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The ecology and diversity of benthic deep-sea fungi

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Chapter 1

Fugal ecology and diversity in benthic deep-sea ecosystems

Abstract: Benthic deep-sea ecosystems host unique and diverse habitats which are characterised by highly diverse biological components. In this review, I analysed the available literature information dealing with fungal diversity in benthic deep-sea habitat and ecosystems worldwide to provide insights into their ecology. Available information based on the use of culture-dependent and -independent approaches indicates that fungi are a diverse and ubiquitous component of the microbial food web in benthic deep-sea ecosystems. Present findings also highlight that benthic deep-sea fungi could be actively involved in critical biogeochemical and ecological processes. Despite this, their quantitative relevance, diversity and ecological role are still far to be elucidated. In particular, while a considerable number of studies have described the diversity of fungi in different benthic deep-sea habitats, their ecological significance and the factors influencing abundance and diversity patterns are almost neglected. Moreover, the different methodologies adopted in the different investigations considerably limits a proper comparison. Thus, this review highlights the need of broad spatial scale investigations thorough standardised methodologies for the assessment of abundance and diversity of benthic deep-sea fungi in order to improve our understanding on their ecological significance and factors influencing their distribution in the largest biome on Earth.

Microbial component in the deep sea

The deep sea accounts for about 95% of oceans volume, and it is the largest and least explored biome of the Earth (Corinaldesi 2015). The deep sea hosts very diverse habitats and incredibly diverse assemblages (Jørgensen and Boetius 2007), where microbes, principally prokaryotes, account for a significant fraction of the total biomass and are critical players of biogeochemical cycles (Azam and Malfatti 2007, Falkowski et al. 2008, Danovaro et al. 2015). However, the Fungi are not yet considered in any model concerning benthic deep-sea ecosystem functioning model.

Acting as decomposers of organic matter, mutualists, or parasites and pathogens, fungi drive carbon cycling and food web dynamics in terrestrial ecosystems. In particular, as decomposers, fungi are known to produce a vast array of organic compounds and enzymes able to decompose even the most recalcitrant fraction of natural and human-made organic materials (Clipson et al., 2006). Fungi are ubiquitous components in marine ecosystems and reported by both culture-dependent and -independent approaches from many of the most remote and extreme deep-sea habitats including hypersaline anoxic basins (Alexander et al. 2009, Bernhard et al. 2014), cold seeps (Nagano and Nagahama 2012, Wang et al. 2014), hydrothermal vents (Gadanho and Sampaio 2005, Burgaud et al. 2009) and superficial and subsurface sediments (Singh et al. 2012, Rédou et al. 2014). Interestingly, the majority of these organisms is genetically similar or identical to known terrestrial taxa (Takishita et al. 2006, Lai et al. 2007, Singh et al. 2011, Zhang et al. 2015).

Recent findings, reporting metabolic activity and capability to thrive at "deep-sea" conditions, highlights an incredible capability of fungi to cope with a variety of environmental conditions. These studies then concluded that fungi might occupy an essential role in benthic deep-sea biogeochemical processes (Singh et al. 2010, Burgaud et al. 2015). Moreover, more than 5 million species of fungi are predicted to be present in the entire Biosphere, but only 5 % have been described (Hawksworth 2001, Blackwell 2011). A large number of unknown fungal sequences has been reported especially from deep-sea habitats highlighting that the majority of fungal diversity is yet to be described (Corinaldesi et al. 2017).

Because of fungal contribution to biogeochemical cycles in terrestrial habitats, marine and marine-derived fungi might play a yet unrecognised role in benthic deep-sea ecosystems processes (Cathrine and Raghukumar 2009, Burgaud et al. 2009, Jebaraj et al. 2010). Increasing evidence indicates that fungi are not only a diversified component of benthic deep-sea microbial assemblages but are also metabolically active, fungal ability to live under deep-sea conditions, and molecular signatures of active metabolisms has been demonstrated (Burgaud et al. 2013, Zhang et al. 2013, Ciobanu et al. 2014, Pachiadaki et al. 2016). Nevertheless, the majority of studies on fungi have focussed on the exploration of their diversity, rather than to investigate their ecological role. In this review, we compile available information on fungal diversity patterns to provide insights into fungal ecology in different benthic deep-sea habitats and ecosystems.

