Alkaline phosphatase (APA)

At 5°C, *M. australis* exhibited a significantly higher V_{max} (8.8 ± 3.7 µmol/g biomass/h) than the other fungal isolates (*t-test*; $p = 0.03$) (Figure II-2B). At 20°C, however, the V_{max} was about 10-fold lower than at 5°C (0.6 ± 0.2 µmol/g biomass/h). In contrast, in all the other fungal strains, V_{max} slightly increased with temperature (Figure II-2B). The Q_{10} values ranging from 2.6 to 1.0 indicated a moderate temperature dependency of APA in all the fungal strains examined, except in *M. australis* (Figure II-5).

The K_m was significantly higher in the Ascomycota than in the Basidiomycota strains (*t-test*; $p = 0.01$) (Figure II-4B). *M. australis* exhibited a higher K_m at 5°C (101.5 ± 43.5 µM) than at 20°C, whereas the K_m in *B. parvus* was higher at 20°C (85.7 ± 10.5 µM) than at 5°C. The other fungal species belonging to the Basidiomycota phylum exhibited K_m

values ranging from 55.1 µM to 4.1 µM (Figure II-4B).

3.2.3. Sulfatase (SUL)

Sulfatase (SUL) activity was generally low compared to the other extracellular enzymatic activities (Figure II-2C). Higher V_{max} values were determined for *B. parvus* and *S. dacryoidea* at 5°C (1.1 and 0.9 µmol/g biomass/h, respectively) (*t-test*; $p = 0.01$) than at 20°C (0.4 and 0.3 µmol/g biomass/h, respectively). In contrast, for *M. australis*, *R. mucilaginosa*, and *R. sphaerocarpa*, the SUL activity increased with temperature. The Q_{10} value in *M. australis* was 1.9 while all the other fungal strains were <1.0 (Figure II-5). With the exception of *R. sphaerocarpa*, all the fungal strains exhibited higher K_m values at 5°C than at 20°C (*t-test*; $p \leq 0.002$) (Figure II-4C).

3.3. Relation between enzyme kinetic parameters, enzyme types, and phylogeny

PCA analysis allowed for the comparison of the five studied marine fungal species and the different extracellular enzymatic activities determined. The explained variance of the dataset was 63.9% (Figure II-6). The minor angle between *R. mucilaginosa* and *R. sphaerocarpa* belonging to the same genera (*Rhodotorula*) indicated very similar activity levels and extracellular enzyme characteristics. In contrast, the other fungal strains substantially differed in their extracellular enzymatic activity and enzyme characteristics, independent of their taxonomic affiliation.

4. Discussion

All the studied marine pelagic fungi species, *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*, produced extracellular enzymes to degrade substrate analogues of carbohydrates such as cellulose, chitin, and xylan. Additionally, they all produced enzymes to cleave off amino acids, phosphate, and sulfate

esters from organic compounds.

4.1. Extracellular enzymatic activities of pelagic fungal isolates

4.1.1. Influence of taxonomy/diversity on the different EEAs

Since each organism has specific enzymatic capabilities and substrate preferences (Arnosti, et al. 2014), extracellular enzymatic activities (EEA) can be used as functional traits to investigate functional diversity (Cullings and Courty 2009). Hence, the different EEAs detected in the studied marine fungi can be used to infer their influence on marine ecological processes. Interestingly, we found that the two species belonging to the genus *Rhodotorula* (*R. mucilaginosa* and *R. sphaerocarpa*) exhibited similar kinetic parameters (V_{max} and K_m) for the majority of extracellular enzymes (Figures II-1, II-2, II-3, II-4, and II-6). This might indicate some degree of trait conservation among organisms on the genus level, although more species of this genus need to be investigated before a firm conclusion can be drawn. Differences were observed, however, in the EEAs of all the other fungal strains.

Polysaccharides, as the most abundant organic compound class, but also the most complex one, require a wide range of enzymes to degrade them (Helbert 2017; Metreveli, et al. 2021). We measured three EEAs responsible for the cleavage of cellulose, xylan, and chitin (Figure II-1). In terrestrial environments, the plant cell wall is composed mainly of cellulose and protected by lignin (Houtman and Atalla 1995). In marine ecosystems, cellulose is present in the algae cell wall and covered by distinct polymers (Domozych, et al. 2012). Nonetheless, as marine cellulose is more accessible, but less frequent than other substrates, only specific microorganisms are capable of degrading cellulose (Arora 1991). Some studies have reported cellulose degradation by marine fungi like *Arthrinium saccharicola* (Hong, et al. 2015) and *Lulworthia floridana* (Meyers and Scott 1968). Other studies have also described cellulose hydrolysis by wider distributed fungal species, for

instance, *Aspergillus niger* (Baraldo Junior, et al. 2014) and *Trichoderma virens* (Ab Wahab, et al. 2019). Vaz, et al. (2011) showed that 76% of the studied marine fungi exhibit cellulolytic activity. In this case, even though all the marine fungal species used were able to cleave cellulose, S. dacryoidea dominated this EEA (Figure II-1A). Hudson (1980) stated that each fungi species have different capacities to decompose cellulose due to diverse enzymatic machinery. *S. dacryoidea* exhibited a high K_m indicating a low affinity to the substrate (Huitema and Horsman 2018). This also suggests that, even though the overall enzymatic activity is high, the substrate dissociates easily from the enzyme (Roussel 2012).

Xylan is a polysaccharide formed by residual monosaccharides called xylose (dos Santos, et al. 2016). Similar to cellulose, in terrestrial environments, xylan can be found in plants (Balabanova, et al. 2018), whereas in the ocean, xylan can be present in algae (Goddard-Borger, et al. 2012; Synytsya, et al. 2015). Fungi can degrade xylose via the oxidoreductase pathway (Domingues, et al. 2021) as it is a primary carbon source (Collins, et al. 2005). Even though the general EEA was low (Figure II-1B), we can deduce that the studied marine fungi are capable of releasing enzymes related to the hydrolysis of xylose. Raghukumar, et al. (1994) identified low xylanase activity rates of fungal coastal strains. Duarte, et al. (2013) also reported xylanase activity of Antarctic fungal strains but highlighted a higher activity of Basidiomycota over Ascomycota strains. In this study, we could not identify a clear difference between these two phyla. Nonetheless, the low K_m suggests a high affinity of the enzyme to the substrate at low concentrations (Figure II-3B). Thus, it seems likely that pelagic marine fungi might use xylose as a carbon source even when present at low concentrations.

Chitin is one of the most abundant naturally occurring polysaccharides, and it is an essential component of the cell wall of fungi, the exoskeleton of arthropods, the radula of mollusks, and the beak of cephalopods (Ruiz-Herrera 1991; Numata and Kaplan 2011). Although the overall chitinase activity was low, we can infer that the studied marine fungi

are capable of degrading chitin (Figure II-1C). The chitin degradation by marine fungi has been reported in species such as *Lecanicillium muscarium* (Fenice 2016) and *Verticillium lecanii* (Fenice, et al. 1998). Interestingly, the number of fungal enzymes involved in the degradation of chitin is related to the fungal chitin content, which varies strongly between fungal species and is dependent on the growth mode (Hartl, et al. 2012). For instance, the hyphae-like fungal cell wall consists of 10 to 20% of chitin (Ruiz-Herrera 1991), whereas yeast-like fungi have a rather low chitin content of 0.5 to 5% (Garcia-Rubio, et al. 2020). The filamentous fungus *B. parvus* exhibited high chitinase activity (Figure II-1C). Moreover, the low K_m obtained for this species suggests that only a low substrate concentration is needed to saturate its chitinase. Thus, pelagic marine fungi likely use chitin as a carbon source even when present at only low concentrations.

Leucine aminopeptidase is a critical biological enzyme due to its key role in the degradation of proteins (Burley, et al. 1990). In the present study, this enzymatic activity was different for each fungal species. Despite the majority of microbial LAP being intracellular, extracellular enzymes have been reported in filamentous fungi (Nampoothiri, et al. 2005). All the species we tested showed LAP activity with *R. sphaerocarpa* exhibiting the highest V_{max}. The substrate concentration needed to achieve half V_{max} varied among species (Figure II-4A). Although *B. parvus* had one of the lowest V_{max}, its K_m was the highest (Figures II-2A and II-4A). In contrast, *R. sphaerocarpa* exhibited high substrate affinity and a low K_m (Figure II-4A) Thus, as the kinetics for LAP varied among the fungal species, the protein hydrolysis in the ocean might be species dependent.

Inorganic phosphate (Pi) is the preferred phosphorus source for microbial uptake. In surface waters, however, Pi frequently limits phytoplankton productivity (Karl 2000). To overcome this P-limitation, microorganisms use dissolved organic phosphorus (DOP) (Srivastava, et al. 2021). For prokaryotic microorganisms, Baltar, et al. (2016) reported that irrespective of the phosphate bioavailability, the activation of alkaline phosphatase was

related to sporadic pulses of organic matter. Thus, a high K_m might be beneficial to allow for high cleavage rates when organic substrate availability is high. The species *R. sphaerocarpa*, *R. mucilaginosa*, and *S. dacryoidea*, belonging to the phylum Basidiomycota, exhibited a low enzymatic activity but a high K_m (Figures II-2B and II-4B). In contrast, the species of the phylum Ascomycota (*B. parvus* and *M. australis*) seem to be more suitable to overcome this P-limitation, but a higher amount of substrate, and hence of organic matter, might be needed for this purpose.

In living organisms, sulfur is the sixth most abundant element as it can be found in amino acids, such as cysteine and methionine, but also in polysaccharides and proteoglycans (Helbert 2017). In contrast to terrestrial polysaccharides, several marine polysaccharides, specially in the cell wall of macroalgae, are highly sulfated (Helbert 2017). The terrestrial fungus *Fusarium proliferatum* was reported to produce sulfatase for the assimilation of sulfated fucoidans of brown algae (Shvetsova, et al. 2015). In the present study, the species of the genus *Rhodotorula* maintained a low sulfatase activity, whereas it varied among the other species. The V_{max} and K_m were high in *S. dacryoidea*, indicating fast hydrolysis and low substrate affinity. As all the fungal species showed different sulfatase activity, we can deduce that these marine fungi might be capable to use sulfated amino acids as well as carbohydrates.

In this study, even though we analyzed just a few hydrolysis possibilities, the functional diversity seems to be broad in marine fungi. As suggested by Berlemont (2017) and Baltar, et al. (2021), marine fungi are potentially involved in carbohydrates' degradation. Based on our results, we can infer that marine pelagic fungi are actively utilizing carbohydrates such as cellulose, chitin, and xylose, as well as some carbohydrates with sulfur content, potentially of algal origin (Balabanova, et al. 2018).

The ocean is a complex and diverse ecosystem composed of microorganisms such as archaea, bacteria, fungi, protists, and viruses. As osmoheterotrophic organisms, fungi

might provide intermediate decomposition products needed for other microorganisms. These also might lead to the proliferation or inhibition of other microbes as well as enzymatic activities. As marine fungi can utilize a wide range of organic substrates (Loque, et al. 2010), nutrient availability can impact the magnitude and distribution of extracellular enzymatic activities (Arnosti, et al. 2014).

4.1.2. Temperature influence

Temperature is considered one of the most important abiotic factors because it influences essentially all biochemical reactions (Georlette, et al. 2004). Enzymes are sensitive to temperature (Roussel 2012) influencing the kinetics along with the substrate binding property and stability (John, et al. 2002; Arnosti, et al. 2014). According to the Van't Hoff rule, a temperature increase of 10°C can double a reaction rate. Q_{10} values lower than 1.0 would indicate a reaction rate completely independent of temperature, whereas values above 1 indicate thermodependency (Collins, et al. 2007).

The results obtained in this study indicate that the majority of enzymatic activities were lower at 5°C than at 20°C (Figures II-1 and II-2). In general terms, at 5°C the species *R. mucilaginosa* and *R. sphaerocarpa* maintained V_{max} values as low as 0.1 µmol/g biomass/h, whereas the other species exhibited only half of the activity at 5°C, particularly for BGL and APA (Figures II-1 and II-2). This reduced enzymatic synthesis at a low temperature might be due to limited transcriptional and translational activity, limited protein folding, and DNA and RNA secondary structures' stabilization (D'Amico, et al. 2006). *B. parvus*, for all the substrates except APA, had a higher K_m at 5°C, which suggests that the affinity of this species for some substrates increases with increasing temperature. Taken together, the effect of temperature on the characteristics of extracellular enzymes depends on the fungal species and the type of enzyme.

Psychrophiles microorganisms have evolved a complex range of adaptation

strategies, such as production of antifreeze proteins (Kawahara, et al. 2007) and exopolysaccharides (EPS) (Krembs, et al. 2002), high levels of unsaturated fatty acids to maintain the membrane fluidity (Los and Murata 2004), and certain enzymes adapted to those temperatures (D'Amico, et al. 2006). Gerday, et al. (2000) described a peculiar type of extracellular enzymes known as "cold-adapted enzymes" produced by microorganisms living at low temperatures. For these enzymes, the reaction rate is dependent on the encounter rate of the enzyme and substrate, so it is controlled mainly by diffusion, and it is temperature independent (Feller and Gerday 1997, 2003).

Aghajari, et al. (1996) suggested that the main structural feature of these "cold-adapted enzymes" is flexibility or plasticity. The structures involved in the catalytic cycle are more flexible, whereas other structures that do not participate in the catalytic cycle might be more rigid (Georlette, et al. 2004; Truongvan, et al. 2016). For instance, the chitinase of *Glaciozyma antarctica* presented fewer salt bridges and hydrogen bonds, which increased its flexibility (Ramli, et al. 2012; Yusof, et al. 2021). Another key structural feature of these enzymes is stability (Feller and Gerday 1997; Gerday, et al. 2000), with for example, amino acids modifications in key regions of the protein (Ramli, et al. 2012; DasSarma, et al. 2013; Michetti, et al. 2017; Baeza, et al. 2021). Nonetheless, there is not a single strategy, as each cold-adapted enzyme can perform different ways to enhance its activity at low temperatures (Feller and Gerday 1997; Yusof, et al. 2021).

Cold-adapted enzymes have been reported from a wide variety of marine fungi (Fenice, et al. 1998; Vaz, et al. 2011; Ramli, et al. 2012; Duarte, et al. 2013; Baharudin, et al. 2014; Fenice 2016; Duarte, et al. 2018; Yusof, et al. 2021). *M. australis* and *R. sphaerocarpa* were one of the few species that showed a noticeable enzymatic activity at 5°C for APA and SUL, respectively (Figures II-2B and II-2C). *M. australis* is an endemic species of Antarctic waters (Fell and Hunter 1968; Fell and Pitt 1969), whereas *R. sphaerocarpa* was originally isolated close to Marguerite Bay on the west side of the

Antarctic Peninsula but has a wider distribution including the Caribbean Sea (Van Uden and Fell 1968) and the Andaman Sea (Hoondee, et al. 2019), among others. Low temperatures exert high selective pressure on endemic organisms (Gerday, et al. 1997), such as alkalinity phosphatase in *M. australis*. For this species, the substrate-binding affinity was lower at 5°C, whereas for *R. sphaerocarpa*, K_m was lower at this temperature (Figure II-4). This suggested that the enzyme–substrate complex of *M. australis* APA is more effective at higher substrate concentration typical for Antarctic waters known as a major high-nutrient low-chlorophyll region in the global ocean (Dugdale and Wilkerson 1991).

Fungal cold-adapted chitinases have been previously reported (Reyes, et al. 1989; Plíhal, et al. 2007; Ramli, et al. 2012). Ramli, et al. (2012) found that the chitinase sequence of *G. antarctica* had a low sequence identity with other chitinases. Moreover, they found that the enzyme flexibility was due to certain amino acids substitutions in the surface and loop regions. In this study, at 5°C, we could only identify a higher chitinase activity for *B. parvus*. For the rest of the species, there was a positive enzymatic activity, but higher at 20°C.

Microorganisms isolated from cold environments can also display kinetic parameters similar to those of their mesophilic counterparts (Collins, et al. 2007; Santiago, et al. 2016). Ito, et al. (2015) deduced that a high Q_{10} value (>2) is due to a conformational change in proteins and indicates the need for high activation energy. We found that only for LAP, all the examined fungal species expressed Q_{10} values higher than 1. Generally, the temperature where an enzyme can achieve its highest activity does not match the optimal growth temperature of the microorganism that is producing it (Baeza, et al. 2021). Apparently, cold-adapted species, such as *B. parvus*, *M. australis*, *R. sphaerocarpa*, and *S. dacryoidea*, can respond to a temperature rise by increasing enzymatic activity. According to the Arrhenius equation, temperature can influence the activation energy needed to initiate a chemical reaction, and hence, its rate. At a higher temperature, the molecules gain energy

to move faster, which also increases the collisions between enzymes and substrates. As a result, elevated extracellular enzymatic activity at increasing temperatures in the surface waters might lead to changes in cleavage and uptake rate of organic matter in oceanic fungi.

5. Conclusions

In the present study, we have shown that different marine fungal strains exhibit varying extracellular enzyme characteristics with V_{max} and K_m values varying over a range of one order of magnitude. Although the fungal species were isolated from coastal Antarctic waters (*B. parvus*, *M. australis*, *R. sphaerocarpa*, and *S. dacryoidea*), and hence are potentially adapted to low temperatures, they exhibited higher extracellular enzymatic activity at 20°C than at 5°C, with some exceptions. While in some fungal strains the K_m values for specific extracellular enzymes were higher at low temperatures, for other enzymes they were lower. Additionally, there was considerable species-specific variability in the extracellular enzymatic activity. Taken together, our study indicates that temperature might be one of most important physical factors controlling marine fungal extracellular enzymatic activity. Thus, species composition and temperature determine the role of marine fungi in organic matter cleavage in the global ocean.

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Chapter III: Influence of salinity on the extracellular enzymatic activities of marine pelagic fungi

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In revision

Abstract

Even though fungi are ubiquitous in the biosphere, the knowledge of the ecology of marine fungi remains rather rudimentary. Extracellular enzymatic activities (EEAs) measurements are widely used to determine the enzymatic capabilities and substrate preferences of heterotrophic microbes. Five marine fungal species belonging to the most abundant pelagic phyla (Ascomycota and Basidiomycota) were grown under non-saline and saline conditions (0 g/L and 35 g/L, respectively). Using eight fluorogenic substrates analogues, enzymatic assays were performed and kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m) were calculated. Substrate analogues were used to determine hydrolytic activity on carbohydrates (β-glucosidase, β-xylosidase, and *N*-acetyl-β-D-glucosaminidase); peptides (leucine aminopeptidase, and trypsin); lipids (lipase); organic phosphorus (alkaline phosphatase) and sulfur compounds (sulfatase). All fungal species investigated were capable to cleave these substrates. The majority of enzymatic activities were reduced in the saline medium, with some exceptions like sulfatase. Moreover, some species were more capable to cleave specific substrates than others. Taken together, our results highlight a strong tolerance of marine fungi to freshwater conditions and indicate that changes in salinity due to freshwater input or evaporation might impact the enzymatic activities of marine fungi and therefore their contribution to the oceanic elemental cycles.

1. Introduction

Marine fungi can live obligatorily or facultatively in saline habitats (Jennings 1983). The obligate fungi grow only in marine environments, whereas facultative marine fungi are species of terrestrial origin that can also grow in marine environments (Raghukumar 2017). Compared to their terrestrial and freshwater counterparts, marine fungi need to tolerate high salinity and limited substrate availability (Ruisi, et al. 2007; Shivaji and Prasad 2009; Gladfelter, et al. 2019). Therefore, marine fungi might have developed unique strategies to survive and thrive in this environment.

Marine fungi have been reported to be abundant in deep water marine snow (Bochdansky, et al. 2017), in subseafloor sediments (Orsi, et al. 2013), and in coastal habitats (Ueda, et al. 2015; Picard 2017). Additionally, marine fungi can be parasites of phytoplankton (Hanic, et al. 2009; Lepelletier, et al. 2014; Hassett and Gradinger 2016; Jephcott, et al. 2016; Scholz, Küpper, et al. 2017; Scholz, Vyverman, et al. 2017) and metazoans (Polglase 1980; McLean and Porter 1982; Bower 1987; Shields 1990; Rahimian 1998). In land-sea transition zones, the role of marine fungi is similar to their terrestrial counterparts acting as decomposers, pathogens, and mutualistic symbionts (Boddy 2016; Gladfelter, et al. 2019). These fungi contribute to the recycling of organic carbon, nitrogen, and phosphorus (Caruso 2010; Arnosti, et al. 2014). However, the role of fungi in the open ocean is less known. Recent studies suggest that open water fungi play an active role in marine biogeochemical cycles (Morales, et al. 2019), and that they are degrading a variety of carbohydrates (Baltar, et al. 2021). Moreover, it has been suggested that fungi are significant components of the marine microbial community involved in the breakdown of organic matter (Gutiérrez, et al. 2011).

The ecology of marine microorganisms such as fungi is crucial to reveal how they will respond to an ocean global change. The desalination of the oceans, also known as ocean freshening, is a freshwater input from ice melting, riverine, and precipitation that exceeds

the evaporation; hence, influencing the salinity of the oceans (Myers, et al. 1990; Munk 2003; Reid, et al. 2009; Hutchins and Fu 2017; Kohler, et al. 2020). Moreover, this freshwater input can intensify stratification which reduces the vertical mixing of the water column (Reid, et al. 2009; Balaguru, et al. 2016; Hutchins and Fu 2017). Drastic salinity changes lead to osmotic and ionic stress on the organism (Gladfelter, et al. 2019). Even though most marine fungi are adapted to tolerate high salinity (Jennings 1983), they might not be halophilic (Gladfelter, et al. 2019). Thus, it remains enigmatic how marine fungi might be influenced by salinity changes.

Proteins are categorized based on their role, hence, as active or structural (Yusof, et al. 2021). Enzymes are active proteins operating as biological catalysts and controlling chemical reactions (Cooper 2000; Yusof, et al. 2021). To acquire critical nutrients, fungi as osmotrophs synthesize different enzymes to break down high molecular weight polymers into smaller monomers that can then be absorbed by the cell wall (Arnosti 2011; Richards, et al. 2012; Muszewska, et al. 2017). The enzymatic capabilities and substrate preferences differ between organisms (Arnosti, et al. 2014). Thus, the study of extracellular enzymatic activities (EEAs) can be used as a functional trait to investigate the potential functional diversity of organisms (Cullings and Courty 2009).