Diversity and ecology of fungi in different benthic deep-sea habitats and ecosystems

1. Hypersaline anoxic basins (DHABs)

DHABs are typically formed at depths greater than 3’000 m below sea level. The high density of the hypersaline water hampers the mixing with the overlying oxygenated seawater (Figure 1). The combination of high salinity, high density, high hydrostatic pressure, the absence of light, and anoxia, makes these basins some of the most extreme habitats on Earth. Moreover, redox boundaries occurring within short vertical distances are zones of intense biogeochemical cycling, involving key elements such as carbon, nitrogen, sulphur, and hydrogen as well as iron and manganese. These habitats are exciting environments to obtain new insights into the microbial diversity and ecosystem functioning and have already extended our knowledge of the environmental factors that define the limits of life (Eder et al. 1999, 2001, Hallsworth et al. 2007, Edgcomb et al. 2016).

Figure 1. Deep Sea Hypersaline Anoxic Basin in the Gulf of Mexico, image credit BBC.

Although most of the studies focused on prokaryotes, eukaryotic ribosomal DNA analyses, as well as microscopic images, revealed the presence of unexpected micro-eukaryotic communities thriving in DHABs (Alexander et al. 2009, Stock et al. 2012, Edgcomb and Bernhard 2013, Bernhard et al. 2014). The eukaryotic communities identified in several DHABs are characterised by a variety of taxa including, ciliates, alveolates, stramenopiles and fungi, suggesting that even under high salinity and permanent anoxic conditions eukaryotic life is not only possible but even diverse (Table 1).

Table 1. Fungal diversity in deep-sea hypersaline anoxic basins. Stars correspond to taxa characterised by large fruiting bodies and strictly terrestrial.

Phylum	Closest relative	Depth (mbsl)	Location	Reference
Ascomycota	*Acremonium*	850	Thuwal	Wang et al. 2014
Ascomycota	*Aspergillus*	3500; 3258	L'Atalante Upper halocline; Thetis brine	Alexander et al. 2009; Stock et al. 2012
Ascomycota	*Candida*	3258	Thetis brine	Stock et al. 2012
Ascomycota	*Cladosporium*	3258	Thetis brine	Stock et al. 2012
Cryptomycota	*LKM11*	3500	L'Atalante Lower Halocline; L'Atalante Upper halocline	Alexander et al. 2009
Basidiomycota	*Lycoperdon* *	3500	L'Atalante Upper halocline	Alexander et al. 2009
Basidiomycota	*Malassezia*	3258	Thetis interface; Bannock interface; Thetis brine; Thetis Lower halocline	Edgcomb et al. 2009; Stock et al. 2012
Basidiomycota	Malasseziomycetes	3582	Discovery Upper Halocline; L'Atalante Lower Halocline; L'Atalante Upper halocline; Urania Halocline	Bernhard et al. 2014
Basidiomycota	Microbotryomycetes	3430	L'Atalante Lower Halocline	Bernhard et al. 2014
Ascomycota	*Penicillium*	3500	L'Atalante Upper halocline	Alexander et al. 2009
Basidiomycota	*Rhodosporidium*	3258	Thetis brine	Stock et al. 2012
Basidiomycota	*Rhodotorula*	3500	L'Atalante Upper halocline; Thetis brine; Thetis interface	Alexander et al. 2009; Stock et al. 2012
Ascomycota	*Sarocladium*	850	Thuwal	Wang et al. 2014
Ascomycota	*Schizosaccharomyces*		Bannock interface; Oxygenated deep-sea water	Edgcomb et al. 2010; Edgcomb et al. 2011
Ascomycota	*Sordaria*	3500	L'Atalante Upper halocline	Alexander et al. 2009
Basidiomycota	Unknown Agaricomycetes *	3258	Thetis brine; Thetis interface	Stock et al. 2012
Basidiomycota	Unknown Tremellomycets *	3258	Thetis brine	Stock et al. 2012