Microbial enzymes are crucial in organic matter processing in the ocean (Caruso and Zaccone 2000). Here we investigated the enzymatic capability of five marine fungi species; two belonging to the phylum Ascomycota, and three to Basidiomycota, the two dominant pelagic fungal phyla (Taylor and Cunliffe 2016; Amend, et al. 2019; Morales, et al. 2019). Moreover, we compared the effect of salinity on their enzymatic repertoire using non-saline and saline media to represent freshwater and the average salinity of the global ocean.

2. Methods

2.1. Culture of fungal species

Blastobotrys parvus (HA 1620), *Metschnikowia australis* (HA 635), *Rhodotorula sphaerocarpa* (HB 738), and *Sakaguchia dacryoidea* (HB 877) were obtained from the Austrian Center of Biological Resources (ACBR). These species were originally isolated from the Antarctic Ocean at salinities of 34.23 (Fell and Statzell 1971), 32.48 to 36.42 (Newell and Fell 1970), N/A (Fell and Hunter 1968), and 34.72, respectively (Fell, et al. 1973). The species *Rhodotorula mucilaginosa* was isolated from the Atlantic Ocean during the Poseidon Cruise in March 2019 at a salinity of 36.97.

Two media were prepared, both containing 2 g/L of glucose, malt extract, peptone, yeast extract, and 0.5 g/L of chloramphenicol. To one medium, 35 g/L of artificial sea salts (S9883 Sigma-Aldrich) were added, whereas to the other medium, no salts were added (0 g/L). Pure isolates cultured on yeast malt extract agar for one week (Wickerham 1939; Wickerham 1951) were diluted in artificial seawater (35 g/L sea salts S9883 Sigma-Aldrich) to achieve an $OD_{660} \approx 1$ (Salazar Alekseyeva, et al. 2021). The optical density at 660 nm wavelength (OD_{660}) was measured with a UV-1800 Shimadzu spectrophotometer, and 1 mL of this diluted fungal culture was added to 100 mL of autoclaved medium. Afterwards, 150 mL of this medium containing fungi were filled in Schott bottles and incubated in triplicate at 5°C on a rotary shaker (Jeio Tech ISS-7100 Incubated Shaker). The growth of the cultures was measured daily via OD_{660} and once the exponential phase was reached, samples with similar OD_{660} values were chosen in triplicate for further analyses (EEAs and biomass).

2.2. Measuring extracellular enzymatic activity and fungal biomass determination

The hydrolysis of the fluorogenic substrate analogues such as 4-methylumbelliferyl β-D-glucopyranoside (M3633 Sigma-Aldrich), 4-methylumbelliferyl β-D-xylopyranoside (M7008 Sigma-Aldrich), and 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (M2133

Sigma-Aldrich) was used to determine the potential activity of the enzymes β-glucosidase (BGL), β-xylosidase (BXY), and *N*-acetyl-β-D-glucosaminidase (NAG) respectively (Table III-1). As these enzymes are capable of hydrolyzing part of cellulose (Pointing, et al. 1998), chitin, and xylan (Sinsabaugh and Moorhead 1994; Collins, et al. 2005; dos Santos, et al. 2016; Ravn, et al. 2021), respectively, they were used as a potential indicator of carbohydrate cleavage. For the potential enzymatic activity of lipase (OLE), alkaline phosphatase (APA), and sulfatase (SUL), the fluorogenic substrate analogues 4-methylumbelliferyl-oleate (75164 Sigma-Aldrich), 4-methylumbelliferyl phosphate (M8883 Sigma-Aldrich), and 4-methylumbelliferyl sulfate potassium salt (M7133 Sigma-Aldrich), respectively were used. OLE mediates the cleavage of fatty acids (Joo, et al. 2012; Prem, et al. 2022), whereas APA and SUL are indicative of the cleavage of phosphate and sulfate esters in molecules (Hoppe 1983), respectively. The hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (L2145 Sigma-Aldrich), and t-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine-7-amido-4-methylcoumarin (3107-v PeptaNova) was used to identify the potential enzymatic activity of leucine aminopeptidase (LAP), and trypsin (TRY), both involved in the cleavage of proteins and peptides (Hoppe 1983; Dubovenko, et al. 2010). Finally, the fluorophores methylcoumaryl amide (MCA) (A9891 Sig-ma-Aldrich) and methylumbelliferone (MUF) (M1381 Sigma-Aldrich) were used to standardize the hydrolytic activity.

Table III-1. Targeted enzymes with fluorogenic substrate analogues and their respective fluorophore standards (MUF- methylumbelliferyl, MCA -methylcoumarylamide).

Type	Code	Name	Standard
Carbohydrates	BGL	β-glucosidase	MUF
	BXY	β-xylosidase	MUF
	NAG	*N*-acetyl-β-D-glucosaminidase	MUF
Lipids, phosphorus and sulfur moieties	OLE	Lipase	MUF
	APA	Alkaline phosphatase	MUF
	SUL	Sulfatase	MUF
Proteins	LAP	Leucine aminopeptidase	MCA
	TRY	Trypsin	MCA

For the enzymatic assays, sterile 96 well microplates with F bottom and low protein binding (XT64.1, Carl Roth) were used. The protocol was based on Hoppe (1983) and Salazar Alekseyeva, et al. (2022). The MCA fluorophore was dissolved in 2-methoxyethanol to a final concentration of 100 µM, 50 µM, 10 µM, and 1 µM, whereas MUF was diluted to a final concentration of 2000 µM, 1000 µM, 100 µM, and 50 µM. Both, MCA and MUF served as fluorescent standards to normalize the fluorescence obtained in the incubations of the fluorogenic substrates.

Each fluorogenic substrate was serially diluted in the microplate to obtain 12 final concentrations ranging from 1000 to 0.5 µM, except trypsin, which ranged from 500 to 0.2 µM. The emitted fluorescence was measured with FluoroLog® Horiba at an excitation wavelength of 365 nm and an emission wavelength of 445 nm. An initial measurement was

performed (T_0) followed by hourly measurements (T_1, T_2, and T_3) over 3 h. The microplates were incubated in the dark at 5°C between measurements.

To determine fungal biomass, 40 mL of the individual fungal cultures were filtered gently onto pre-weighed and combusted (450°C for 6 h) Whatman GF/F filters (WHA1825047 Sigma-Aldrich, 47 mm diameter). The filters with the collected fungal biomass were dried at 80°C for 3 d. Thereafter, the samples were weighed again, and the biomass was determined.

To determine the cell-specific biomass, 1.5 mL of sample were filtered with a pluriStrainer Mini (43-10040-50 pluriSelect, 40 µm mesh size) to obtain a single-cell suspension. The sample was fixed in the dark with 0.5% (final conc.) glutaraldehyde for 10 min, and subsequently frozen at −80°C until further processing. Due to the multicellular structures of the filamentous species *Blastobotrys parvus*, its cell abundance could not be determined. For the other species, depending on the optical density, 10 to 40 µL of the sample was diluted with TE to obtain a final volume of 500 µL which was later stained with 5 µL SYBR® Green 100x (S9430, Sigma-Aldrich). The cell abundance was determined using a BD Accuri™ C6 Plus Flow Cytometry set at 'Run with limits' of 10,000 events and 'Medium' and the cell abundance was obtained with the BD Accuri C6 Software.

2.3. Determination of the enzyme kinetics

The increased fluorescence over time in the samples with the fluorogenic substrate added was converted into hydrolysis rate [µmol L^{-1} h^{-1}] using the standard calibration with MCA and MUF. The obtained hydrolysis rates were fitted directly to the Michaelis–Menten equation using nonlinear least-squares regression analysis with R software (Huitema and Horsman 2018). The kinetic parameters maximum velocity (V_{max}) and half-saturation constant (K_m) were calculated. For the biomass-specific activity, the V_{max} was normalized to the dry weight [µmol/h/g biomass]. The cell-specific activity was obtained by normalizing

the hydrolysis rate to the cell abundance and is given in amol/h/cell.

2.4. Statistical analysis

For the kinetic parameters V_{max} and K_m, one-way Analysis of Variance (ANOVA) was used to determine whether the differences found between fungal species were significant. Tukey's Honestly Significant Difference (Tukey's HSD) was performed to identify significance at species level. Finally, Student-T test was used to test the normal distribution of the significant data.

3. Results

All the fungal strains used in this study were able to hydrolyze all the fluorogenic substrates targeting carbohydrates (Figure III-1), lipids, phosphorus and sulfur moieties (Figure III-2), and peptides (Figure III-3). The results obtained suggest that the substrate concentrations needed to achieve half of the maximum velocity were variable among the different fungal strains (Figure III-4). Generally, the extracellular enzymatic activities (EEAs) were higher in the non-saline than in the saline medium (Figure III-5).

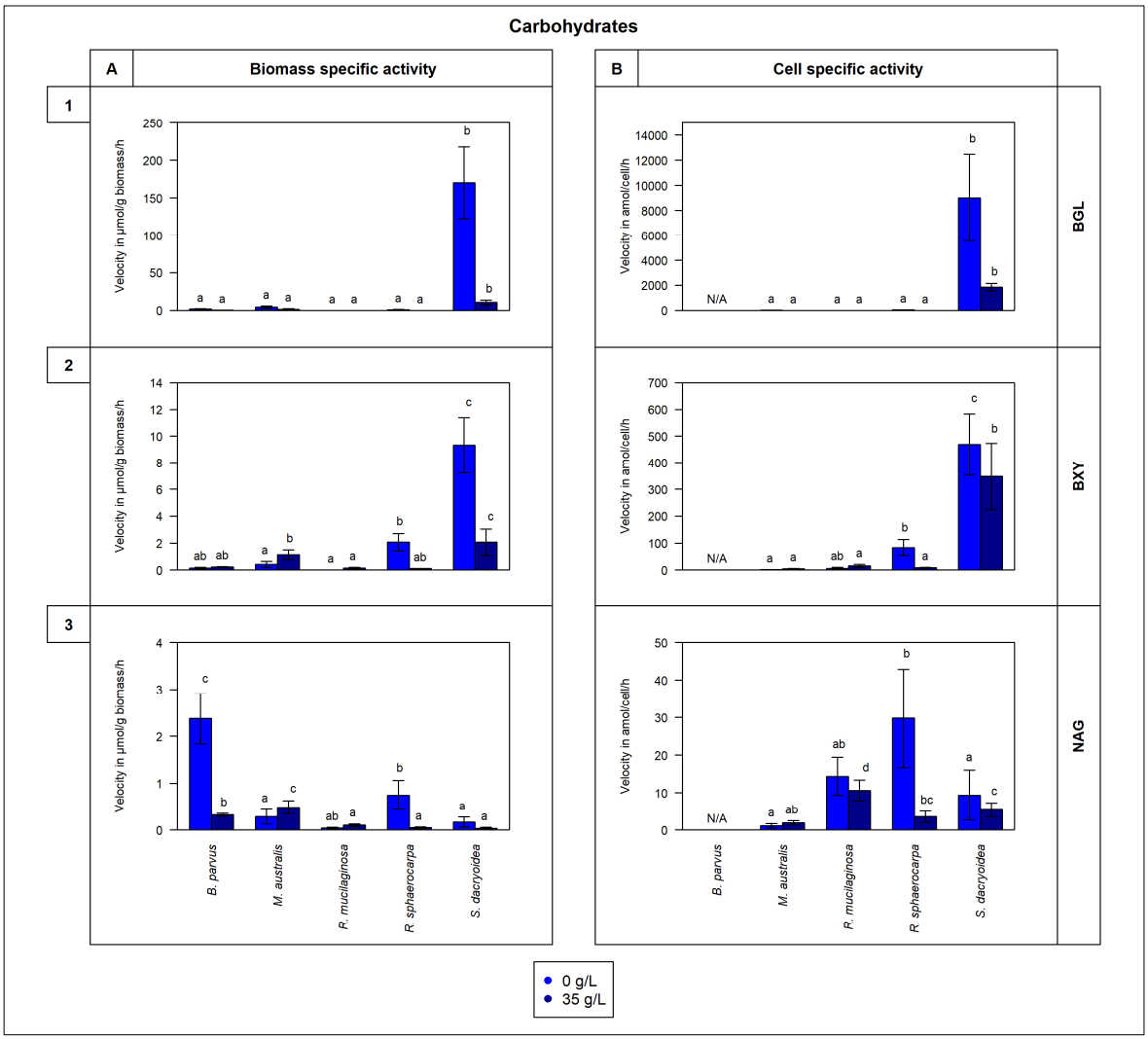

Figure III-1. V_{max} in (**A**) µmol/g biomass/h and in (**B**) amol/cell/h obtained from the total enzymatic activity of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown in non-saline (0 g/L) and saline (35 g/L) media. The substrates used represented the hydrolysis of carbohydrates by (**1**) β-glucosidase (BGL), (**2**) β-xylosidase (BXY), and (**3**) *N*-acetyl-β-D-glucosaminidase (NAG). However, due to the filamentous structure of *B. parvus*, its cell abundance was not possible to calculate. Tukey's HSD was performed to test for significance ($p < 0.05$) between the extracellular enzymatic activity in the non-saline and saline medium where bars not sharing any letter (a, b, and c) are significantly different, and bars sharing a letter (ab and bc) are not significantly different.

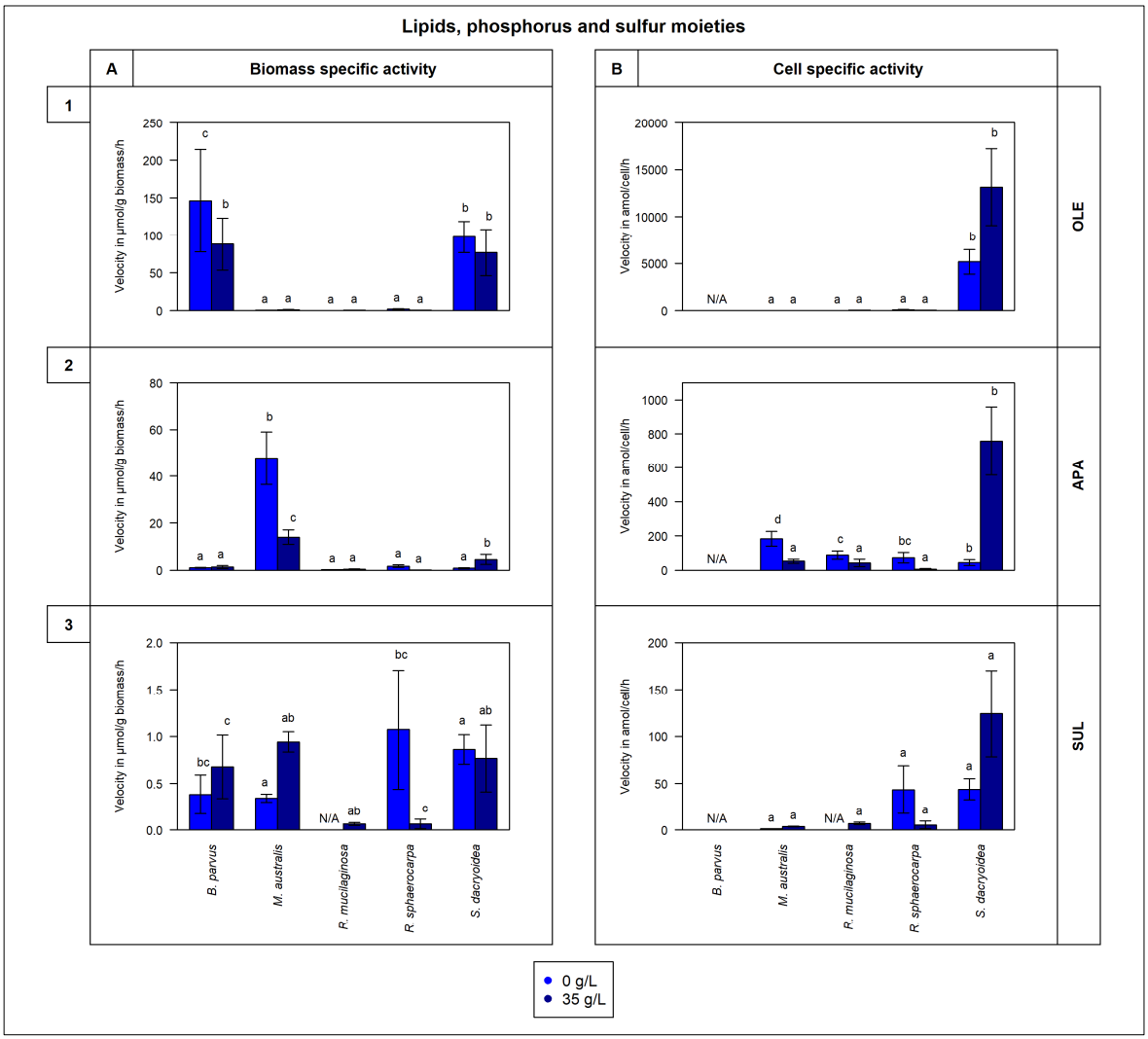

Figure III-2. V_{max} in (**A**) µmol/g biomass/h and in (**B**) amol/cell/h obtained from the total enzymatic activity of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* in non-saline (0 g/L) and saline (35 g/L) media. The substrates used represented the hydrolysis of lipids, phosphorus and sulfur moieties by (**1**) lipase (OLE), (**2**) alkaline phosphatase (APA), and (**3**) sulfatase (SUL) respectively. However, due to the filamentous structure of *B. parvus*, its cell abundance was not possible to calculate. *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, indicated by "N/A". Tukey's HSD was performed to test for significance ($p < 0.05$) between the extracellular enzymatic activity in the non-saline and saline medium where bars not sharing any letter (a, b, c, and d) are significantly different, and bars sharing a letter (ab and bc) are not significantly different.

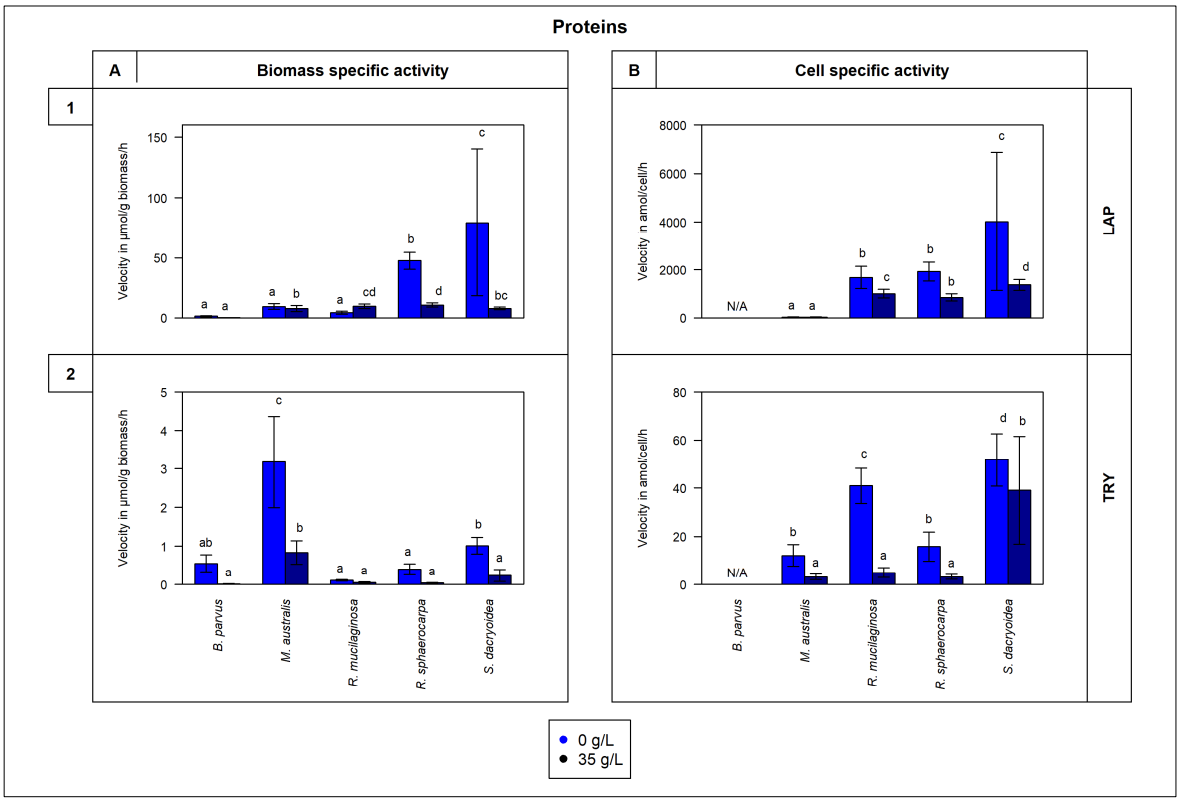

Figure III-3. V_{max} in (**A**) µmol/g biomass/h and in (**B**) amol/cell/h obtained from the total enzymatic activity of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown in non-saline (0 g/L) and saline (35 g/L) media. The substrates used represented the hydrolysis of proteins by (**1**) leucine aminopeptidase (LAP), and (**2**) trypsin (TRY). However, due to the filamentous structure of *B. parvus*, its cell abundance was not possible to calculate. Tukey's HSD was performed to test for significance ($p < 0.05$) between the extracellular enzymatic activity in the non-saline and saline medium where bars not sharing any letter (a, b, c, and d) are significantly different, and bars sharing a letter (ab, bc, and cd) are not significantly different.