Among eukaryotes, molecular investigations of environmental SSU rDNA have identified fungi as the most diverse taxonomic group in the brine of the Thetis basin (Stock et al. 2012). Some of the retrieved fungal taxa were closely related to described species, widely distributed in the deep sea, such as *Rhodotorula*, *Cladosporium* and *Aspergillus* genera. However, the majority of molecular signatures were only distantly related to described species and potentially representing novel taxa even at high taxonomic level. Also, the hypoxic/suboxic brine layer near the Thuwal cold seeps in the Red Sea is characterised by abundant fungal signatures mostly affiliating with *Acremonium* genus which include several saprophytic species (Wang et al. 2014). The study also succeeded in isolating fungal cultures similar or

identical to the *Sarocladium strictum* and *Acremonium* sp. previously identified from the seafloor of the Arabian Sea (Jebaraj et al. 2010, Wang et al. 2014). While Thetis and Thuwal DHABs were characterised by abundant fungal genera belonging to Ascomycota (Stock et al. 2012, Wang et al. 2014), rRNA analyses of some Mediterranean DHABs (i.e. Discovery, Urania, L'Atalante) showed a large share of reads affiliating to Basidiomycota (Bernhard et al. 2014).

Furthermore, Bernhard and colleagues (2014) were also able to visualise fungal hyphae within sediments, including the lower halocline samples. Concerning fungal molecular diversity, they found the *Malassezia* signature to account for a significant proportion of OTUs in the lower haloclines of both Discovery and L' Atalante. Interestingly, *Malassezia* can be found almost everywhere on Earth (Amend 2014) and *Malassezia* clones retrieved from Discovery and L'Atalante (Bernhard et al. 2014) were also found in the anoxic lower halocline water layer of the Thetis basin (Stock et al. 2012), the anoxic marine Cariaco Basin (Edgcomb et al. 2011b), the anoxic fjord Saanich Inlet (Orsi et al. 2012) and deep sub-surface marine sediments of Peru Margin (Edgcomb et al. 2009, 2011a). However, this species of *Malassezia* remain uncultured, and it is not possible to infer much about on its ecology. Nevertheless, the diversity of habitats in which *Malassezia*-like organisms are found suggests this group may have a variety of trophic strategies ranging from saprotrophy to biotrophy (Amend 2014).

The majority of the studies regarding DHAB eukaryotic communities aimed to identify species able to live in such hostile habitats. However, there is almost no information regarding the drivers of fungal diversity and abundance, thus limiting our understanding of their ecology. The overall phylotype richness in hypersaline systems is, however, lower than in the surrounding seawater suggesting that only a few taxa can thrive in such harsh environmental conditions (Behnke et al. 2006). Nonetheless, molecular diversity surveys sometimes identified fungal to account for small but significant fractions of total microbial diversity (Stoeck et al. 2006). Moreover, SSU rRNA and microscopic observations have detected fungi up to 360‰ salinity (Edgcomb et al. 2009, Alexander et al. 2009, Stock et al. 2012). Some authors suggested that fungi might be a significant member of microbial communities (Jebaraj et al. 2010), and that their signatures in DHABs lower haloclines where organic detritus accumulate might indicate that fungi may be involved in organic matter degradation (Pachiadaki et al. 2014). Consistently, meta-transcriptomic analyses support this conclusion indicating that fungi

are not only present but also a metabolically active component of benthic microbial communities in DHABs systems (Bernhard et al. 2014, Edgcomb et al. 2016). In particular, the Urania middle halocline and Discovery lower halocline had high metabolic potential, with most transcripts affiliating with Malasseziomycetes, Dothideomycetes and Microbotryomycetes as well as to *Aspergillus* and *Penicillium* genera and various yeasts (Bernhard et al. 2014, Edgcomb et al. 2016). The quantitative relevance of transcripts belonging to saprophytic fungi in the Discovery and Urania basin (Edgcomb et al., 2016), further support their crucial role in carbon cycling. While these findings shed light on the potential ecological role and diversity of fungal communities in Urania and Discovery, L'Atalante had rare or absent fungal transcripts suggesting that the elevated salinity of the lower halocline may represent a barrier for most fungi (Edgcomb et al., 2016). There is also some evidence that competition can occur among members of the microbial community (Edgcomb et al., 2017). For example, expression of antibiotic production or resistance genes, including fusaric acid produced by the genus *Fusarium* has been reported in the Discovery upper and lower halocline and the Urania middle halocline (Edgcomb et al., 2017). Indeed, while fungi might not be abundant or hyperdiverse, they could be involved in both biological and carbon and nutrient dynamics in some of the most adverse ecosystem on Earth.

2. Cold seeps

Cold seeps are geologically diverse reducing environments (Boetius and Wenzhöfer 2013) located near to tectonically active and passive continental margin (German et al. 2011). Generally, seep systems originate where reduced chemicals such as methane and sulphide subsurface interstitial fluids, resulting from biogenic or thermogenic origins, are released as a result of tectonic activity, differential compaction of organic-rich sediments, gas hydrate dissociation and subsurface salt migration (Figure 2). Here, chemosynthetic production provides nutrition to a variety of benthic and planktonic heterotrophic species which depends on the oxidation of reduced of sulphur and methane by microorganisms (Levin et al. 2016) while hard and biotic substrates produced by prokaryotic-driven carbonate precipitation, provide critical habitat for several chemosynthetic and heterotrophic organisms (Levin et al. 2016). When seepage ceases, these areas may still host a few species characteristic of seep habitats, but attract new assemblages including cold-water corals (Cordes et al. 2008).

Figure 2. Cold seep assemblages. Okeanos methane seep (image credit NOAA) and Carbonate pavement (image credit MARUM).

In the last three decades, extensive research has been carried out to investigate the diversity of bacterial, archaeal and metazoan assemblages living in cold seeps ecosystems (Levin et al. 2016). However, studies on the micro-eukaryotic component account for only a small number of studies. Nevertheless, these studies have revealed that a vast diversity of microbial eukaryotes including novel lineages and endemism which could be found in these habitats, including fungi (Table 2).

Table 2. Fungal diversity in deep-sea cold seeps. Stars correspond to taxa characterised by large fruiting bodies and strictly terrestrial.

Phylum	Closest relative	Depth (mbsl)	Location	Reference
Other	Basal Clone Group I	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	*Candida*	1170; 850–1200	Sagami Bay; Sagami-Bay	Takishita et al. 2007; Nagahama et al. 2011
Ascomycota	*Cladosporium*	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	*Coniothyrium*	850–1200	Sagami-Bay	Nagahama et al. 2011
Basidiomycota	*Cryptococcus curvatus*	9cm bsf; 650; 1170	Kuroshima Knoll; Sagami Bay)	Takishita et al. 2006; Takishita et al. 2007
Ascomycota	DSF-group1	2400	Alaminos Canyon 601 methane seep (Gulf of Mexico)	Thaler et al. 2012
Basidiomycota	*Entorrhiza fineranae*	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	*Geomyces*	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	*Hypocrea*	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	*Kluyveromyces nonfermentans*	850–1200	Sagami-Bay	Nagahama et al. 2011
Cryptomycota	LKM11	850–1200	Sagami-Bay	Nagahama et al. 2011
Basidiomycota	*Lopharia mirabilis**	850–1200	Sagami-Bay	Nagahama et al. 2011
Basidiomycota	*Malassezia restricta*	850–1200	Sagami-Bay	Nagahama et al. 2011

Table 2. Continued.

Phylum	Closest relative	Depth (mbsl)	Location	Reference
Ascomycota	*Metschnikowia*	850–1200	Sagami-Bay	Nagahama et al. 2011
Microsporidia	*Nematocenator marisprofundi*	587–810	Nematode; sulphide-oxidising bacterial mats at Hydrate Ridge	Sapir et al. 2014
Ascomycota	*Penicillium*	850–1200	Sagami-Bay	Nagahama et al. 2011
Basidiomycota	*Phallus hadriani* *	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	*Phoma herbarum*	850–1200	Sagami-Bay	Nagahama et al. 2011
Basidiomycota	*Rhodosporidium sphaerocarpum*	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	Soil Clone Group I	850–1200	Sagami-Bay	Nagahama et al. 2011
Basidiomycota	*Trechispora alnicola* *	850–1200	Sagami-Bay	Nagahama et al. 2011
Ascomycota	Uncultured	2400	Alaminos Canyon 601 methane seep; Gulf of Mexico	Thaler et al. 2012
Basidiomycota	Uncultured	2400	Alaminos Canyon 601 methane seep; Gulf of Mexico	Thaler et al. 2012
Chytridiomycota	Uncultured	2400	Alaminos Canyon 601 methane seep; Gulf of Mexico	Thaler et al. 2012
Chytridiomycota and Zygomycota	Unknown	1170	Sagami Bay	Takishita et al. 2007
Ascomycota	Unknown Saccharomycotina	850–1200	Sagami-Bay	Nagahama et al. 2011