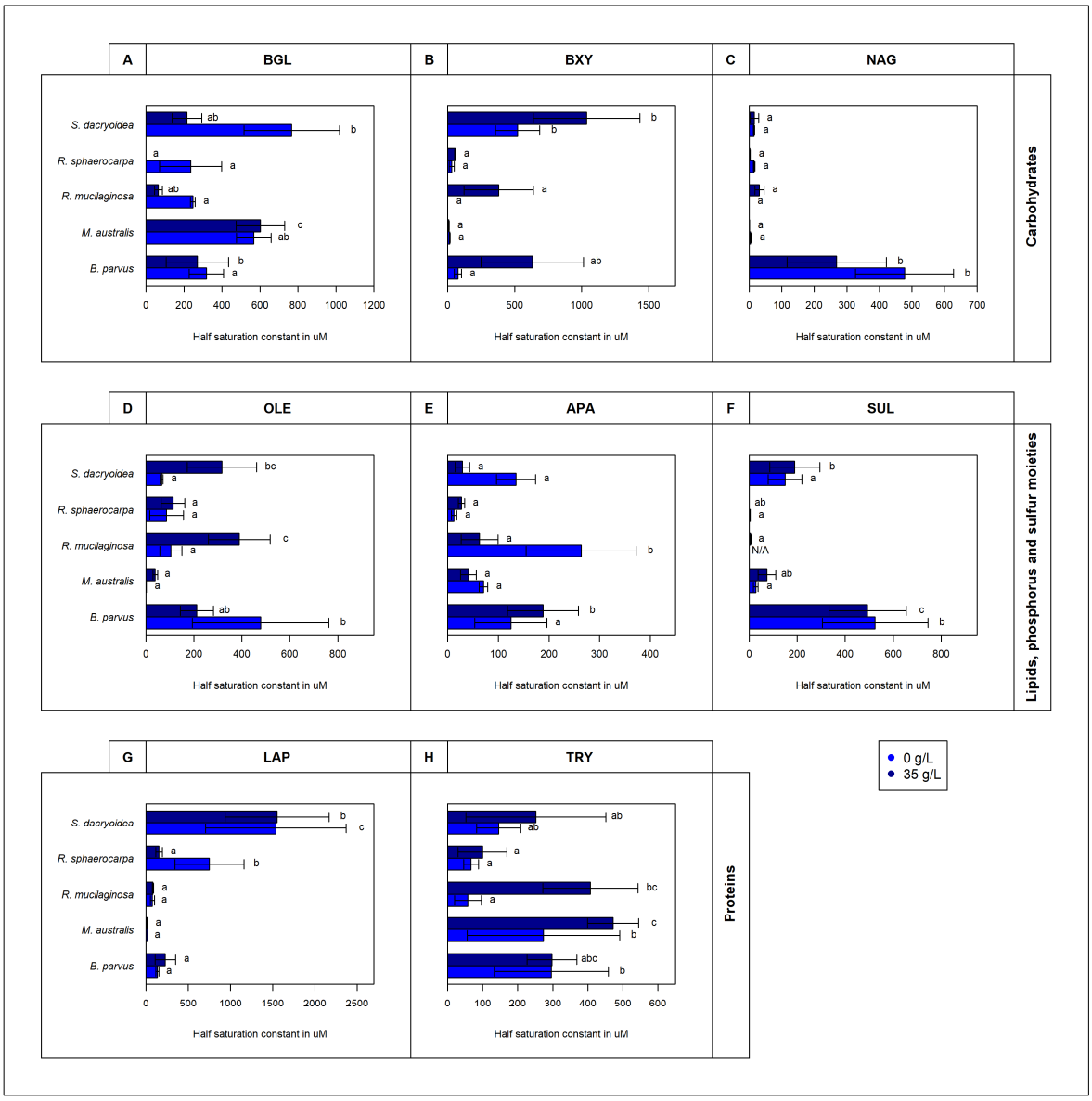

Figure III-4. Half saturation constant (K_m) in µM obtained from the total enzymatic activity of five marine fungal isolates *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown in non-saline (0 g/L) and saline (35 g/L) media, for substrates representatives of carbohydrates: (**A**) β-glucosidase (BGL), (**B**) β-xylosidase (BXY), and (**C**) *N*-acetyl-β-D-glucosaminidase (NAG); lipids, phosphorus and sulfur moieties: (**D**) lipase (OLE), (**E**) alkaline phosphatase (APA), and (**F**) sulfatase (SUL) respectively; and proteins: (**G**) leucine aminopeptidase (LAP), and (**H**) trypsin (TRY). Tukey's HSD was performed to test for significance ($p < 0.05$) between the extracellular enzymatic activity in the non-saline and saline medium where bars not sharing any letter (a, b, and c) are

significantly different ($p < 0.05$) and bars sharing a letter (ab, bc, and abc) are not significantly different.

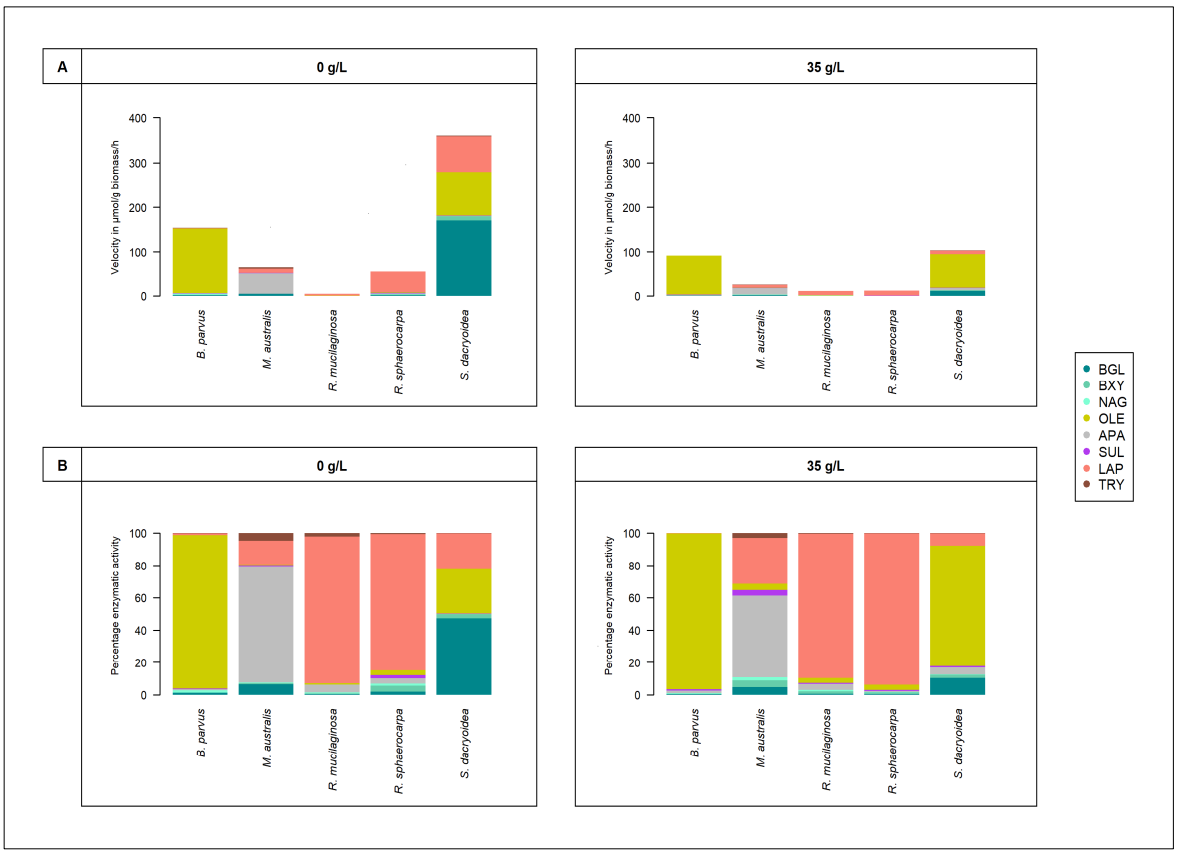

Figure III-5. Individual enzymatic contribution of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* in (**A**) µmol/g biomass/h and in (**B**) percentage for the enzymes β-glucosidase (BGL), β-xylosidase (BXY), *N*-acetyl-β-D-glucosaminidase (NAG), lipase (OLE), alkaline phosphatase (APA), sulfatase (SUL), leucine aminopeptidase (LAP), and trypsin (TRY).

3.1. Carbohydrate cleavage

3.1.1. β-glucosidase (BGL)

Compared to the other fungal species, *S. dacryoidea* exhibited a significantly higher V_{max} for BGL (*t-test*; $p < 0.001$; Figure III-1.1). This was significantly higher at a salinity of 0 g/L (170.1 ± 47.7 µmol/g biomass/h and 9015.0 ± 3453.3 amol/cell/h) than at 35 g/L (10.7

± 3.2 µmol/g biomass/h and 1853.4 ± 283.6 amol/cell/h) (*t-test*; $p < 0.001$). Even though the other fungal species exhibited a generally low BGL hydrolysis rate, this was significantly higher at a salinity of 0 g/L (*t-test*; $p < 0.001$), except for *R. mucilaginosa*. Interestingly, this was the only species that exhibited a higher BGL activity at a salinity of 35 g/L (0.07 ± 0.02 µmol/g biomass/h and 7.6 ± 2.4 amol/cell/h) compared to 0 g/L (0.02 ± 0.00 µmol/g biomass/h and 7.0 ± 1.3 amol/cell/h) (*t-test*; $p < 0.001$).

S. dacryoidea exhibited a higher BGL activity and a higher K_m (767.3 ± 251.3 µM) at a salinity of 0 g/L than all the other fungal strains tested (*t-test*; $p < 0.001$; Figure III-4A). The K_m values of the other Basidiomycota species (*R. mucilaginosa* and *R. sphaerocarpa*) were also higher in the non-saline than in the saline medium (*t-test*; p= 0.001). In contrast, there was no clear influence of salinity on the K_m of the Ascomycota strains *B. parvus* and *M. australis* (*t-test*; p= 0.2).

3.1.2. β-xylosidase (BXY)

For BXY, *S. dacryoidea* also exhibited a significantly higher V_{max} than the other fungal strains (*t-test*; $p < 0.001$; Figure III-1.2). The V_{max} of this species was significantly higher in the non-saline (9.3 ± 2.1 µmol/g biomass/h and 468.9 ± 114.8 amol/cell/h) than in the saline medium (2.1 ± 1.0 µmol/g biomass/h and 348.3 ± 124.4 amol/cell/h) (*t-test*; p= 0.01). Similarly, *R. sphaerocarpa* exhibited a significantly higher V_{max} at a salinity of 0 g/L (*t-test*; $p< 0.001$) than at 35 g/L. The two Ascomycota species *B. parvus* and *M. australis* and the Basidiomycota species *R. mucilaginosa* showed low BXY activity.

All the fungal strains, except *M. australis*, displayed a significantly higher K_m at a salinity of 35 g/L than at 0 g/L (*t-test*; p= 0.004; Figure III-4B). *S. dacryoidea* exhibited the highest K_m value (1036.3 ± 397.9 µM) at 35 g/L of all the fungal strains tested followed by *B. parvus* also at 35 g/L (631.8 ± 382.4 µM). In the non-saline medium, the K_m for *S. dacryoidea* was only about half (521.9 ± 165.7 µM) of the one determined in the saline

medium.

3.1.3. *N*-acetyl-β-D-glucosaminidase (NAG)

Even though all the fungal strains were capable to cleave chitin, the overall NAG activity rates were low (Figure III-1.3). The V_{max} of *B. parvus* at a salinity of 0 g/L (2.4 ± 0.5 μmol/g biomass/h) was significantly higher than the V_{max} of the other fungal species (*t-test*; $p < 0.001$). Also, the species of the Basidiomycota phylum, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* exhibited a higher V_{max} in the non-saline medium (*t-test*; p= 0.001), whereas *M. australis* exhibited a higher V_{max} in the saline medium (*t-test*; p= 0.009).

In the non-saline medium, *B. parvus* exhibited the highest NAG activity and with 477.4 ± 150.6 μM also the highest K_m (Figure III-4C), so it was significantly higher than the K_m for NAG of the other fungal strains (*t-test*; p= 0.004). The K_m of the other fungal strains varied between 46.7 and 0.01 μM.

3.2. Hydrolytic activity on organic compounds containing lipids, phosphorus and sulfur moieties

3.2.1. Lipase (OLE)

When normalizing OLE activity to biomass, *B. parvus* and *S. dacryoidea* exhibited significantly higher V_{max} values than the other species (*t-test*; $p < 0.001$; Figure III-2.1). Nevertheless, when OLE activity was normalized to cell-specific activity, *S. dacryoidea* exhibited the highest V_{max} of all the species (*t-test*; $p < 0.001$), specially in the saline medium (13159.9 ± 4080.3 amol/cell/h). *M. australis*, *R. mucilaginosa*, and *R. sphaerocarpa* were also capable to cleave lipids, but at much lower rates ranging from 2.9 to 0.03 μmol/g biomass/h and 127.6 to 0.5 amol/cell/h, and there was not a clear pattern on the effect of salinity (*t-test*; p= 0.1).

Generally, in all fungal strains the K_m was significantly higher in the saline than in the non-saline medium except in *B. parvus* (*t-test*; $p < 0.001$; Figure III-4D) where the K_m was significantly higher in the non-saline medium (*t-test*; $p = 0.01$), and was 478.3 ± 284.7 μM. In all the other fungal strains the K_m varied between 172.8 and 0.6 μM in the non-saline medium and from 700.7 to 29.1 μM in the saline medium.

3.2.2. Alkaline phosphatase (APA)

Normalizing APA activity to biomass revealed that the V_{max} of *M. australis* in the non-saline medium was significantly higher than the V_{max} of the other species (*t-test*; $p < 0.001$; Figure III-2.2). When the V_{max} was normalized by the cell abundance, *S. dacryoidea* exhibited the highest V_{max} in the saline medium (757.2 ± 201.1 amol/cell/h) compared to the other fungal strains (*t-test*; $p < 0.001$), followed by *M. australis* with a V_{max} of 182.4 ± 43.1 amol/cell/h in the non-saline medium. Similarly, *R. mucilaginosa* and *R. sphaerocarpa* showed higher V_{max} in the non-saline medium (*t-test*; < 0.001) than the other fungal strains, whereas *B. parvus* exhibited a higher V_{max} in the saline medium (*t-test*; p= 0.02).

Under non-saline conditions, *R. mucilaginosa* exhibited a significantly lower V_{max} than the other fungal strains (*t-test*; $p < 0.001$; Figure III-2.2), while its K_m (506.2 ± 103.8 μM) was the highest (*t-test*; $p < 0.001$; Figure III-4E). While in *M. australis* and *S. dacryoidea* the K_m was higher in non-saline conditions than in saline media (*t-test*; $p < 0.001$). The K_m values of *B. parvus* and *R. sphaerocarpa* were not significantly different between both conditions (*t-test*; p= 0.2).

3.2.3. Sulfatase (SUL)

All fungal strains studied were capable to cleave sulfate esters, however, at low rates (Figure III-2.3). All the species, except *R. sphaerocarpa*, showed a significantly higher V_{max} in saline than in non-saline medium (*t-test*; p= 0.05). The highest V_{max} values were

detected in *S. dacryoidea* in saline medium (0.8 ± 0.4 µmol/g biomass/h and 124.1 ± 45.9 amol/cell/h) while in *R. sphaerocarpa* the V_{max} was higher in the non-saline than in the other medium (*t-test*; $p < 0.001$).

However, as shown in Figure III-4F, there was not a clear effect of salinity on the K_m of sulfatase (*t-test*; p= 0.3). Significantly higher values were found for both salinities in *B. parvus* than in the other strains with K_m values of 524.7 ± 220.6 µM in the non-saline and 493.3 ± 160.6 µM in the saline medium.

3.3. Proteins cleavage

3.3.1. Leucine aminopeptidase (LAP)

Comparing all the fungal species, the V_{max} for LAP was significantly higher in the non-saline than in the saline medium (*t-test*; p= 0.004; Figure III-3.1). The species of the phylum Basidiomycota, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*, exhibited higher V_{max} values than the Ascomycota species *B. parvus* and *M. australis* (*t-test*; $p < 0.001$). The V_{max} values of the Basidiomycota species ranged from 208.5 to 2.9 µmol/g biomass/h, and between 10101.1 to 604.4 amol/cell/h.

The K_m was significantly higher in the Basidiomycota than in the Ascomycota strains (*t-tes*t; $p < 0.001$; Figure III-4G). Compared to the other enzymes, the LAP K_m values were the highest. Even though there was no clear influence of the salinity on the K_m (*t-tes*t; p= 0.5), *S. dacryoidea* exhibited high K_m values (1536.7 ± 832.8 µM in the non-saline and 1415.4 ± 615.3 µM in the saline medium).

3.3.2. Trypsin (TRY)

Similar to LAP, the V_{max} values for TRY in all the fungal strains were significantly higher in the non-saline than in the saline medium (*t-test*; $p < 0.001$; Figure III-3.2). Comparing the biomass-specific TRY activity with the cell-specific activity, the overall

pattern did not change. However, when calculating the V_{max} based on the biomass-specific TRY activity, *M. australis* exhibited a significantly higher V_{max} (3.2 ± 1.2 µmol/g biomass/h) in the non-saline medium (*t-test*; $p < 0.001$) than all the other strains, while the V_{max} calculated from the cell-specific activity, was higher in *S. dacryoidea* in the non-saline medium (51.8 ± 10.9 amol/cell/h; *t-test*; $p < 0.001$) than in all the other fungal strains.

For all the fungal strains except *B. parvus*, K_m values were significantly higher in the saline medium (*t-test*; $p < 0.001$; Figure III-4H). For this species, there was not a clear influence of the salinity on the K_m (*t-test*; p= 1.0). The highest K_m detected corresponded to *M. australis* and *R. mucilaginosa* in the saline medium (471.7 ± 72.7 µM and 407.0 ± 136.0 µM, respectively).

4. Discussion

The use of fluorogenic substrate analogues allowed the determination of potential extracellular enzymatic activities of marine pelagic fungal isolates. The fungal strains studied, *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* exhibited a broad spectrum of extracellular enzymes as they were able to enzymatically hydrolyze part of carbohydrates like cellulose, chitin, and xylan, and proteins such as peptide chains, and leucine residues. Moreover, these fungal strains expressed enzymes to hydrolytically cleave lipids, phosphate-containing compounds, and sulfate esters.

4.1. Extracellular enzymatic activities of pelagic fungal strains

The enzymatic capabilities and substrate preferences differ between organisms (Arnosti, et al. 2014), so extracellular enzymatic activities (EEAs) can be used as a functional trait to investigate potential functional diversity (Cullings and Courty 2009). Moreover, according to Martínez-García, et al. (2022), the number of substrates used can be suggestive of the functional metabolic potential and diversity of microbial communities.

4.1.1. Release of enzymes hydrolytically cleaving carbohydrates

In marine environments, wood is an important substrate that comes mainly from dead trees and human-made constructions (Raghukumar 2017), but is also present in mangroves (Bucher, et al. 2004). More than 80% of wood is composed of lignocellulose which is a complex of cellulose, hemicellulose, and lignin (Hyde, et al. 1998; Ab Wahab, et al. 2019). Thus, the degradation of these complex compounds requires a wide range of enzymes (Metreveli, et al. 2021).

As cellulose is the most common natural polysaccharide (Brown, et al. 2019), its degradation is crucial in the carbon cycle (Norkrans 1963). Several microorganisms, specially fungi, perform cellulolytic activities with three major classes of enzymes such as endoglucanases, exoglucanases, and β-glucosidases (Pointing, et al. 1999; Kuhad, et al. 2011; Lindenmuth and McDonald 2011; van den Brink and de Vries 2011). The hydrolysis of cellulose has been reported by a wide variety of marine fungi (Meyers and Scott 1968; MacDonald, et al. 1985; Pointing, et al. 1999; Elyas, et al. 2010; Baraldo Junior, et al. 2014; Hong, et al. 2015; Ab Wahab, et al. 2019; Lee, et al. 2019; Pilgaard, et al. 2019; Salazar Alekseyeva, et al. 2022), but their capability to decompose cellulose depends on their enzymatic machinery (Hudson 1980). Vaz, et al. (2011) and Gonçalves, Paço, et al. (2021) showed that 76% and 68%, respectively, of their studied marine fungi were able to release enzymes related to cellulolytic activity. Similar to the study of Salazar Alekseyeva, et al. (2022), all our fungal isolates were able to hydrolytically cleave cellulose with *S. dacryoidea* exhibiting the highest cellulolytic activity among all the strains tested in this study (Figure III-1.1). Moreover, all the species exhibited K_m values > 97.0 µM, except the two species belonging to the genus *Rhodotorula* under saline conditions (Figure III-4A). Thus, even though the studied species are capable of using this type of cellulolytic enzyme, the K_m values obtained indicate that relatively high concentrations of cellulose are preferred. In marine environments, cellulose is present in mangroves and associated with hemicellulose

and lignin (Bucher, et al. 2004; Behera, et al. 2017), but it is also present in some algal species (Domozych, et al. 2012). As the organic matter of terrestrial origin is more available in coastal areas, and less abundant in the open ocean (Pakulski and Benner 1994; Opsahl and Benner 1997; Hernes and Benner 2006) only specific microorganisms might be able to use cellulose (Arora 1991) and dominate its decay there.

Hemicellulose is mainly composed of xylan (Biely 1985; Hyde, et al. 1998), and this, in turn, is composed of the residual monosaccharide xylose (dos Santos, et al. 2016; Domingues, et al. 2021). Due to xylan's heterogeneity and complexity, several enzymes with diverse specificities are required to cleave it (Biely 1985; Sunna and Antranikian 1997; Beg, et al. 2001; Knob, et al. 2010). Thus, the xylanolytic activities are performed by xylanases, β-xylosidases, α-arabinosidases, α-glucuronidases, and esterases (Bajpai 1997; Sunna and Antranikian 1997). In Antarctic environments, xylan is limited, but it can be found in the cell walls of green and red algae (Turkiewicz, et al. 2000). Possibly, the xylanase activity is needed to permeabilize the cell walls of these algae allowing better access to the cellulose and other polysaccharides (Collins, et al. 2002; Li, et al. 2022). Widely distributed fungal species such as *Trichoderma viride* and *Emericella nidulans* exhibit β-xylosidase activity (Matsuo and Yasui 1988). La Grange, et al. (2001) reported that *Aspergillus niger* can produce two types of β-xylosidases, encoded in a single gene. Biely, et al. (1980) reported low β-xylosidase activity for *Cryptococcus alhidus*, but high xylanase activity, so 1,4 β-xylanase was highlighted as the main "xylan-attacking enzyme". Salazar Alekseyeva, et al. (2022) reported low β-xylosidase activity by pelagic marine fungi, which is consistent with the present study, where the two species of the phylum Ascomycota, *B. parvus* and *M. australis*, and one species of Basidiomycota, *R. mucilaginosa*, also exhibited generally low xylosidase activity (Figure III-1.2). *R. sphaerocarpa* and *S. dacryoidea*, however, exhibited higher enzymatic activity in a non-saline than in the saline medium. As shown in Figure III-4B, in the saline medium, all the fungal strains except *M. australis* exhibited a high K_m,

indicative of a low affinity to the substrate (Huitema and Horsman 2018) and an inefficient organism to obtain resources present at low concentrations (Mulder and Hendriks 2014).

Chitin is an essential component of the cell wall of fungi, the exoskeletons of arthropods, the radula of mollusks, and the beaks of cephalopods (Ruiz-Herrera 1991; Seidl 2008; Numata and Kaplan 2011). After cellulose, chitin is the second most abundant polysaccharide (Ruiz-Herrera 1991; Duo-Chuan 2006; Numata and Kaplan 2011; Brown, et al. 2019) and is composed of monomers of *N*-acetyl-D-glucosamine (GlcNAc) linked by β-glycosidic bond (Slámová, et al. 2010; Chen, Shen, et al. 2011; Suresh and Anil Kumar 2012; Gaderer, et al. 2017). Hexosaminidases are enzymes catalyzing the cleavage of these glycosidic bonds (Duo-Chuan 2006; Zhang, et al. 2010). Chitin is abundant in marine environments (Arnosti 2011), so several marine fungal species are efficient chitin degraders (Fenice, et al. 1998; Fenice 2016; Pilgaard, et al. 2019; Gonçalves, Paço, et al. 2021; Salazar Alekseyeva, et al. 2022) and together with bacteria, they are the main chitin degraders (Gaderer, et al. 2017). Unlike bacteria, fungi can hydrolyze chitin and use it as a building block for new chitin synthesis (Edson and Brody 1976) or as a carbon and nitrogen source (Gaderer, et al. 2017). Nonetheless, dissimilarities between hyphae-like fungi and yeast chitinolytic enzymes have been reported (Plíhal, et al. 2007). The amount of chitinases released by fungi is related to their chitin content, so it is also dependent on the growth mode (Hartl, et al. 2012). As hyphae-like fungal cell walls contain 10 to 20% of chitin (Ruiz-Herrera 1991), these fungi typically have 10 to 30 chitinases (Seidl 2008; Kubicek, et al. 2011). In contrast, yeast cell walls have a chitin content as low as 0.5 to 5% (Garcia-Rubio, et al. 2020). *B. parvus*, a filamentous fungus, had the highest chitinase V_{max} detected, under non-saline conditions (Figure III-1.3), and also presented the highest K_m (Figure III-4C). In filamentous fungi, chitinolytic enzymes are important for cell wall regeneration and hyphae formation (Reyes, et al. 1989). For the species not forming hyphae, their low V_{max} and K_m indicate a high affinity of the enzyme to the substrate at low concentrations (Mulder and

Hendriks 2014). Hence, we conclude that all the studied species can use chitin (either as a carbon and/or nitrogen source), even when present at low concentrations, but hyphae-like species (*B. parvus*) might dominate fungal chitinase activity in the ocean.

4.1.2. Release of enzymes cleaving lipids, phosphorus and sulfur moieties

Lipase is the third largest enzyme group that can catalyze both, hydrolysis and synthesis of esters and fatty acids (Basheer, et al. 2011; Geoffry and Achur 2017). In marine environments, lipids offer a high energetic reserve which represents at least two-thirds more energy per gram than carbohydrates or proteins (Parrish 2013), and can also be used as building blocks (Bergé and Barnathan 2005). Lipases have been widely reported in marine fungi like *Hortaea werneckii*, *Trimmatostroma salinum* (Zalar, et al. 2005), *Rhodotorula glutinis* (Papaparaskevas, et al. 1992), *Yarrowia lipolytica* (Papanikolaou, et al. 2007; Louhasakul, et al. 2016), *Leucosporidium scottii* (Duarte, et al. 2021), and in some Antarctic Ocean (Duarte, et al. 2013) and China Sea strains (Wang, et al. 2007). Additionally, a fungus isolated from the Arabian Sea, *Aspergillus awamori*, is a lipase producer, degrading 92% of the fat and oil content of polluted effluents (Basheer, et al. 2011). A species also included in the present study, *R. mucilaginosa* was suggested to be a promising fungal strain for lipase production (Potumarthi, et al. 2008), similar to Wang, et al. (2007). Nonetheless, compared to the other fungal strains, *R. mucilaginosa* had lower lipase activity (Figure III-2.1). *B. parvus* and *S. dacryoidea* are efficient producers of lipases (Figure III-2). Although the overall enzymatic activity was high, the substrate dissociates easily from the enzyme (Figure III-4F), so a considerable amount of substrate was needed to saturate the enzyme (Roussel 2012). Therefore, it seems that all the fungal species are capable to release enzymes related to OLE, but *B. parvus* and *S. dacryoidea* showed the highest OLE activity among the fungal strains tested in this study.

Phosphorus is an essential element required for the synthesis of important biological

molecules such as adenosine triphosphate, nucleic acids, phospholipids, and metabolites (Colman, et al. 2005; Paytan and McLaughlin 2007; Brembu, et al. 2017; Lockwood, et al. 2022). Inorganic phosphate (Pi, PO_4^{3-}) is the preferred phosphorus source by microorganisms (Chrost, et al. 1984; Karl 2000), though, in surface waters, it is readily used by bacteria and phytoplankton leading to low PO_4^{3-}concentrations in vast areas of the global ocean (Karl 2000; Colman, et al. 2005; Paytan and McLaughlin 2007; Brembu, et al. 2017). To overcome this P-limitation, microorganisms release alkaline phosphatase to use a range of dissolved organic phosphorus (DOP) (Hassan and Pratt 1977; Colman, et al. 2005; Srivastava, et al. 2021). This enzyme is ubiquitous and catalyzes the cleavage of phosphate monoesters (Deng, et al. 2009; Chen, Lu, et al. 2011; Sharma, et al. 2014). APA has been reported in marine fungi species like *Debaryomyces hanseni* (Adler 1978), and some species of Ascomycota and Basidiomycota phylum (Salazar Alekseyeva, et al. 2022). Also, Marchese, et al. (2020) reported that the marine echinoderm *Holothuria poli* that was widely colonized by fungi like *Penicillium chrysogenum* and *Penicillium citrinum*, showed an increase in APA. According to Baltar, et al. (2016), the release of alkaline phosphatase by prokaryotic microorganisms might be related to sporadic pulses of organic matter. Deng, et al. (2009) also suggested that the APA was related to the use of phosphate from organic matter. Hence, at a sporadic high organic matter concentration, a high K_m might be advantageous allowing higher hydrolysis rates, for instance, for species like *B. parvus* (Figures III-2.2 and III-4E). Interestingly, *S. dacryoidea* exhibited the highest hydrolysis rate, but a relatively low $K_{m;}$ so it seems this species is suitable to overcome the P-limitation at times when organic matter is low.

Sulfur is another essential element required for the growth of some microorganisms, as well as it is required as a hydrogen donor and acceptor (Starkey and Temple 1956). It is one of the most abundant elements and can be found in diverse biological molecules such as cysteine, methionine, polysaccharides, and proteoglycans (Klotz, et al. 2011; Helbert 2017).

In contrast to terrestrial polysaccharides, numerous marine polysaccharides are highly sulfated (Kloareg and Quatrano 1988; Wang, et al. 2016; Helbert 2017). For marine organisms, sulfated polysaccharides might be a physiological adaptation to the high ionic strength of their environment (Kloareg and Quatrano 1988; Aquino, et al. 2005; Aquino, et al. 2011; Ciancia, et al. 2020). Nonetheless, microorganisms need to remove the sulfate groups to gain access to the carbohydrates (Schultz-Johansen, et al. 2018; Hettle, et al. 2022). Hence, many microorganisms can use sulfur from sulfates (Starkey and Temple 1956). Choline sulfate occurs broadly in marine fungi which suggests similar mechanisms for its synthesis and utilization in fungi (Catalfomo, et al. 1973). The widely distributed fungus *Aspergillus nidulans* was reported to produce cystine from sulfate (Hockenhull 1949). Moreover, the terrestrial fungus, *Fusarium proliferatum* (Shvetsova, et al. 2015), and the marine fungus *Paradendryphiella salina* (Pilgaard, et al. 2019) assimilate sulfate from brown algae. In genomic studies, *Emericellopsis atlantica* contained six putative sulfatases (Hagestad, et al. 2021). In the present study, all the marine fungal species, except *R. sphaerocarpa*, exhibited a higher sulfatase activity in the saline medium (Figure III-2.3). The K_m was low for the species of the genus *Rhodotorula* (Figure III-4F), but for the other examined species, the K_m was high indicating low substrate affinity (Roussel 2012). Consequently, all the species exhibited different sulfatase activity. As it was generally higher in saline conditions, marine fungi might be adapted to use sulfates from polysaccharides which are common in marine environments.

4.1.3. Release of enzymes hydrolytically cleaving proteins

Protease is an enzyme group ubiquitously produced by prokaryotic and eukaryotic organisms (Razzaq, et al. 2019) catalyzing the hydrolysis of peptide bonds in proteins (Cooper 2000; Souza, et al. 2015). Based on the catalytic action, proteases can be aspartic, cysteine, metallo, and serine proteases (Dubovenko, et al. 2010; dos Santos Aguilar and Sato

2018; Mamo and Assefa 2018). These last ones, based on the substrate specificity, can further be classified as chymotrypsin-like, elastase-like, subtilisin-like, trypsin-like, etc. (Gupta, et al. 2002; Barzkar, et al. 2021; Naeem, et al. 2022). A salt-tolerant marine fungi species, *Engyodontium album* has been reported as a protease producer (Chellappan, et al. 2006). Furthermore, among 97 fungal strains isolated from Antarctica, *R. mucilaginosa*, a species also studied here, was the main releaser of proteases (Duarte, et al. 2013). Nonetheless, in another study, the extracellular protease production of this species was related to the culture medium composition (Lario, et al. 2020).

Leucine aminopeptidase (LAP) is a ubiquitous enzyme produced by prokaryotic and eukaryotic organisms, and it is involved in the hydrolysis of peptides (Burley, et al. 1990; Matsui, et al. 2006; Wahid, et al. 2008). The production of these enzymes depends on the nitrogen source (Bonugli-Santos, et al. 2015). In marine environments, the microbial production of LAP might indicate the use of nitrogen from suspended and sinking organic matter (Caruso and Zaccone 2000; Huston and Deming 2002; Baltar and Aristegui 2017), so LAP is a key player in the carbon and nitrogen recycling (Matsui, et al. 2006). Gutiérrez, et al. (2011) associated an increase in LAP and BGL activities in surface waters with the availability of new organic matter during periods of high phytoplankton biomass production. Thus, microorganisms might respond to changing substrate availability by producing LAP (Huston and Deming 2002). In the present study, due to higher V_{max} values detected in the non-saline medium, it seems that in marine environments the fungal strains produce less LAP than in freshwater environments (Figure III-3.1). In the study of Caruso and Zaccone (2000), changes in LAP activity of marine microbial communities were related to physical (salinity and temperature), chemical (nutrients composition), and microbiological variables. In our study, Basidiomycota species expressed a higher LAP activity than the Ascomycota species. As the substrate concentration needed to achieve half the maximum velocity is considerably high (Figure III-4G), in pelagic environments where the organic matter is

readily used within the water column (Middelburg, et al. 1993), and decreases with depth (Druffel, et al. 1992), the activity of LAP by marine fungi might be preferably associated to particles environments with relatively high organic matter content.

Comparable to leucine aminopeptidase, the production of trypsin also depends on the nitrogen source (Souza, et al. 2015; Kumar 2020), so it is important in the carbon and nitrogen cycles (Bong, et al. 2013). *Aspergillus ustus*, a deep-sea isolate from the Central Indian Basin, was a high producer of serine protease (Damare, et al. 2006). In the present study, we found that *R. mucilaginosa* had a high trypsin activity, but also other species such as *S. dacryoidea*, specially in the non-saline medium (Figure III-3.2). Only *R. sphaerocarpa* presented relatively low K_m values in our study (Figure III-4H). Therefore, the cleavage of proteins specifically at the carboxyl end of lysine and arginine (Olsen, et al. 2004) or trypsin activity, might be an extracellular enzymatic activity performed by marine fungi species, but high inputs of substrates might be required.

4.2. Influence of salinity on extracellular enzymatic activity

High salt concentrations can cause osmotic and ionic stress to cells (Plemenitaš, et al. 2014), which leads to reduced survival (Śliżewska, et al. 2022). Marine microorganisms perform several strategies to overcome them. As biological membranes are permeable to water, marine microorganisms need to maintain the cytoplasm slightly hyperosmotic (Oren 1999, 2002). In bacteria, the strategy seems to be the “salt in” which consists of equalizing internal and external salt concentrations usually with KCl, but this requires special adaptation of the intracellular systems (Oren 1999; Madern, et al. 2000). In fungi, this strategy has also been reported by Norkrans and Kylin (1969), together with the “compatible solute” strategy (Gostinčar, et al. 2011). The “compatible solute” strategy is based on polyols, which are organic molecules that do not interfere with vital cellular functions (Luard 1983; Gladfelter, et al. 2019). A conserved pathway, known as the high-osmolarity glycerol

(HOG), accumulates these polyols intracellularly to balance the osmotic pressure; hence, preserving internal salt concentrations below toxic levels without any special adaptation of the intracellular systems (Luard 1983; Blomberg and Adler 1992; Oren 1999; Dihazi, et al. 2004; Gostinčar, et al. 2011; Gladfelter, et al. 2019). Nonetheless, energy and carbon are required to synthesize these compatible solutes, while more energy is also needed to export ions (Blomberg and Adler 1992; Gostinčar, et al. 2011). Other strategies have also been reported in fungi such as Na + /H+ antiporters that expel sodium ions from the cell and allow a turgor pressure within the extracellular environment (Oren 1999). Additionally, for fungi, the cell wall is a crucial structure that protects them from environmental stresses like osmotic pressure (Velez, et al. 2015). Changes in the cell wall structure can preserve the integrity and function of the stressed cells (Gostinčar, et al. 2011). For example, as observed in *Candida albicans* by Ene, et al. (2015), under osmotic stress, the β-glucan–chitin layer of the cell wall can undergo a reversible increase of thickness, while maintaining sufficient rigidity of the cell morphology. Therefore, some fungi do not require salts to grow (Plemenitaš, et al. 2014), but as reported by Jones and Jennings (1964), salinity might not affect remarkably the growth of fungi in marine environments compared with freshwater and terrestrial environments. Similar results were found by Arfi, et al. (2013), but we found that salinity might exert an important effect on the kinetics of certain enzymes (Figures III-1, III-2, III-3, and III-4).

In marine environments, Caruso and Zaccone (2000) stated that salinity was an important variable affecting microbial enzymatic activities. In soil studies, salinity increase lead to the exponential decrease of some microbial enzymatic activities such as alkaline phosphatase and β-glucosidase (Rietz and Haynes 2003). For the halotolerant species *Debaryomyces hansenii*, high salinity resulted in low alkaline phosphatase activity (Adler 1978). In the present study, we also found that the majority of extracellular enzymatic activities are reduced at higher salinity (Figure III-5). The alkaline phosphatase, β-

glucosidase, and *N*-acetyl-β-D-glucosaminidase activities were lower for all the species, except *S. dacryoidea* for alkaline phosphatase, *R. mucilaginosa* for β-glucosidase, and *M. australis* for *N*-acetyl-β-D-glucosaminidase (Figures III-1 and III-2.1). Also, the hydrolysis of proteins and peptides by leucine-aminopeptidase and trypsin (Hoppe 1983; Dubovenko, et al. 2010), were lower in the saline medium for all the studied species (Figure III-3).

Proteins are tightly associated with water as it tends to bind to the hydrophobic groups in the protein surface (Saenger 1987; Kornblatt and Kornblatt 2002; Zaccai 2004). Water is incorporated inside the protein which makes it an important part of its native structure; hence its proper function (Kuntz Jr 1971; Saenger 1987; Karan, et al. 2012). Nevertheless, high salt concentrations affect water structure and dynamics, as well as protein solubility and stability due to ionic and hydrophobic effects (Baxter 1959; Lanyi 1974; Karan, et al. 2012). Salt ions tend to sequester water into other hydrated ionic structures, which diminishes intermolecular hydrogen bonds, and also the availability of free water molecules (Danson and Hough 1997; Mountain and Thirumalai 2004). Under these conditions, enzymes can be subjected to a "salting-out" effect (Frankenberger Jr and Bingham 1982). As free water molecules are limited, salt ions remove water from protein surfaces dehydrating it and altering its native structure and solubility (Frankenberger Jr and Bingham 1982; Karan, et al. 2012). As stated by Sinha and Khare (2014), the salinity effect depends on the salt concentration along with the chemical composition of the protein. Based on our results, we suggest that salinity influenced differently the kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m) (Figures III-1, III-2, III-3, III-4, and III-5). The majority of V_{max} (APA, BGL, BXY, LAP, and TRY) were reduced in the saline conditions, whereas some enzymes (BXY, and TRY) exhibited a lower affinity under saline conditions. For the remaining enzymes, there was no clear influence of the salinity on the K_m detectable. According to Blomberg and Adler (1992), under osmotic stress, the capacity for substrate uptake limits growth. This might lead to a decrease in the activation

energy, and hence, increasing and maximizing the reaction rate (Feller and Gerday 1997).

Some enzymes, however, are salt-tolerant or halophilic (Larsen 1967). Compared to other enzymes, the halophilic ones have a surface with a higher amount of amino acid residues that tend to bind to hydrated cations (Lanyi 1974), which results in a substantially larger multi-layered hydration shell (Karan, et al. 2012). This reduces the enzyme hydrophobicity, prevents aggregation (Karan, et al. 2012; DasSarma and DasSarma 2015), and also allows the halophilic enzyme to adopt a conformational structure that is flexible (Baxter 1959; Hutcheon, et al. 2005; Sinha and Khare 2014; Karan, et al. 2020). In this study, we detected potentially halophilic enzymes which were APA and OLE for *S. dacryoidea*, and SUL for all species except *R. sphaerocarpa* (Figure III-2). Interestingly, *R. mucilaginosa* was the only species that did not exhibit a sulfatase activity in the non-saline medium, but displayed one in the saline medium. As mentioned above, as sulfate esters are less common in terrestrial plants (Anderson, et al. 1965), sulfated polysaccharides might be a physiological adaptation to the high ionic strength of the marine environment (Kloareg and Quatrano 1988; Aquino, et al. 2005; Aquino, et al. 2011; Ciancia, et al. 2020). Velez, et al. (2015) reported that *Corollospora marítima* encodes choline sulfatase, which might be related to osmotic regulation. Even though, the overall rates of sulfatase were low compared to other enzymes, the studied marine fungi use it, which might be an advantage in marine environments to access monosaccharides from sulfated polysaccharides. Similarly, Gladfelter, et al. (2019) and Gonçalves, Hilário, et al. (2021) supported that as fungal nutritional sources are at high environmental osmolyte concentrations, fungi might have evolved enzymatic activities to compete for its uptake. In addition, we did not find differences in the K_m between the saline and non-saline medium (Figure III-4F). Hence, the affinity to sulfate esters might depend on the species rather than on salinity.

The results obtained in this study suggest that the salinity effect on the enzyme kinetics of marine fungi depends on the species as well as on the enzyme type, specially for

the K_m (Figure III-4). For V_{max}, the studied marine fungi seem to be adapted to various levels of salinity. Generally, however, extracellular enzymatic activity was higher in the non-saline medium (Figure III-5). For mangrove-associated fungal species, secretomics studies also supported high protein diversity with 75 proteins specific for non-saline, and 33 specific for saline conditions (Arfi, et al. 2013). Cellulase and xylanase produced by *Pestalotiopsis sp*. in the absence of salts exhibited lower activities when they were added to a saline medium and *vice versa* (Arfi, et al. 2013). As suggested by Zaccai (2004) it seems that each protein has evolved a stable form for a particular environment. This was also proposed for another mangrove fungal species that secreted two enzymes in the presence of salts, and another one in absence of salt (Li, et al. 2002). Thus, the release of isoenzymes might be an adaptation mechanism to salinity (Arfi, et al. 2013). As all the studied fungal species displayed enzymatic activities in both media, we suggest that different enzymes might catalyze the same reaction (isoenzymes) and allow them to perform their activities at a wide range of salinities.

4.3. Life strategies

The spectrum of secreted enzymes by an organism determines its physiological needs (Cooper 2000), as well as its adaptation to nutrition sources (Mulder and Hendriks 2014; Muszewska, et al. 2017; Semenova, et al. 2017; Kohler, et al. 2020). The biochemical versatility of fungi might not be related only to adaptation, but also to their capability to use resources that suddenly become available (Leger, et al. 1997). This keeps them on a consistent state of "alert" to be opportunistic saprophyte/pathogen (Cotty, et al. 1990). So by releasing the necessary enzymes for the hydrolysis of polymers present in their respective environments (Leger, et al. 1997), fungi are capable to change between different lifestyles (Muszewska, et al. 2017) Some marine fungi that released algal degrading enzymes such as cellulose were related to the development of ice-ice disease in cultivated red seaweed (Solis,

et al. 2010). The wide spectrum of secreted enzymes for the hydrolysis of polysaccharides and proteins by some pathogenic fungal strains is indicative of less specialized nutritional requirements (Leger, et al. 1997). In the present study, we found that some species are more specialized in cleaving certain substrates while others appear to be more generalists (Figure III-5). *S. dacryoidea* exhibited the highest extracellular enzymatic activity of all the tested fungal strains. Allen, et al. (2004) found that while the percentage of the total yeast community present on healthy leaves of bentgrass was <9.9%, their biomass increased to 32.0–44.7% on infected leaves. This included yeast species such as *S. dacryoidea*. Furthermore, proteases are one of the largest enzyme groups and are involved in normal and pathological processes (Razzaq, et al. 2019). In terrestrial environments, trypsin-like enzymes are characteristic of plant pathogens (Leger, et al. 1997; Gzogyan, et al. 2005; Dubovenko, et al. 2010; Semenova, et al. 2017). The outstanding combination of cellulase, xylosidase, and trypsin-like activities of *S. dacryoidea* in the non-saline and saline medium suggests less specialized nutritional requirements and potential pathogenicity that could affect plants in terrestrial environments as well as algae in aquatic systems.