Initial studies on fungal diversity in cold-seeps suggested that overall fungal diversity was low and the saprophytic basidiomycetous yeast *Cryptococcus curvatus* as the dominant eukaryotic component (Takishita et al. 2006). Consistently, microscopic observation provided further evidence of the quantitative importance of yeast-like cells (Takishita et al. 2007). Similarly, *C. curvatus* was dominant also in the sediments of a deep-sea methane cold seep (1170 mbsl) in Sagami Bay, Japan (Takishita et al. 2007). However, later studies identified a wider array of fungal diversity where *Candida* and phylotypes related to Chytridiomycota and Zygomycota with no affiliation with any sequence of known fungal species were also observed (Takishita et al. 2007). In addition, fungal-specific PCR-based analysis of environmental DNA in the deep-sea methane cold-seep area in Sagami-Bay (depth 850–1200m) identified 35 phylotypes, including 12 early diverging clones and 23 phylotypes within Dikarya (Nagahama et al. 2011) in which *Penicillium chrysogenum* was the dominant phylotypes (Lai et al. 2007, Bass et al. 2007). Similarly, a total of 39 fungal sequences were recovered from deep-sea methane seeps (2400 mbsl) at Alaminos Canyon in the Gulf of Mexico (Thaler et al. 2012). Consistently, Ascomycota accounted for the majority of recovered sequences, followed by Basidiomycota, and Chytridiomycota and the number of recovered sequences was highest at the sediment redox boundary where the most intense carbon and nutrient cycling take place (Thaler et al. 2012).

Authors suggest that fungi might contribute in a significant way to several processes within seep systems. Several fungal sequences retrieved from seep systems are known to have important degradation capabilities, while others, such as *Rhodosporidium sphaerocarpum* and *M. restricta,* are known to establish parasitic relationships with a broad set of organisms (López-García et al. 2007, Lai et al. 2007, Bass et al. 2007, Le Calvez et al. 2009). For example, many species of the order Saccharomycetales have a fermentative metabolism and the commonly retrieved *Candida* genus can grow under strictly anaerobic conditions consistent to those encountered in seep systems (Nagahama et al. 2011). Other taxa belonging to *Penicillium*, *Phoma*, *Cladosporium* and *Geomyces* genera possess enzymatic activity for lignocellulosic degradation (Junghanns et al. 2009) and perhaps involved in the degradation of recalcitrant compounds such as lignin and other carbohydrates (Damare et al. 2006b). *Cryptococccus* and its teleomorphs have been found to be common across various oceanic regions, and *C. curvatus* is a known opportunistic pathogen of animals, including humans, and has been suggested to be a possible pathogen of seeps animals as well (Takishita et al. 2006). *Malassezia* is known causative agents of skin diseases in mammals and invertebrates and also soil nematodes (Amend 2014). Therefore, it has been suggested that *Malassezia* species retrieved from seeps and other deep-sea systems might be associated with small marine invertebrates, such as nematodes or polychaetes (Nagahama et al. 2011, Amend 2014). However, the ecological relevance of fungi in biological and biogeochemical dynamics are still unclear and not often sufficiently addressed.

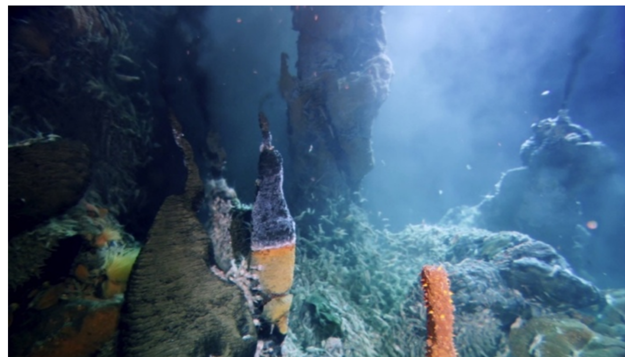
A particular exception is portrayed by the microsporidia Nematocenator marisprofundi. This genus was found infecting benthic nematodes at the Hydrate Ridge methane seeps site (Sapir et al. 2014). The infection was described as species-specific and temporally and spatially stable indicating an ecologically consistent host-parasite interaction with potentially significant consequences on nematodes population dynamics (Sapir et al. 2014). Supporting the authors' conclusions, Microsporidia fungi have been shown by ultrastructural and molecular approaches to be true parasites of several hosts in various marine habitats (Ardila-Garcia and Fast 2012).