Chitinases and trypsin inhibitors have been highlighted as antifungal proteins that are released by the host to prevent or fight against an infection (Mauch, et al. 1988; Duo-Chuan 2006). The trypsin inhibitor competes with trypsin for the catalytic site, which inhibits its activity (Tripathi, et al. 2011). However, in the case of chitinase, it can occur in a wide range of organisms (Duo-Chuan 2006). For instance, fungi can produce them for nutritional, symbiotic, or parasitic purposes (Gooday, et al. 1992; Seidl 2008; Slámová, et al. 2010), while bacteria can use chitin as a carbon and energy source (Park, et al. 1997). Unlike bacteria, many fungi can penetrate solid substrates (Raghukumar 2017), specially filamentous ones (Souza, et al. 2015), so hyphal growth is an important feature to colonize substrates (Zalar, et al. 2005). In the present study, *B. parvus* was the only species that had a filamentous structure. Even though, the overall rate of NAG was low when compared to

other enzymes, *B. parvus* had the highest NAG (Figure III-1.3). Some fungi live on the surface of other organisms as ectobionts, or live within them (Raghukumar 2017). Our results suggest that the high release of chitinases by the filamentous species *B. parvus* might be an advantage to penetrate and use this solid substrate. Moreover, the hydrolysis of chitin by the other studied marine fungi might indicate the capability to degrade organic matter of animal origin, as well as other fungi, even if it is performed at low rates.

Some marine fungi species are obligatory marine, whereas others were introduced by river runoff, wind, or other organisms animals (Duarte, et al. 2013; Boddy 2016). Thus, facultative marine fungi are frequent (Raghukumar 2017). A comparative study using *Paradendryphiella salina* and its terrestrial relative *Septoria lycopersic* showed that the latter had more genes for cellulose and hemicellulose degradation, but both harbored an equal amount of genes for chitin hydrolysis (Pilgaard, et al. 2019). Nonetheless, *P. salina* was capable to degrade most components of brown algae, seagrasses, as well as terrestrial plant material (Pilgaard, et al. 2019). Hence, this species has a broad and saprobic lifestyle, and also a colonizes terrestrial and marine environments (Pilgaard, et al. 2019). *S. dacryoidea* has a worldwide distribution and has been reported from the coast of Portugal (Gadanho, et al. 2003), New Zeeland (Francis, et al. 2016), and grasslands in Georgia, USA (Allen, et al. 2004), but was originally isolated from the Antarctic Peninsula (Fell, et al. 1973). This wide distribution together with our results suggests that the high enzymatic activity of *S. dacryoidea* in both, saline and non-saline media is related to a potential facultative marine species that is adapted to different salinities (Figure III-5). Moreover, it seems that *S. dacryoidea* still conserves enzymes to degrade carbohydrates of terrestrial origin such as cellulose, and xylose, which might be an advantage over obligatory marine species like *M. australis* (Fell and Hunter 1968; Fell and Pitt 1969).

4.4. Potential environmental implications

Our results suggest that the release of certain enzymes might be favored in freshwater. Similar results were reported by Caruso and Zaccone (2000), where higher microbial LAP enzymatic activities were found in the upper layers of marine waters associated with freshwater input. During the second half of the 20th century, more than 80% of the Antarctic Peninsula glaciers have retreated (Cook, et al. 2005), so freshwater input has lowered the salinity of coastal waters (Meredith, et al. 2013). Consistently, salinity variations have been reported in the Arctic (Dickson, et al. 1988). Rojas-Jimenez, et al. (2019) stated that salinity variation influences the fungal community composition. Our results indicate that the studied marine fungal species are adapted to different salinities. However, extracellular enzymatic activities of the investigated fungal species were generally higher under freshwater conditions (Figure III-5). Hence, in regions where the ocean is freshening, the species with broader enzymatic capabilities at lower salinities might thrive, whereas, others might disfavored. The potential stratification of the water column might also influence this activity, as the critical transport of deep-water nutrients might be affected (Balaguru, et al. 2016; Hutchins and Fu 2017). This might lead to an expression of different enzymes, which might influence the role of marine fungi in the biogeochemical cycles of the surface ocean.

5. Conclusions

As stated by Dubovenko, et al. (2010), environmental conditions as well as the fungal lifestyle can determine the array of enzymes secreted. Collectively, the results presented here expand our current knowledge of the enzymatic activities of marine fungi. Moreover, the extracellular enzymatic activity detected indicates the potential hydrolysis of complex living and non-living substrates suggesting a great biochemical versatility of marine fungi. Finally, based on our results, the salinity changes might affect the enzymatic

activities of marine fungi, which might also affect their role in the oceanic biogeochemical cycles.

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Chapter IV: Release of cell-free enzymes by marine pelagic fungal strains

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Abstract

Fungi are ubiquitous organisms that secrete different enzymes to cleave large molecules into smaller ones, and thus be able to assimilate the substrate. Recent studies suggest that fungi are also present in the oceanic water column harboring the necessary enzymatic repertoire to cleave carbohydrates and proteins. In marine prokaryotes, the cell-free fraction is an important contributor to the oceanic extracellular enzymatic activities (EEAs), but the release of cell-free enzymes by marine fungi remains unknown. Here we used five strains of marine fungi that belong to the most abundant pelagic phyla (Ascomycota and Basidiomycota). To also study the potential influence of salinity on the fungal cell-free EEAs, the isolates were grown under non-saline and saline conditions (0 g/L and 35 g/L, respectively). Then, the biomass was separated from the medium by filtration (0.2 μm), and the filtrate was used to perform fluorogenic enzymatic assays and calculate kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m). Substrate analogues were used to determine the breakdown of carbohydrates by β-glucosidase (BGL), β-xylosidase (BXY), and *N*-acetyl-β-D-glucosaminidase (NAG); lipids by lipase (OLE); organic phosphorus by alkaline phosphatase (APA); sulfur moieties by sulfatase (SUL); and proteins by leucine aminopeptidase (LAP), and trypsin (TRY). The release of cell-free enzymes differed between the fungal species, but represented up to 85.1% of the total EEA. In the saline medium, the release of cell-free enzymes degrading carbohydrates was reduced compared to the non-saline medium, but the ones degrading lipids and sulfur moieties were increased. For the remaining substrates, there was not a clear influence of the salinity. Taken together, our results suggest that marine fungi are potential contributors to the oceanic dissolved (i.e., cell-free) enzymatic pool. Our results also suggest that, under salinity changes, a potential effect of global warming, the hydrolysis of organic matter by marine fungal cell-free enzymes might be affected and hence, their potential contribution to the oceanic biogeochemical cycles.

1. Introduction

Low-molecular-weight (LMW) molecules are operationally defined as molecules with a molecular weight below 1000 Da, whereas high-molecular-weight (HMW) molecules compromise those larger than 1000 Da (Benner, et al. 1992; Amon and Benner 1996; Benner 2002). In marine ecosystems, the majority of dissolved organic matter (DOM) is composed of LMW molecules, but the bioavailable ones are mainly HMW molecules (Wheeler 1976; Amon and Benner 1994, 1996; Benner 2002). In order to take them up, microorganisms need to hydrolyze them into smaller molecules (<600 Da) (Weiss, et al. 1991). Osmotrophy is a feeding strategy that involves the secretion of different enzymes to transform these large molecules into smaller ones which can then be absorbed by osmosis (Richards, et al. 2012; Muszewska, et al. 2017). For fungi, osmotrophy has allowed them to use largely inaccessible nutrients and colonize diverse environments (Richards and Talbot 2013), including marine systems.

Total extracellular enzymatic activities (EEAs) are a combination of cell-attached and cell-free enzymatic activities (Wetzel 1991; Baltar 2018). Operationally defined, cell-attached enzymes are those retained on a 0.2 μm filter, whereas cell-free enzymes pass this filter (Baltar 2018). Cell-associated enzymes are tightly linked to the cell and represent, therefore, a chemical communication with the surrounding environment (Chróst 1990). In contrast, cell-free enzymes are released by the cells into the surrounding environment (Priest 1977; Chróst 1990; Wetzel 1991). As these are not linked anymore to the cell, they are not metabolically controlled by the cell (Kamer and Rassoulzadegan 1995).

The majority of marine EEAs were originally believed to be cell-attached rather than cell-free (Hoppe 1983; Rego, et al. 1985; Chróst 1990; Chróst and Rai 1993; Hoppe, et al. 2002). Contrary, other studies suggested that the cell-free EEAs can be similar to or even higher than the cell-attached enzymatic pool (Kamer and Rassoulzadegan 1995; Keith and Arnosti 2001; Baltar, et al. 2010; Duhamel, et al. 2010; Allison, et al. 2012; Baltar, et al.

2013; Baltar, et al. 2016; Baltar 2018). Additionally, some studies pointed out that the sources of marine cell-free enzymes can be numerous (Kamer and Rassoulzadegan 1995; Allison, et al. 2012; Baltar, et al. 2013), but believed to be mostly of bacterial origin (Hollibaugh and Azam 1983; Chróst 1990; Hoppe and Ullrich 1999; Obayashi and Suzuki 2008b; Baltar, et al. 2010; Bong, et al. 2013; D'ambrosio, et al. 2014; Li, et al. 2019). However, in a study on the upwelling ecosystem of Chile, Gutiérrez, et al. (2011) found potential evidence of an active contribution of marine fungi to the total enzymatic pool, suggesting that they could also be involved in the breakdown of organic matter in the ocean.

Due to anthropogenic and natural causes, salinity fluctuations have been reported in different oceanic regions (Skliris, et al. 2014). These changes can lead microorganisms to experience osmotic and ionic stress (Gladfelter, et al. 2019), and influence their extracellular enzymatic activities (Chróst 1990; Caruso and Zaccone 2000; Salazar-Alekseyeva, et al. 2022 [*in revision*]). Marine fungi seem to tolerate a wide range of salinities (Jennings 1983), but they are probably not halophilic (Gladfelter, et al. 2019). Hence, it is currently unknown how changes in oceanic salinities might affect fungi and their EEAs, specially, the cell-free fraction.

Compared to bacteria, marine fungi are less studied, so here we investigated their secretion of cell-free enzymes using five species as representatives of the most dominant marine pelagic fungal phyla: Ascomycota, and Basidiomycota (Taylor and Cunliffe 2016; Amend, et al. 2019; Morales, et al. 2019). These species were grown in non-saline and saline media to resemble conditions of freshwater and marine environments, respectively. The cell-free fraction was determined and the potential effect of salinity on the kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m) was analyzed.

2. Material and methods

The marine fungal species *Blastobotrys parvus* (HA 1620), *Metschnikowia*

australis (HA 635), *Rhodotorula sphaerocarpa* (HB 738), and *Sakaguchia dacryoidea* (HB 877) were obtained from the Austrian Center of Biological Resources (ACBR), but were originally isolated from the Antarctic Ocean (Fell and Hunter 1968; Newell and Fell 1970; Fell and Statzell 1971; Fell, et al. 1973). In the case of *Rhodotorula mucilaginosa*, this was isolated from the Atlantic Ocean during the Poseidon Cruise in March 2019. To culture these species, the protocols of Salazar Alekseyeva, et al. (2021) and Salazar-Alekseyeva, et al. (2022) [*in revision*] were followed. One medium containing 2 g/L of glucose, malt extract, peptone, yeast extract, and 0.5 g/L of chloramphenicol was prepared and further divided into two media. The first one contained 35 g/L of artificial sea salts (S9883 Sigma-Aldrich), and the second one did not contain salts (0 g/L). A one-week-old isolate grown on yeast malt extract agar (Wickerham 1939; Wickerham 1951) was diluted in autoclaved artificial seawater (35 g/L sea salts S9883 Sigma-Aldrich) until an optical density of ≈ 1 at 660 nm wavelength (OD_{660}) measured with a UV-1800 Shimadzu spectrophotometer was reached. Per every 1 L of the autoclaved medium, 0.01 L of this fungal dilution was added. Afterwards, 150 mL of this mixture (medium and fungal dilution) were put in Schott bottles and incubated at 5°C on a rotary shaker (Jeio Tech ISS-7100 Incubated Shaker) until the exponential phase was reached. Three bottles with similar OD_{660} values were chosen for further analyses (EEAs and biomass).

To estimate the fungal biomass that was releasing cell-free enzymes, 40 mL of the sample was vacuum filtered onto a pre-weighed and combusted (450°C for 6 h) Whatman GF/F filter (WHA1825047 Sigma-Aldrich, 47 mm diameter), and for 3 days, this filter was dried at 80°C. Finally, the filter was weighed again, and the fungal biomass was estimated from the difference between the pre-weighted filter and the dried one.

To estimate the fungal abundance that was releasing cell-free enzymes, 1.5 mL of the sample was fixed with a final concentration of 0.5% glutaraldehyde for 10 min, and then frozen at −80°C until further processing. For a single-cell suspension, the thawed sample

was filtered onto a pluriStrainer Mini (43-10040-50 pluriSelect, 40 µm mesh size). As *B. parvus* has a filamentous structure, its abundance was not possible to estimate with this method. For the other species, depending on the OD_{660} value, 10 to 40 µL of the sample and 5 µL of SYBR® Green 100x (S9430, Sigma-Aldrich) were added, and completed with TE to obtain a final volume of 500 µL. Finally, the sample was measured with a BD Accuri™ C6 Plus Flow Cytometry set at 'Run with limits' of 10,000 events and 'Medium', and the cell abundance was estimated with the BD Accuri C6 Software.

To obtain the cell-free fraction, the protocols of Hollibaugh and Azam (1983) and Kim, et al. (2007) together with the suggestion of Obayashi and Suzuki (2008a) were followed. This was obtained by separating the biomass through filtration on Whatman Track-Etched Membranes with a pore size of 0.22 µm (WHA10417012 Sigma-Aldrich, 47 mm diameter). To estimate the enzymatic activity, the protocols of Hoppe (1983), Salazar Alekseyeva, et al. (2022), and Salazar-Alekseyeva, et al. (2022) [*in revision*] were followed. The fluorogenic substrate analogues 4-methylumbelliferyl β-D-glucopyranoside (M3633 Sigma-Aldrich), 4-methylumbelliferyl β-D-xylopyranoside (M7008 Sigma-Aldrich), 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (M2133 Sigma-Aldrich), 4-methylumbelliferyl-oleate (75164 Sigma-Aldrich), 4-methylumbelliferyl phosphate (M8883 Sigma-Aldrich), 4-methylumbelliferyl sulfate potassium salt (M7133 Sigma-Aldrich), N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (L2145 Sigma-Aldrich), and t-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine-7-amido-4-methylcoumarin (3107-v PeptaNova) were used to determine the potential activity of the enzymes β-glucosidase (BGL), β-xylosidase (BXY), *N*-acetyl-β-D-glucosaminidase (NAG), lipase (OLE), alkaline phosphatase (APA), sulfatase (SUL), leucine aminopeptidase (LAP), and trypsin (TRY), respectively (Table IV-1). According to the targeted substrate, the enzymes were classified as cleaving carbohydrates (BGL, BXY, and NAG); lipids, phosphorus and sulfur moieties (OLE, APA, and SUL, respectively), and proteins (LAP and TRY).

Table IV-1. Enzymes targeted with fluorogenic substrate analogues of three categories, and their respective fluorogenic standards (MUF methylumbelliferyl, and MCA methylcoumarylamide). The abbreviations of the respective enzyme used throughout the text are given in the column ID.

Category	ID	Enzyme	Standard
Carbohydrates	BGL	β-glucosidase	MUF
	BXY	β-xylosidase	MUF
	NAG	*N*-acetyl-β-D-glucosaminidase	MUF
Lipids, phosphorus and sulfur moieties	OLE	Lipase	MUF
	APA	Alkaline phosphatase	MUF
	SUL	Sulfatase	MUF
Proteins	LAP	Leucine aminopeptidase	MCA
	TRY	Trypsin	MCA

To calculate the kinetic parameters such as maximum velocity (V_{max}) and half-saturation constant (K_m), the hydrolysis rates obtained from the change of fluorescence over time in the samples with the fluorogenic substrate, were fitted directly to the Michaelis–Menten equation using nonlinear least-squares regression analysis (Huitema and Horsman 2018). For the biomass-specific activity, the V_{max} was normalized to the dry weight [µmol/h/g biomass], and for the cell-specific activity, it was normalized to the cell abundance [amol/h/cell]. The V_{max} and K_m values provided in this study refer only to the cell-free fraction, as the total EEA values are from Salazar-Alekseyeva, et al. (2022) [*in revision*]. The percentage of the cell-free fraction to the total EEA (cell-attached plus cell-free) was

calculated by comparing the cell-free rates from this study to the total rates from the mentioned study of Salazar-Alekseyeva, et al. (2022) [*in revision*]. Finally, the significance of these kinetic parameters and percentages was analyzed with one-way Analysis of Variance (ANOVA), Tukey's Honestly Significant Difference (Tukey's HSD), and Student-T test.

3. Results

Remarkably, all the studied fungal species were capable to release cell-free enzymes to enzymatically hydrolyze carbohydrates (Figure IV-1) lipids, phosphorus and sulfur moieties (Figure IV-2), and proteins (Figure IV-3). Generally, the contribution of cell-free enzymes to the total EEA (V_{max}) varied among the fungal species, as well as between the non-saline and saline conditions (Figure IV-4 and Table IV-2), similar to the K_m values (Figure IV-5). The percentage of cell-free enzyme secretion represented up to 85.1% of the total EEA (Figure IV-6).

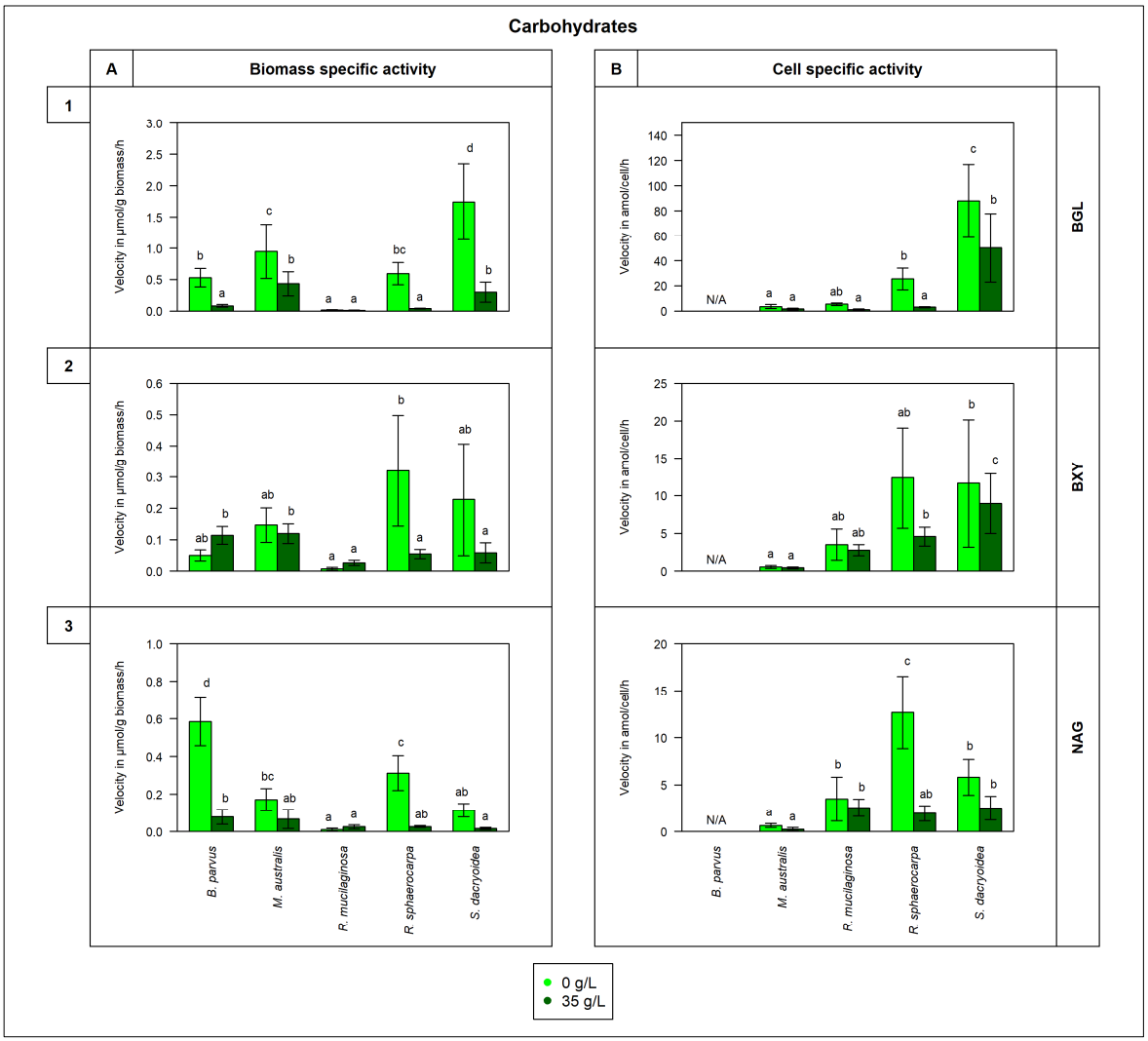

Figure IV-1. Maximum velocity (V_{max}) of the cell-free enzymatic activity obtained from the filtrate of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown under non-saline and saline conditions and normalized by the (**A**) dry weight in µmol/g biomass/h and by the (**B**) cell abundance in amol/cell/h. For *B. parvus*, **B** was not possible to calculate, so it is represented by "N/A". The substrates hydrolyzed denoted the use of carbohydrates by (**1**) β-glucosidase (BGL), (**2**) β-xylosidase (BXY), and (**3**) *N*-acetyl-β-D-glucosaminidase (NAG). Moreover, Tukey's HSD was calculated by salinity where bars not sharing any letter (a, b, c, and d) are significantly different ($p < 0.05$) and bars sharing a letter (ab, and bc) are not significantly different.

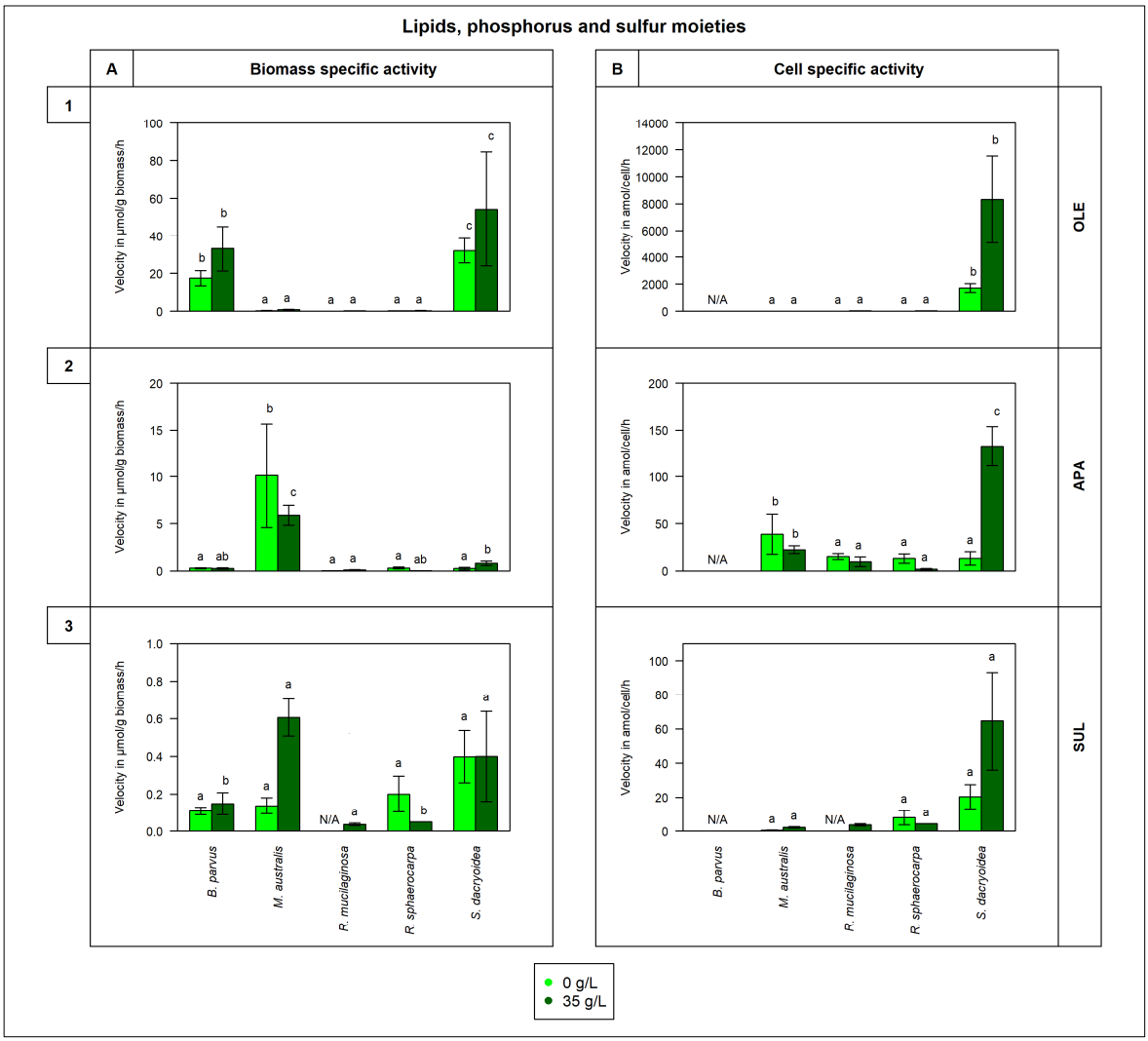

Figure IV-2. Maximum velocity (V_{max}) of the cell-free enzymatic activity obtained from the filtrate of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown under non-saline and saline conditions and normalized by the (**A**) dry weight in µmol/g biomass/h and by the (**B**) cell abundance in amol/cell/h. For *B. parvus*, **B** was not possible to calculate, so it is represented by "N/A". The substrates hydrolyzed denoted the use of lipids, phosphorus and sulfur moieties by (**1**) lipase (OLE), (**2**) alkaline phosphatase (APA), and (**3**) sulfatase (SUL), respectively. *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, so it is also represented by "N/A". Moreover, Tukey's HSD was calculated by salinity where bars not sharing any letter (a, b, and c) are significantly different ($p < 0.05$) and bars sharing a letter (ab) are not significantly different.

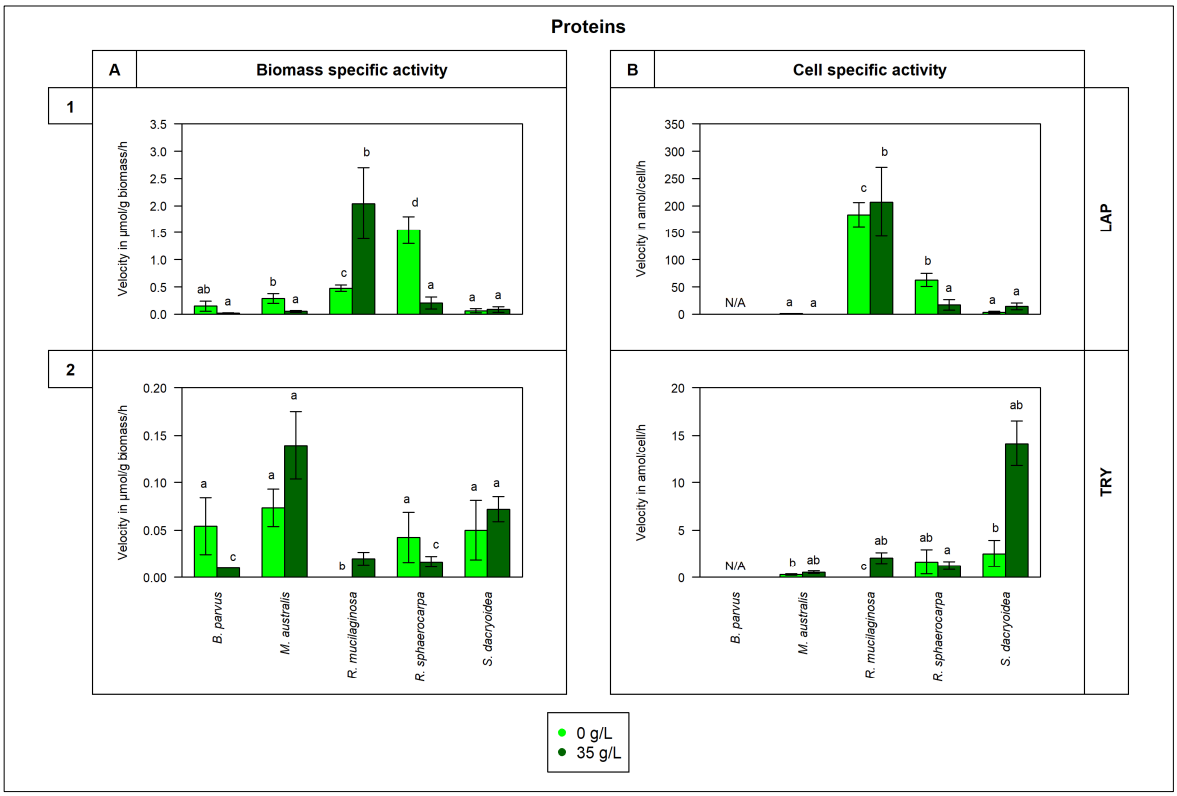

Figure IV-3. Maximum velocity (V_{max}) of the cell-free enzymatic activity obtained from the filtrate of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown under non-saline and saline conditions and normalized by the (**A**) dry weight in µmol/g biomass/h and by the (**B**) cell abundance in amol/cell/h. For *B. parvus*, **B** was not possible to calculate, so it is represented by "N/A". The substrates hydrolyzed denoted the use of proteins by (**1**) leucine aminopeptidase (LAP), and (**2**) trypsin (TRY). Moreover, Tukey's HSD was calculated by salinity where bars not sharing any letter (a, b, c, and d) are significantly different ($p < 0.05$) and bars sharing a letter (ab) are not significantly different.

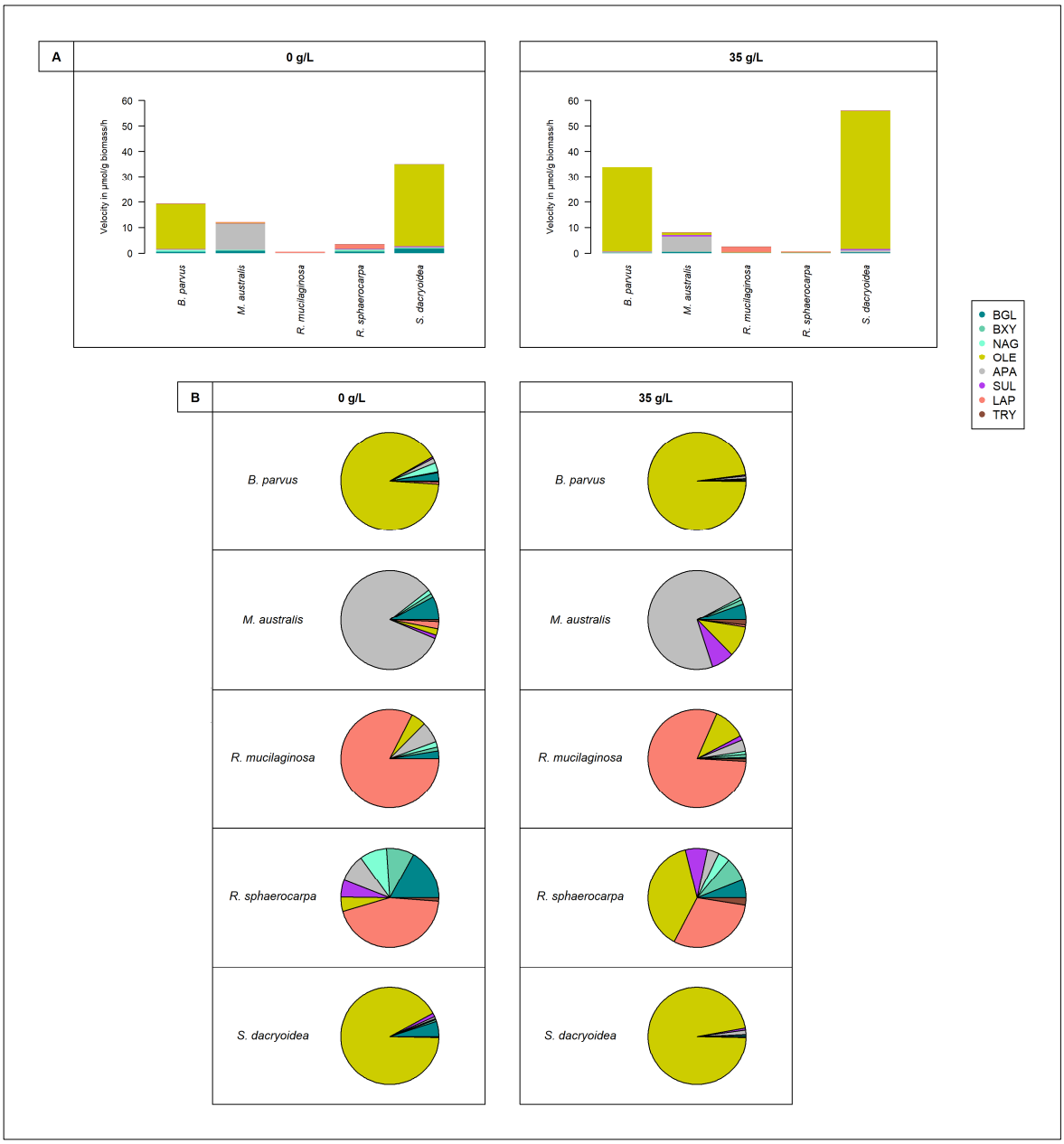

Figure IV-4. Cell-free enzymatic pool by fungal species: *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* for β-glucosidase (BGL), β-xylosidase (BXY), *N*-acetyl-β-D-glucosaminidase (NAG), lipase (OLE), alkaline phosphatase (APA), sulfatase (SUL), leucine aminopeptidase (LAP), and trypsin (TRY), under non-saline (0 g/L), and saline (35 g/L) conditions. (**A**) Sum of all cell-free V_{max} in µmol/g biomass/h and (**B**) Percentage of all cell-free V_{max} in %.

Table IV-2. Main location (≥50%), cell-attached (CA) or cell-free (CF), of the enzymes degrading carbohydrates by β-glucosidase (BGL), β-xylosidase (BXY), and *N*-acetyl-β-D-glucosaminidase (NAG); lipids, phosphorus and sulfur moieties by lipase (OLE), alkaline phosphatase (APA), and sulfatase (SUL), respectively; and proteins by leucine aminopeptidase (LAP) and trypsin (TRY). These were investigated in five marine fungal isolates *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown under non-saline (N) and saline conditions (S). *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, so it is indicated below by "N/A".

	Carbohydrates						Lipids, phosphorus and sulfur moieties						Proteins			
Enzyme	BGL		BXY		NAG		APA		SUL		OLE		LAP		TRY	
Condition	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S
B. parvus	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CF
M. australis	CA	CA	CA	CA	CF	CA	CA	CA	CA	CF	CF	CF	CA	CA	CA	CA
R. mucilaginosa	CF	CA	CF	CA	CA	CA	CA	CA	N/A	CF	CF	CF	CA	CA	CA	CA
R. sphaerocarpa	CF	CF	CA	CF	CA	CF	CA	CA	CA	CF	CA	CF	CA	CA	CA	CA
S. dacryoidea	CA	CA	CA	CA	CF	CA	CA	CA	CA	CA	CA	CF	CA	CA	CA	CA

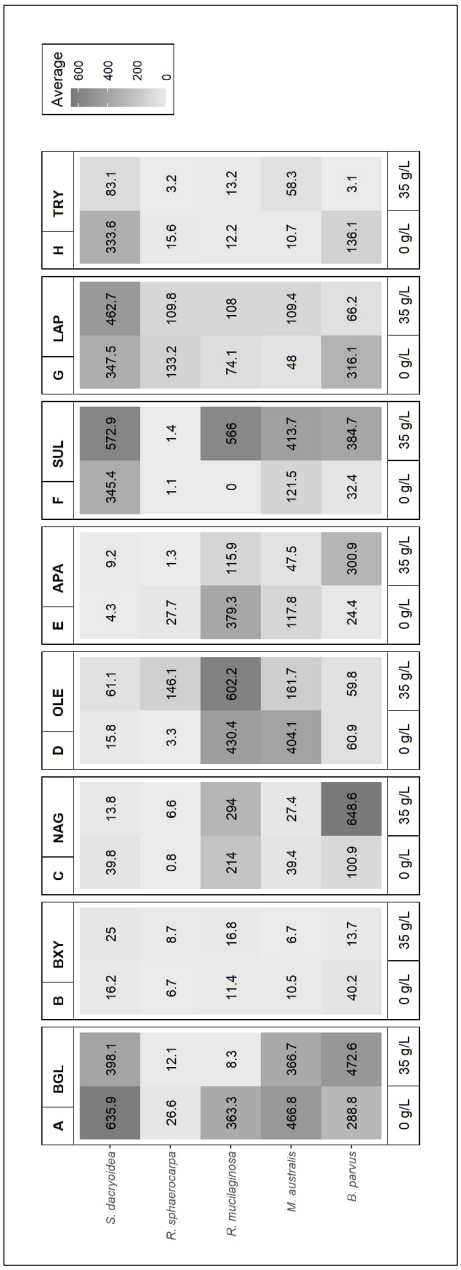

Figure IV-5. Half saturation constant (K_m) in µM calculated from the cell-free enzymatic activity fraction of five marine fungal isolates *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea* grown under non-saline and saline conditions. The substrates used were indicative of hydrolysis of carbohydrates by (**A**) β-glucosidase (BGL), (**B**) β-xylosidase (BXY), and (**C**) *N*-acetyl-β-D-glucosaminidase (NAG); lipids, phosphorus and sulfur moieties by (**D**) lipase (OLE), (**E**) alkaline phosphatase (APA), and (**F**) sulfatase (SUL), respectively; and proteins by (**G**) leucine aminopeptidase (LAP), and (**H**) trypsin (TRY).

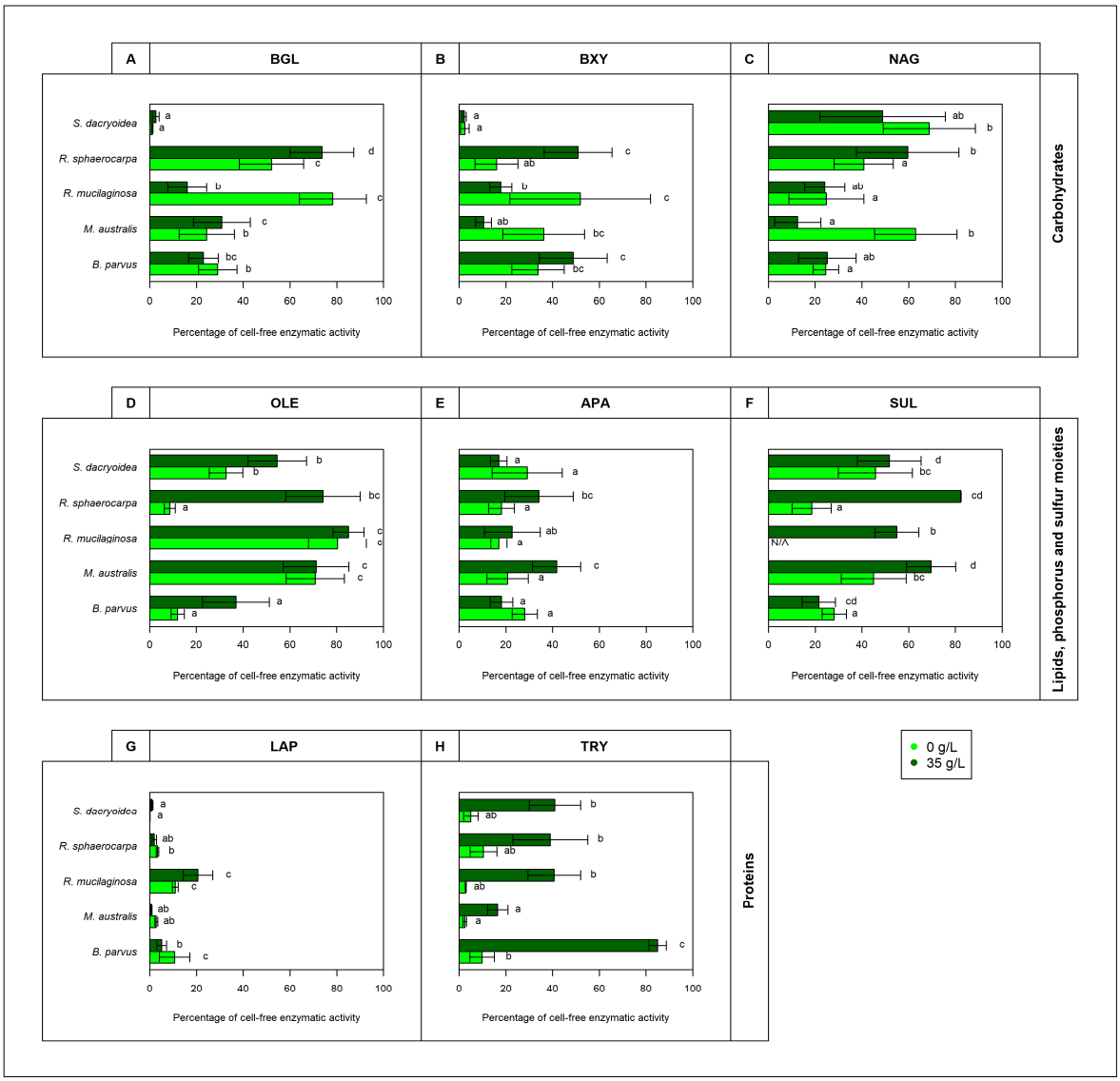

Figure IV-6. Contribution of cell-free enzymatic activity as a percentage of the total extracellular enzymatic activity for the enzymes (**A**) β-glucosidase (BGL), (**B**) β-xylosidase (BXY), (**C**) *N*-acetyl-β-D-glucosaminidase (NAG), (**D**) lipase (OLE), (**E**) alkaline phosphatase (APA), (**F**) sulfatase (SUL), (**G**) leucine aminopeptidase (LAP), and (**H**) trypsin (TRY) of the fungal strains *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*. The species *R. mucilaginosa* did not exhibit any SUL activity under non-saline conditions, so it is represented by "N/A". According to the salinity, Tukey's HSD was performed and bars not sharing any letter (a, b, c, and d) are significantly different ($p < 0.05$), whereas bars sharing a letter (ab, bc, and cd) are not significantly different.

3.1. Cleavage of carbohydrates

3.1.1. β-glucosidase (BGL)

In all the fungal species studied, the V_{max} of cell-free BGL was significantly higher in the non-saline than in the saline medium (*t-test*; $p < 0.001$; Figure IV-1.1), but there was no significant difference in the K_m (*t-test*; p= 0.3; Figure IV-5A). The highest V_{max} value was detected in *S. dacryoidea* under non-saline conditions (*t-test*; $p < 0.001$; 1.7 ± 0.6 µmol/g biomass/h and 87.9 ± 29.0 amol/cell/h). The percentage of cell-free relative to the total BGL by *S. dacryoidea* only represented 1.0 ± 0.4% and 2.6 ± 1.5% in the non-saline and saline medium, respectively (Figure IV-6A). In contrast, in the two species of the genus *Rhodotorula*, *R. mucilaginosa* under non-saline conditions and *R. sphaerocarpa* under saline conditions, the BGL rates were low, but the proportion of cell-free fraction represented 78.3 ± 14.3%, and 73.6 ± 13.7%, respectively, of the total BGL. For the remaining species, the contribution of cell-free to the total BGL activities ranged between 16.0% and 52.1%.

3.1.2. β-xylosidase (BXY)

Similar to BGL, in all the fungal strains, the V_{max} of cell-free BXY was significantly higher under non-saline than under saline conditions (*t-test*; p= 0.005; Figure IV-1.2), but no significant difference was detected in the K_m (*t-test*; p= 0.5; Figure IV-5B). In the non-saline medium, the highest V_{max} values were detected for *R. sphaerocarpa* and *S. dacryoidea* (*t-test*; p= 0.001), with values of 0.3 ± 0.2 µmol/g biomass/h and 12.4 ± 6.7 amol/cell/h, and 0.2 ± 0.2 µmol/g biomass/h and 11.7 ± 8.5 amol/cell/h, respectively. Consistent with what was observed for BGL, even though *S. dacryoidea* exhibited one of the highest V_{max}, the contribution of the cell-free fraction to the total EEA was low with percentages of 2.4 ± 1.9% and 2.2 ± 0.8% under non-saline and saline conditions, respectively (Figure IV-6B). For the remaining fungal strains, the contribution of cell-free to the total BXY varied between 10.4 and 51.8%.

3.1.3. *N*-acetyl-β-D-glucosaminidase (NAG)

Similar to BGL and BXY, the V_{max} of cell-free NAG was significantly higher in the non-saline than in the saline medium for all the studied species (*t-test*; $p < 0.001$; Figure IV-1.3). However, as shown in Figure IV-5C, there was no significant difference in the K_m for all the species (*t-test*; p= 0.3), except for *B. parvus*, where it was significantly higher under saline conditions (*t-test*; p= 0.02; 631.5 ± 141.5 µM). When the V_{max} was normalized by the biomass, *B. parvus* had the highest V_{max} under non-saline conditions (*t-test*; $p < 0.001$; 0.6 ± 0.1 µmol/g biomass/h). But when the V_{max} was normalized to the cell abundance, *R. sphaerocarpa* exhibited the highest V_{max} also in this medium (*t*-test; p= 0.003; 12.7 ± 3.8 amol/cell/h). The contribution of the cell-free fraction to the total NAG varied from 12.5 to 68.8% (Figure IV-6C).

3.2. Cleavage of lipids, phosphorus and sulfur moieties

3.2.1. Lipase (OLE)

The V_{max} values were significantly higher in the saline than in the non-saline medium for all the fungal strains (*t-test*; p= 0.05; Figure IV-2.1). The K_m, however, was not significantly different between both media (*t-test*; p= 0.4; Figure IV-5D). *S. dacryoidea* exhibited significantly higher V_{max} than the other species (*t-test*; $p < 0.001$), with values of 32.1 ± 6.5 µmol/g biomass/h and 1676.5 ± 333.4 amol/cell/h under non-saline conditions, and 54.2 ± 30.3 µmol/g biomass/h and 8319.8 ± 3229.3 amol/cell/h under saline conditions. The contribution of cell-free to the total OLE oscillated from 8.6 to 85.1% where the highest percentages corresponded to species with generally low OLE activity, such as *M. australis, R. mucilaginosa*, and *R. sphaerocarpa* (Figure IV-6D).

3.2.2. Alkaline phosphatase (APA)

The V_{max} of cell-free APA was significantly higher under non-saline than under

saline conditions for all the fungal strains (*t-test*; p< 0.001; Figure 2.2), but no significant difference was detected in the K_m (*t-test*; p= 0.6; Figure IV-5E). Normalizing APA activity to the biomass revealed a significantly higher V_{max} in *M. australis* under both, non-saline and saline conditions (*t-test*; p < 0.001; 10.1 ± 5.5 µmol/g biomass/h and 5.9 ± 1.1 µmol/g biomass/h, respectively). Normalizing the V_{max} to the cell abundance exposed *S. dacryoidea* in the saline medium as the species with the highest V_{max} (*t-test*; p < 0.001; 132.5 ± 21.1 amol/cell/h). The contribution of cell-free to the total APA varied between 16.9 and 41.7% (Figure IV-6E).

3.2.3. Sulfatase (SUL)

All the marine fungal strains expressed cell-free SUL activity with no significant difference between the V_{max} in the non-saline and saline medium (*t-test*; p= 0.5; Figure IV-2.3). The highest SUL activity detected among all the fungal strains was in *S. dacryoidea* under saline conditions (*t-test*; p= 0.04; 0.4 ± 0.2 µmol/g biomass/h and 64.6 ± 28.6 amol/cell/h). Contrary, in the case of the K_m, these values were significantly higher under saline than under non-saline conditions for all the species (*t-test*; p< 0.001; Figure IV-5F). The proportion of the cell-free fraction of SUL to the total SUL activity varied between 18.5 and 82.3% (Figure IV-6F).

3.3. Cleavage of proteins and peptides

3.3.1. Leucine aminopeptidase (LAP)

There was no significant difference in the V_{max} nor the K_m of cell-free LAP between the non-saline and saline medium for none of the fungal species (*t-test*; p= 0.4; Figure IV-3.1 and *t-test*; p= 0.9; Figure IV-5G, respectively). The highest LAP activity detected among all the fungal strains was in *R. mucilaginosa* under saline conditions (*t-test*; p < 0.001; 2.0 ± 0.7 µmol/g biomass/h and 206.8 ± 63.6 amol/cell/h). The highest contribution of cell-free

LAP to the total LAP activity was also detected in *R. mucilaginosa* (20.6 ± 6.3%). The other fungal species exhibited generally a low contribution of cell-free LAP activity to the total LAP activity ranging from 0.1 to 10.9% (Figure IV-6G).

3.3.2. Trypsin (TRY)

There was no significant difference between the V_{max} obtained in the non-saline and the saline medium (*t-test*; p= 0.08; Figure IV-3.2). However, as illustrated in Figure IV-5H, *M. australis*, *R. mucilaginosa*, and *R. sphaerocarpa* exhibited a significantly higher K_m in the saline than in the non-saline medium (*t-test*; p= 0.04). The contrary occurred for *B. parvus* and *S. dacryoidea* (*t-test*; p= 0.003). Similar to APA, TRY activity normalized to biomass resulted in a significantly higher V_{max} for *M. australis* in both media than for the remaining species (*t-test*; p< 0.001). However, normalizing the V_{max} to cell abundance revealed that *S. dacryoidea* in the saline medium exhibited a higher V_{max} than the other fungal species (*t-test*; p < 0.001; 14.1 ± 2.4 amol/cell/h). Interestingly, TRY was the only enzyme studied where a significant difference in the contribution of cell-free TRY activity to the total TRY activity was detectable between the non-saline and saline medium (Figure IV-6H). The contribution of the cell-free fraction to the total TRY activity was significantly higher under saline with 16.5 to 84.9% than under non-saline conditions with 2.5 to 10.4% (*t-test*; p < 0.001).

3.4. Cell-free enzymatic contribution

Compared to the other EEAs tested, the contribution of cell-free OLE to the cell-free enzymatic pool was significantly higher (*t-test*; p < 0.001; Figure IV-4). Cell-free OLE exhibited the highest V_{max} values, and these were mainly found in *S. dacryoidea* and *B. parvus*. Contrarily, the contribution of cell-free TRY to the cell-free enzymatic pool was significantly lower than the other EEAs (*t-test*; p < 0.001; Figure IV-4).

4. Discussion

4.1. Release of cell-free enzymes by pelagic fungal strains

It is remarkable that all the studied species of marine pelagic fungi, *B. parvus*, *M. australis*, *R. mucilaginosa*, *R. sphaerocarpa*, and *S. dacryoidea*, released cell-free enzymes. These species seem to be versatile as they exhibited a spectrum of cell-free enzymes capable of hydrolytically cleaving carbohydrates, proteins, lipids, and moieties of phosphorus and sulfur at a distance from the cell that originally produced them. Nonetheless, species and enzymes were affected differently by salinity changes.

4.1.1. Release of cell-free enzymes cleaving carbohydrates

Cellulose is a polymeric substrate that cannot be transported across the fungal cell wall, requiring at least partial extracellular hydrolysis (MacDonald and Speedie 1982). The release of cell-free β-glucosidase by fungi has been reported in terrestrial species like *Sporotrichum thermophile* (Bhat, et al. 1993; Gaikwad and Maheshwari 1994) and marine species associated with decaying estuarine and marine plants (MacDonald and Speedie 1982), driftwood (MacDonald and Speedie 1982), mangroves (Pointing, et al. 1999), sediments (Elyas, et al. 2010), and macroalgae (Lee, et al. 2019). In the present study, the level of secretion of cell-free β-glucosidase differed among the studied species (Table IV-2). Compared with the rest of the fungal species, *S. dacryoidea* was the species that exhibited the highest V_{max} of cell-free (Figure IV-1.1), but the lowest cell-free contribution to the total BGL (Figure IV-6A). This low cell-free contribution to the total β-glucosidase activity has also been reported for species like *Trichocladium achrasporum* (MacDonald, et al. 1985). For the other fungal species tested except the ones of the genus *Rhodotorula*, *R. mucilaginosa* and *R. sphaerocarpa*, the cell-free fraction represented up to 30.8% of the total EEA suggesting that the β-glucosidase activity is mainly cell-associated (Table IV-2 and Figure IV-6A). This is consistent with previous reports on other marine fungi species

(MacDonald and Speedie 1982; MacDonald, et al. 1985; Pointing, et al. 1999).

Like cellulose, xylan also cannot penetrate the cell due to its polymeric structure (Biely 1985; Lenartovicz, et al. 2003; Collins, et al. 2005), so β-xylosidases can be cell-attached or cell-free (Lenartovicz, et al. 2003). Reese, et al. (1973) reported that in the early growth stages of fungal cultures, xylosidases were cell-attached, but later on, these enzymes were released into the medium either by true secretion or cell lysis. Interestingly, similar to BGL, although *S. dacryoidea* displayed one of the highest V_{max} values (Figure IV-1.2), cell-free β-xylosidase was low compared to the total EEA (Figure IV-6B). Hence, we conclude that β-xylosidases released by the studied marine fungi species were also mostly cell-attached (Table IV-2). This was also found for some widespread fungi species like *Cryptococcus albidus* (Defaye, et al. 1992), *Aspergillus fumigatus* (Lenartovicz, et al. 2003), *Thermomyces lanuginosus* (Singh, et al. 2003), *Aureobasidium pullulans* (Ohta, et al. 2010), and some other species (Reese, et al. 1973).

In the study of Matsumoto, et al. (2004), *Verticillium lecanii*, originally isolated from *Lecanium corni*, produced extracellular *N*-acetyl-β-D-glucosaminidase from shrimp waste. Fungi can degrade chitin and use it as a carbon and nitrogen source (Gaderer, et al. 2017), but in contrast to bacteria, fungi can also use it as a building block for the synthesis of new chitin (Edson and Brody 1976). Thus, the number of chitinases produced by fungi has been related to their chitin content and growth mode (Hartl, et al. 2012). For instance, as the cell wall of filamentous fungi consists of 10 to 20% of chitin (Ruiz-Herrera 1991), the number of chitinases is normally 10 to 30 (Seidl 2008; Kubicek, et al. 2011). The cell wall of yeast fungi consists of only 0.5 to 5% of chitin (Garcia-Rubio, et al. 2020), so the number of chitinases might be lower in yeast than in filamentous fungi. Even though the overall *N*-acetyl-β-D-glucosaminidase activity was low, compared with other species, *B. parvus*, a filamentous species, exhibited the highest V_{max} of cell-free (Figure IV-1.3), similar to what was reported by Salazar Alekseyeva, et al. (2022) and Salazar-Alekseyeva, et al. (2022) [*in*

revision]. As shown in Table IV-2, the main location of *N*-acetyl-β-D-glucosaminidase might be cell-attached.

In marine environments, carbohydrates are the largest macromolecular compound class of DOC (Benner, et al. 1992). According to Biely (1985), microorganisms that compete for carbon sources, secrete enzymes that are mainly cell-attached. As mentioned above, cellulose, chitin, and xylan are polymeric structures, so for these carbohydrates, fungal cell-attached enzymes might dominate the hydrolytic cleavage of this abundant macromolecular compound class.

4.1.2. Release of cell-free enzymes cleaving lipids, phosphorus and sulfur moieties

Lipids are high energy sources (Parrish 2013), and also building blocks for organisms (Bergé and Barnathan 2005), so the degradation of lipids like phospholipids might be a means to obtain both, carbon and phosphorus (Celussi and Del Negro 2012). According to Singh and Mukhopadhyay (2012) and Duarte, et al. (2021), the majority of lipases in fungi are released into the extracellular medium. In the studies of Papanikolaou, et al. (2007) and Louhasakul, et al. (2016), the marine yeast *Yarrowia lipolytica*, produced only cell-attached lipase, but in the study of Scioli and Vollaro (1997) this same species produced both, cell-attached and cell-free OLE. In another study, other 9 marine yeast strains, including one used in our study, *R. mucilaginosa*, produced cell-attached lipase, and only *Aureobasidium pullulans* produced cell-free lipase (Wang, et al. 2007). Table IV-2 and Figure IV-6D indicate that the lipase activity of *M. australis* and *R. mucilaginosa* was mainly cell-free, whereas for the species *R. sphaerocarpa*, and *S. dacryoidea* the cell-free fraction depended mainly on the salinity, where higher percentages of cell-free enzymatic activity were found under saline conditions. *B. parvus* was the only species where the majority of lipase activity was cell-attached. Hence, we suggest that the release of cell-free lipase might be species-specific and dependent on the salinity.

Phosphorus is an essential element required for many biological processes (Colman, et al. 2005; Paytan and McLaughlin 2007; Brembu, et al. 2017; Lockwood, et al. 2022). In aquatic environments, low phosphate concentrations have been related to the expression of alkaline phosphatase (Hassan and Pratt 1977; Colman, et al. 2005; Srivastava, et al. 2021). However, in carbon-limited environments, microorganisms might use APA not only to obtain phosphate, but also to access the carbon moieties from organic matter (Hoppe and Ullrich 1999; Colman, et al. 2005). In bacteria, APA is generally located in the periplasmatic space, whereas in fungi, it is generally attached to the cell surface (Chrost, et al. 1984). In soil studies, six fungal species, one of them a widespread fungus as it is *Aspergillus niger*, released approximately 22% of extracellular alkaline phosphatase (Tarafdar, et al. 2002). These authors suggested that this low release was related to the fungal structure, and a low membrane permeability for this enzyme (Tarafdar, et al. 2002). Our study on marine fungi also suggests that APA activity is mostly cell-attached (Table IV-2), as the activities of the cell-free APA fraction amounted to at most 41.7% of the total APA activity (Figure IV-6E). The capability to secrete extracellular APA might be important for microorganisms that cannot hydrolyze dissolved organic phosphorus compounds on the surface of the ocean (Luo, et al. 2009).

Sulfur is also an essential element required for numerous biological molecules (Klotz, et al. 2011; Helbert 2017). In marine environments, polysaccharides can be highly sulfated (Kloareg and Quatrano 1988; Wang, et al. 2016; Helbert 2017), probably as a physiological adaptation to the high environmental ionic strength (Kloareg and Quatrano 1988; Aquino, et al. 2005; Aquino, et al. 2011; Ciancia, et al. 2020). In these environments, the cleavage of sulfate groups might be necessary not only to obtain sulfur, but also to access the carbohydrates (Schultz-Johansen, et al. 2018; Hettle, et al. 2022). For the widespread fungi species *Neurospora crassa*, cell-attached, and cell-free sulfatase were reported by Scott and Metzenberg (1970). We also found these two types of extracellular enzymes in our

studied marine fungi species. Interestingly, all the species, except *B. parvus*, released a higher percentage of cell-free enzymes in the saline than in the non-saline medium (Table IV-2 and Figure IV-6F). Therefore, fungi might be capable to secrete cell-free sulfatase as a strategy to remove sulfates from marine sulfated polysaccharides and access the carbohydrates (Salazar-Alekseyeva, et al. 2022 [*in revision*]).

4.1.3. Release of cell-free enzymes cleaving proteins and peptides

Grazing and viral lysis are the main sources of proteins and peptides released into the seawater (Repeta 2015). Proteases hydrolytically cleave them to obtain both, carbon and nitrogen (Li, et al. 2019), and this can take place inside or outside the cell (Pantoja, et al. 1997; Gupta, et al. 2002). Nitrogen is an essential element, specially for the growth and function of enzyme-dependent microorganisms (Allison 2005). Hydrolysis rates are mainly influenced by the size and chemical structure of the substrate, where peptides are hydrolyzed much faster than proteins (Pantoja and Lee 1999). Bacterial leucine aminopeptidase has been widely reported to be mainly cell-attached in seawater (Pantoja, et al. 1997; Obayashi and Suzuki 2008b; Bong, et al. 2013), as well as in freshwater (Chróst and Rai 1993; Millar, et al. 2015). Remarkably, in all the marine fungal strains studied, this was the only enzyme where the majority of enzymatic activity was cell-attached (up to 79.4%) in both non-saline and saline medium (Table IV-2 and Figure IV-6G). *S. dacryoidea*, however, exhibited the lowest contribution of cell-free to the total LAP activity (Figure IV-6G). Trypsin also exhibited a high cell-attached enzymatic activity (up to 89.6%), but only under non-saline conditions (Figure IV-6H).

4.2. Influence of salinity on cell-free enzymes in marine fungi

Diverse environmental factors can influence microbial cells, and also the subsequent secretion of enzymes (Chróst 1990). In the case of cell-free enzymes, as these

are released from the cell into the ambient water, their fate will depend on the conditions of the water (Hoppe and Ullrich 1999; Baltar 2018). Therefore, cell-free enzymes might be susceptible to degradation and chemical changes (MacDonald and Speedie 1982; Wetzel 1991).

Enzymes are strongly associated with water as it tends to bind to the hydrophobic groups located on the enzyme surface (Saenger 1987; Kornblatt and Kornblatt 2002; Zaccai 2004; Rezaei, et al. 2007). This allows the enzyme to maintain its native structure, and hence, it can function properly (Kuntz Jr 1971; Saenger 1987; Karan, et al. 2012). As salts promote ionic and hydrophobic effects, salinity is considered an important environmental factor that can influence the solubility and stability of enzymes (Baxter 1959; Lanyi 1974; Karan, et al. 2012) and thus, their functions (King 1986; Caruso and Zaccone 2000). Though, the magnitude of these effects will depend on the salt concentration as well as the chemical composition of the enzyme (Sinha and Khare 2014). Some marine studies have reported salinity as an important environmental factor influencing microbial enzymatic activity (Caruso and Zaccone 2000; Salazar-Alekseyeva, et al. 2022 [*in revision*]). However, the influence of salinity on fungal cell-free enzymatic activities has not been reported yet. Based on our results, we suggest that the salinity effect on the kinetic parameters such as maximum velocity (V_{max}) depends on the enzyme as well as on the species (Figures IV-1, IV-2, IV-3, IV-4, and IV-5). Under saline conditions simulating marine environments, we found that the V_{max} of the enzymes BGL, BXY, NAG, and APA was reduced, with only one exception, the APA of *S. dacryoidea*. This reduction was different for each species, similar to what was reported for the total EEA by Salazar-Alekseyeva, et al. (2022) [*in revision*].

Certain enzymes can be salt-tolerant or halophilic (Larsen 1967). Here, amino acid residues located on the enzyme surface tend to bind to hydrated cations (Lanyi 1974; Jin, et al. 2019). This creates a large multi-layered shell that keeps the enzyme hydrated (Karan, et al. 2012), and also allows it to adopt a flexible conformational structure (Baxter 1959;

Hutcheon, et al. 2005; Sinha and Khare 2014; Karan, et al. 2020). In the present study, under saline conditions, we detected potentially halophilic cell-free enzymes which were APA, SUL, and TRY for *S. dacryoidea*, SUL for *R. mucilaginosa*, and OLE for all the species (Figures IV-2, and IV-3). *S. dacryoidea* is a widespread species (Fell, et al. 1973; Gadanho, et al. 2003; Allen, et al. 2004; Francis, et al. 2016), that has been identified as a potentially facultative marine species adapted to varying salinities Salazar-Alekseyeva, et al. (2022) [*in revision*]. As the available nutritional sources are found in high concentrations of osmolytes, fungi probably evolved enzymes to successfully compete for their uptake with other microorganisms (Gladfelter, et al. 2019; Gonçalves, et al. 2021). Curiously, cell-free lipase was the only enzyme where the V_{max} of all the studied fungal species was enhanced under saline conditions (Figure IV-2.1). Comparable results were supported by Kiran, et al. (2009) showing that the production of lipase and other biosurfactants by the marine fungi *Aspergillus ustus* isolated from a symbiosis with a marine sponge, was higher under saline conditions. On the other hand, interestingly, trypsin was the only enzyme that exhibited a higher percentage of cell-free activity under saline than under non-saline conditions (Figure IV-6H). In coastal seawater, cell-free trypsin has been reported to be between 40 to 80% of the total trypsin activity (Obayashi and Suzuki 2008b). As a result, the release of cell-free enzymes into the surrounding water might be an advantage in marine environments allowing fungi to access substrates away from them.

In the open ocean, microbial cell-free enzymatic activities have been reported to be lower than in nearshore waters (Li, et al. 2019). The reason for this might be the input of terrestrial substrates (Allison, et al. 2012; Millar, et al. 2015) and other organisms that stimulate the enzymatic activities in coastal waters (Li, et al. 2019). For marine aggregates, Ziervogel and Arnosti (2008) stated that salinity or other chemical and physical factors can affect the lifetime of cell-free enzymes, as longer active lifetimes were found in nearshore waters than in offshore waters of the Gulf of Mexico. Our results indicate that salinity can

influence the kinetics of cell-free enzymes, but marine fungi might be capable to produce different enzymes adapted to different salinities. For instance, in a study of fungi inhabiting mangrove forests, one fungal species was able to secrete two enzymes under saline conditions, and a different one in the absence of salt (Li, et al. 2002). As proposed by Zaccai (2004), each enzyme might evolve a stable form specific to an environment, so isoenzyme expression might be a strategy to adapt to different salinities (Arfi, et al. 2013; Salazar-Alekseyeva, et al. 2022 [*in revision*]). From our study, it appears that the marine fungi species are adapted to a wide range of salinities, probably with different enzymes, also known as isoenzymes, capable to perform the same reaction under different salinities.

4.3. Advantages and disadvantages of cell-free enzymes in marine environments

According to Hoppe, et al. (2002), microbial EEAs contribute significantly to the breakdown of organic substrates. Extracellular enzyme production, however, can be energetically expensive (Allison 2005; Thomson, et al. 2019) and their location influences the degradation and subsequent utilization of the cleavage products (Parawira, et al. 2005). Cell-attached enzymes can hydrolyze polymeric compounds until they can be assimilated by the cells (MacDonald and Speedie 1982; Biely 1985; Grant and Rhodes 1992; Confer and Logan 1998; Lenartovicz, et al. 2003; Collins, et al. 2005). As end products are released close to the cell surface, a high concentration might facilitate its rapid uptake by an active transport system (Chróst, et al. 1989; Chróst and Rai 1993). Moreover, as cell-attached enzymes are connected to the cell, they represent a chemical communication between it and the surrounding environment, so these enzymes tend to respond to substrates outside the cell (Chróst 1990). However, when the substrate becomes limited, cell-free enzymes can be used as a strategy “to find food fast” (Chandrasekaran and Kumar 1997), and also to utilize other polymeric compounds which are otherwise non-usable (Chróst 1990, 1992). As shown by Vetter and Deming (1999), cell-free enzymes act at a distance from the microorganism that

originally released them, and in the absence of dissolved organic matter, these enzymes used particulate organic matter which provided enough hydrolysate to support microbial growth. Therefore, cell-free enzymes might be secreted to increase the chance of survival of the cell that originally produced them (Chróst 1986; Chróst and Overbeck 1987).

In aquatic environments, cell-free enzymes might be transported away from the secreting cell (MacDonald and Speedie 1982), so this release might seem ineffective as enzymes and hydrolysates can be lost by diffusion (Obayashi and Suzuki 2008b). Additionally, as hydrolysates are available to a wide range of microorganisms (Hoppe, et al. 2002), the original producer might not benefit from the hydrolytic activity of the released enzymes (Allison, et al. 2012). Thereby, the capability of the original producer to assimilate the hydrolysates reduces as the distance increases (Wetzel 1991). Nonetheless, the release of cell-free enzymes might be an advantage when the hydrolytic cleavage by the enzymes is performed at a well-defined distance from the original producer (Vetter and Deming 1999). This has been estimated to be a distance of 500 µm from the cell surface (Wetzel 1991). Thus, compared to the cell-attached enzymes that produce hydrolysates for the cells that originally synthesized them, cell-free enzymes might benefit also other cells of the surrounding environment (Luo, et al. 2009).

The occurrence of cell-free enzymes in marine environments might be crucial as these can access distant substrates, and influence the kinetics of organic matter (Kamer and Rassoulzadegan 1995). This mobilization might also result in an improvement in substrate availability (Wetzel 1991). As coined by Baltar (2018), cell-free enzymes are a kind of "living dead", as they are not attached anymore to the cell, but can still perform their respective function. Moreover, as suggested by Arnosti (2011), cell-free enzymes can influence the carbon cycle at different times and spaces where they were originally produced. Therefore, a dissociation between marine microorganisms and enzymatic activities might exist (Arnosti 2011; D'ambrosio, et al. 2014; Baltar, et al. 2016; Muszewska, et al. 2017;

Thomson, et al. 2019).

In marine environments, as bacteria and fungi might have similar functions like decomposers and also live in close spatial proximity, could lead to antagonistic or synergistic interactions (Velicer 2003; Romaní, et al. 2006). In marine bacteria, the direct release of cell-free enzymes has been reported as a response to the presence of substrate (Alderkamp, et al. 2007), starvation (Albertson, et al. 1990; Alderkamp, et al. 2007; Bong, et al. 2013), changes in the cell permeability (Chrost, 1990), or due to cell decay (Baltar, et al. 2019). Therefore, cell-free enzymes are released by living cells or via lysis of microorganisms (Chróst 1990), but the secretion of cell-free enzymes by marine fungi remains unknown. In freshwater studies, Millar, et al. (2015) reported contributions of microbial cell-free enzymatic activities of 15.5, 32.6, 32.9, 82.5, and 24.4% for BGL, NAG, APA, SUL, and LAP, respectively, of the total EEA. In contrast, Kamer and Rassoulzadegan (1995) and Baltar (2018) suggested that the marine microbial cell-free fraction can represent up to 100% of the total EEA. These authors reported microbial cell-free enzymatic activities ranging from 0, 37, and 34% for BGL, APA, and LAP, respectively, and up to 100% of the total EEA (Baltar, et al. 2010; Baltar, et al. 2013; Baltar, et al. 2016). In the present study, we report that depending on the species and also on the salinity, the fungal cell-free fraction can vary from 0.1 to 85.1% of the total EEA with some enzymes more likely to produce cell-free enzymes like OLE and SUL.

5. Conclusions

Based on our results, the studied marine fungi species are capable to secrete an array of cell-free enzymes, and this can represent up to 85.1% of the respective total EEA. The release of these extracellular enzymes can be influenced by environmental parameters such as salinity, despite being species-specific. As fungi are undeniably widespread in marine environments, the fungal cell-free enzymes might also be an important part of the oceanic

enzymatic pool.

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General discussion

This thesis provides an ergosterol extraction method adapted to low fungal abundance in marine environments (Chapter I). Additionally, it contributes to the knowledge of extracellular enzymes produced by marine fungi capable of cleaving carbohydrates, proteins, lipids, phosphorus and sulfur moieties, and the influence that important environmental parameters such as temperature and salinity might exert on the kinetic parameters V_{max} and K_m (Chapters II and III). The contribution of the cell-free fraction to the total EEAs (i.e., cell-attached plus cell-free) is also provided (Chapter IV). Therefore, the subsequent sections aim to synthesize the findings of the presented four chapters and highlight their importance for microbial oceanography and climate change research.

1. Ergosterol as a proxy to estimate marine fungal biomass (Chapter I)

Microscopic quantification has been reported to underestimate (Ingham and Horton 1987) or overestimate (Schnürer 1993) fungal biomass. Moreover, as fungi are a highly diverse group, their morphological and physiological differences hinder the visual quantification (Stahl and Parkin 1996). As a solution, ergosterol has been widely used as a proxy to estimate fungal biomass in cereal grains (Seitz and Mohr 1977; Seitz, et al. 1979), plant material (Martin, et al. 1990; Gessner and Chauvet 1993), soil (Grant and West 1986; Stahl and Parkin 1996; Montgomery, et al. 2000), and freshwater (Mille-Lindblom, et al. 2004; Gessner 2020). In the case of marine environments, ergosterol has been used to quantify fungal biomass in decaying plant material (Lee, et al. 1980; Newell, et al. 1988), pure cultures (Padgett and Posey 1993), and sea ice and seawater (Hassett, et al. 2019). All the extractions used in the above-mentioned studies were based on Soxhlet (Lee, et al. 1980), and reflux (Newell, et al. 1988; Padgett and Posey 1993) methods which require special equipment, and are also time-consuming. A method based on chloroform-methanol originally developed for the extraction of lipids from fish tissues (Bligh and Dyer 1959), was

adapted to determine low concentrations of ergosterol in oceanic waters (Chapter I). This adaptation allowed a ~86% retrieval of the ergosterol standards, so the lost ergosterol was either retained in the used material, or removed within the upper phase. When this adaptation was applied to fungal culture samples, 98% of the extracted ergosterol was detected in the lower phase, and the remaining percentage in the upper phase. To overcome the loss of ergosterol, re-extractions were performed. A second extraction allowed a ~10% retrieval of the total ergosterol extracted, whereas a third extraction resulted in only ~1%. The oceanic samples extracted with this adapted method yielded values as low as 0.12 pM of ergosterol, much lower than expected based on the average reference value of Hassett, et al. (2019). Accordingly, a combination of high filtration volume, re-extraction, and low resuspension volume of the sample allowed us to scale up the low fungal biomass of oceanic samples to concentrations that were possible to detect with current quantification methods such as Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LC-MS/MS) (Ory, et al. 2020). This represents an important step in the ecological research of marine fungi and a useful tool to determine their abundance in marine habitats.

2. Total extracellular enzymatic activities (EEAs) utilized by marine fungi (Chapters II and III):

Functional traits are measurable morphological, phenological, or physiological characteristics of an organism that can influence its fitness, so they can be taken as a response to the properties of the ecosystem where it inhabits (Violle, et al. 2007). Generally, each organism expresses specific enzymatic capabilities and substrate preferences (Arnosti, et al. 2014), so extracellular enzymatic activities (EEAs) can be used as a functional trait to infer the potential functional diversity of an organism (Cullings and Courty 2009; Martínez-García, et al. 2022). In Chapters II and III, different fluorogenic substrate analogues were used to estimate the potential degradation of carbohydrates, proteins, lipids, phosphorus and

sulfur moieties by five marine fungal species belonging to the two most abundant marine fungal phyla reported to date, Ascomycota and Basidiomycota (Taylor and Cunliffe 2016; Amend, et al. 2019; Morales, et al. 2019).

The majority of enzymes are efficient within what are considered "standard" or mesophilic conditions (Karan, et al. 2012). Nonetheless, more than 70% of the Earth's surface is covered by seawater (Jannasch and Taylor 1984; Russell 1990), and does not meet these conditions (Karan, et al. 2012). Usually, marine environments have high salinity, low temperature, low nutrient availability, among others, but also sudden and repeated variations, which make these environments challenging for organisms (Feller and Gerday 2003; Zhang and Kim 2010; Vonnahme, et al. 2020). Despite this, different organisms are functional under these circumstances (Hough and Danson 1999). In the case of marine fungi, as nutritional sources occur within these challenging conditions, they probably evolved enzymes successfully adapted to compete for their substrates (Gladfelter, et al. 2019; Gonçalves, et al. 2021). These enzymes, also known as "extremozymes", catalyze reactions where their mesophilic counterparts are reduced or inhibited (Hough and Danson 1999; Karan, et al. 2012). In Chapter II, although all the studied fungal enzymes exhibited activity under extreme conditions, at low temperature (5°C), APA of *M. australis*, and SUL of *B. parvus* and *S. dacryoidea* were enhanced relative to "standard" conditions (i.e., 20°C). While in Chapter III, at high salinity (35 g/L of sea salts), BGL of *R. mucilaginosa*, NAG of *M. australis*, APA of *B. parvus* and *S. dacryoidea*, and SUL of all the species except *R. sphaerocarpa* were increased relative to "standard" conditions (i.e., 0 g/L of sea salts). Enzymes can also display polyextremophilicity which means that they can be efficient in more than one extreme condition (Karan, et al. 2012). Sulfatase of the species *B. parvus* and *S. dacryoidea* presented a potential polyextremophilicity as their enzymatic activities were higher under both extreme conditions i.e., low temperature (5°C) and high salinity (35 g/L of sea salts), than under "standard" conditions i.e., high temperature (20°C) and low salinity

(0 g/L of sea salts), as shown in Chapters II and III.

In the temperature experiment, APA, BGL, BXY, NAG, and LAP activities were higher at 20°C than at 5°C (Chapter II). In the salinity experiment, the activities of the same enzymes together with TRY were higher under non-saline conditions than under saline conditions (Chapter III). At low temperatures, water molecules freeze (Siddiqui and Cavicchioli 2006), and at high salinities, salt ions sequester water molecules into other hydrated ionic structures (Danson and Hough 1997; Mountain and Thirumalai 2004). In both cases, as water activity is perturbed, water becomes limited to enzymes with further consequences to their structure and/or function (Karan, et al. 2012). The production of isoenzymes or "different proteins with similar enzymatic activity" (Latner 1967; Griffin 1996) might be an adaptation mechanism to changing temperatures (Russell 1990) and salinities (Arfi, et al. 2013). These enzymes are produced due to 1) primary or genetic causes and 2) secondary or post-translational causes (Rider 2013). In the case of the first one, the organism holds several genes that encode different types of enzymes, whereas in the second one, a single gene produces an assortment of enzyme subunits (Rider 2013). Consequently, the release of isoenzymes by marine fungi could be a suitable strategy that allows them to inhabit a wide range of temperatures and salinities (Chapters II and III).

In marine environments, facultative marine fungal species are common (Raghukumar 2017). The wide distribution of the studied Basidiomycota species such as *R. mucilaginosa* (Harrison 1928; Mackinnon and Artagaveytia-Allende 1945; Di Menna 1955; Fell, et al. 1960), *R. sphaerocarpa* (Newell and Fell 1970; Butinar, et al. 2005; Gadanho and Sampaio 2005), and *S. dacryoidea* (Fell, et al. 1973; Gadanho, et al. 2003; Allen, et al. 2004; Francis, et al. 2016), together with their enzymatic capabilities at different temperatures and salinities described in Chapters II and III, might suggest that these species are facultatively marine. In contrast, the distribution of the studied Ascomycota species like *B. parvus* (Fell and Statzell 1971; Parasuraman, et al. 2020), and *M. australis* (Fell and Hunter 1968; Fell

and Pitt 1969) in marine habitats, together with the activity of key marine enzymes such as SUL detailed in Chapters II and III, might suggest a potential marine origin, but also a probable adaptation of these species to extreme and fluctuating conditions, which are common traits of these environments (Feller and Gerday 2003; Zhang and Kim 2010; Vonnahme, et al. 2020).

3. Cell-free enzymatic activities in marine fungi (Chapter IV):

The location of extracellular enzymes either cell-attached or cell-free, impacts both, the hydrolysis and assimilation of a substrate (Parawira, et al. 2005). The size and chemical structure of the substrate can also influence these processes (Pantoja and Lee 1999). Marine microorganisms require diverse substrates which are normally polymeric (Wang, et al. 2016). Cell-attached enzymes have been commonly reported to degrade polymeric compounds until they can be assimilated (MacDonald and Speedie 1982; Biely 1985; Grant and Rhodes 1992; Confer and Logan 1998; Lenartovicz, et al. 2003; Collins, et al. 2005). In Chapter IV, the location of the majority of extracellular enzymes was cell-attached. Nonetheless, at high salinity (35 g/L of sea salts), this changed to cell-free in key marine enzymes like SUL. Compared to their terrestrial counterparts, marine polysaccharides are highly sulfated (Kloareg and Quatrano 1988; Wang, et al. 2016; Helbert 2017), which is potentially a physiological adaptation to the high ionic strength of these environments (Kloareg and Quatrano 1988; Aquino, et al. 2005; Aquino, et al. 2011; Ciancia, et al. 2020). The cell-free sulfatase might be used to eliminate these sulfate groups and allow access to the desired substrate (Schultz-Johansen, et al. 2018; Hettle, et al. 2022). Additionally, spores as part of the fungal reproduction cycle, have allowed fungi to conquer new and diverse habitats (Webster and Weber 2007). The release of enzymes by these cells have been reported in terrestrial (Hepper, et al. 1986; Saito 1995), and aquatic (Camargo, et al. 1967) species. Consequently, the mobilization of fungal biological resources like cell-free enzymes

and spores might be a strategy to reach distant substrates (Webster and Weber 2007), which also enhances substrate availability in marine environments (Wetzel 1991).

The location of extracellular enzymes might also depend on the lifestyle (Traving, et al. 2015). For particle-associated microorganisms, cell-free enzymes could be beneficial due to their proximity to the substrates (Vetter, et al. 1998). In contrast, for free-living microorganisms, cell-attached enzymes could be more profitable (Chróst 1990; Traving, et al. 2015) as the substrates in marine environments are usually highly diluted (McCarthy, et al. 1996; Amon and Benner 2003). In the case of marine fungi, the location of extracellular enzymes might also be related to their lifestyle (Dubovenko, et al. 2010). Baltar, et al. (2021) related the high secretion of CAZymes by marine fungi with a preferential particle-associated lifestyle. As presented in Chapter IV, the majority of enzymes produced by the studied marine fungi were cell-attached, suggestive of a free-living lifestyle. Nonetheless, under specific conditions, certain enzymes switched to cell-free. For instance, under non-saline conditions, the release of SUL and OLE was mainly cell-attached, but under saline conditions, these enzymes were cell-free. At high salinity (35 g/L of sea salts), substrates like sulfur moieties and lipids might induce the expression of cell-free SUL and OLE, indicative of a potential particle-associated lifestyle. This also suggests that the fungal lifestyle in marine habitats might depend on the available substrates.

4. Final remarks

Fungi are well-known decomposers of organic matter (Webster and Weber 2007). In transition zones between land and sea, fungi perform similar ecological roles as their terrestrial counterparts (Boddy 2016; Gladfelter, et al. 2019). However, their ecological role in the oceanic water column remains poorly understood. Some recent studies have highlighted the potential of marine fungi to degrade carbohydrates (Baltar, et al. 2021), and proteins (Breyer, et al. 2022). These studies together with their broad enzymatic repertoire

described in Chapters II and III, might suggest that their ecological role is similar to their terrestrial and freshwater counterparts. As shown in Figure 1, marine fungi might be able to break down organic matter like carbohydrates, proteins, lipids, phosphorus and sulfur moieties, among others (Chapters II and III). This could be performed with both, cell-attached and cell-free enzymes (Chapter IV).

Marine microbial extracellular enzymes are essential contributors to the degradation of organic matter (Hoppe, et al. 2002; Traving, et al. 2015). Chapters II and III suggest that marine fungi might also be contributors to the oceanic microbial enzymatic pool as stated by Gutiérrez, et al. (2011). Importantly, Chapter IV supports the view that the cell-free fraction of marine fungi might also be an essential contributor to the ubiquitous oceanic microbial cell-free pool. Therefore, these microorganisms might play a key role in the oceanic biogeochemical cycles, consistent with what was stated by Morales, et al. (2019).

The array of enzymes displayed by fungi depends on environmental conditions (Dubovenko, et al. 2010). As discussed in Chapters II, III, and IV, fluctuations in temperature and salinity might influence the EEAs of marine fungi and thus, also their ecological role. Nonetheless, additional studies are needed to investigate the limitations of this potential ecological role, and in what manner this might be influenced by present and future oscillations of other important environmental parameters associated to climate change.

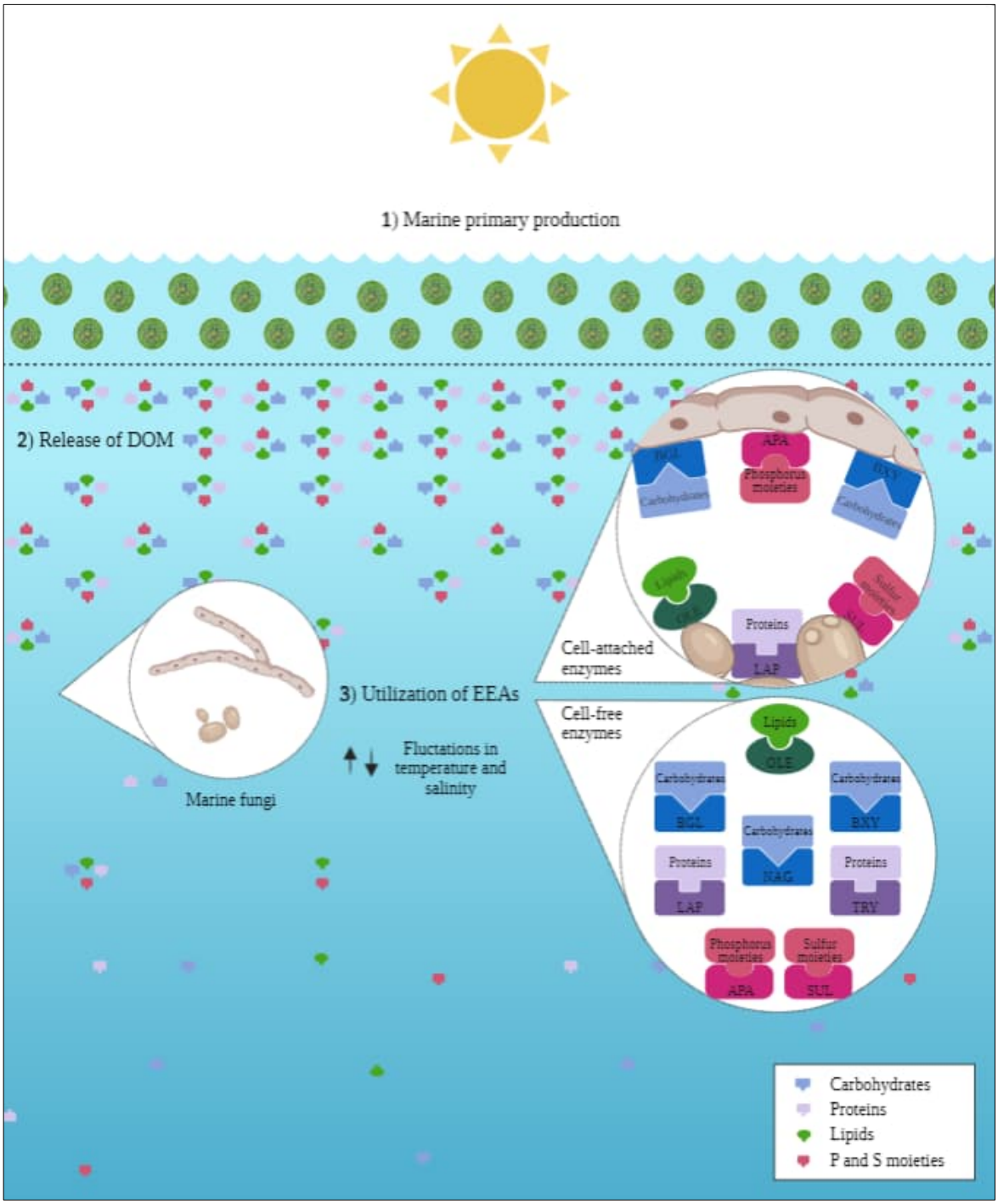

Figure 1. Potential ecological role of fungi, one of the microbial groups that inhabit the oceanic water column. **1**) Phytoplankton fixate atmospheric inorganic carbon and convert it into organic matter (Falkowski 1994; Falkowski, et al. 2000). **2**) Phytoplankton and other living and dead cells release dissolved organic matter (DOM) into the surrounding environment (Azam, et al. 1983). This DOM is composed mainly of carbohydrates (blue), proteins (purple), lipids (green), phosphorus and sulfur moieties (pink), among others

(Biersmith and Benner 1998; Repeta 2015). **3**) Marine fungi might utilize extracellular enzymatic activities (EEAs) with both, cell-attached and cell-free enzymes, to degrade the above-mentioned substrates and this might be influenced by fluctuations in temperature and salinity (Chapters II, III, and IV). The cleavage of carbohydrates might be done by β-glucosidase (BGL), β-xylosidase (BXY), and *N*-acetyl-β-D-glucosaminidase (NAG); proteins by leucine aminopeptidase (LAP) and trypsin (TRY); lipids by lipase (OLE); phosphorus and sulfur moieties by alkaline phosphatase (APA), and sulfatase (SUL), respectively. It is important to mention that oceans are complex and diverse ecosystems, so other microorganisms such as archaea, bacteria, protists, etc. also contribute to the decay of DOM.

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