3. Hydrothermal Vents

Hydrothermal vents are highly productive ecosystems, and by hosting giant tubeworms, large mussel and clam beds, and dense shrimp and crab aggregations are also among the most diverse deep-sea ecosystems (Levin et al. 2016). These geomorphological features occur in a vast range

of geological settings such as ridges, seamounts, back-arc basins, margins and trenches, where they originate from the outflow of chemically altered seawater, circulating from the ridge flanks through the crust near shallow magmatic intrusions at elevated temperature and pressure (Levin et al. 2016). Here, hard and biotic substrates produced at vents are used by benthic fauna for attachment, shelter and food, while microbial communities by chemosynthetic production provide nutrition to a variety of benthic and planktonic heterotrophic species (Figure 3; Levin et al. 2016).

Figure 3. Mid Atlantic Ridge hydrothermal vent systems, image credit BBC.



Probably owing to their diversity and centrality in biogeochemical cycles, extensive research has been carried out to explore hydrothermal vent communities. Fungi have been reported to have an essential role in C cycling in terrestrial vent systems (Pang and Mitchell 2005, Le Calvez et al. 2009) and can contribute to weathering processes of silicates and volcanic rocks (Etienne and Dupont 2002).

Active seamounts and ridges offer several low- and high-temperature hydrothermal habitats that are characterised by fluids with high contents of reduced metals such as Fe (II) and Mn(II) (Statham et al. 2005). In these environments, rich fungal communities have been reported in the actively growing Fe-oxide mats and basalt rock surfaces from the active volcano of the Vailulu'u Seamount (Connell et al. 2009). Connell and colleagues (2009) found that yeasts and yeast-like species were dominant, and fungal assemblage compositions significantly differed between in mat rather than rock substrates. Yeasts and filamentous fungi, including new species, have also been retrieved associated with hydrothermal water and sediment samples (Burgaud et al. 2009, 2010; Table 3), and strikingly, a significant portion of fungi has been recovered from hydrothermal shrimps and mussels (Burgaud et al. 2011). Notably, among the isolated fungi, *Aspergillus sydowii*, the causal agent of disease among sea fan corals (Soler-Hurtado et al. 2016), was detected suggesting possible interactions with cold-water corals.

Furthermore, Burgaud et al. (2009) retrieved fungi of the Chaetothyriales order from deep-sea mussel samples. This order has been associated with diseases of deep-sea mussels (Van Dover et al. 2007). These results confirmed earlier findings which suggest that the deep-sea hydrothermal systems of the Mid-Atlantic Ridge are characterised by the presence of abundant yeast community (Gadanho and Sampaio 2005). Again, molecular investigations targeting the SSU rRNA gene sequences identified many unknown sequences, even at high taxonomic levels in the Chytridiomycota, Ascomycota, and Basidiomycota phyla (Xu et al. 2017). Supporting previous findings regarding Chytridiomycota phylotypes affiliating to species that have not been described (Le Calvez et al. 2009).

Table 3. Fungal diversity in deep-sea hydrothermal vents. Stars correspond to taxa characterised by large fruiting bodies and strictly terrestrial.

Phylum	Closest relative	Depth (mbsl)	Location	Reference
Ascomycota	*Acremonium*	881	Mat from depression	Connell et al. 2009
Ascomycota	*Aspergillus*	2770; 707; 700; 2300	South MAR; Mat from Nafauna; Lost City (Siliceous sponge); Rainbow (Rimicaris)	Xu et al. 2017; Connell et al. 2009; Burgaud et al. 2009
Ascomycota	*Aureobasidium*	750; 2770	Lost City; South MAR	Lopez-Garcia et al. 2007; Xu et al. 2017
Ascomycota	*Botryotinia*	2770	South MAR	Xu et al. 2017
Ascomycota	*Candida*	2620; 800-3150; 2770; 2300	Lau Basin; MAR; South MAR; Rainbow	Burgaud et al. 2010; Gadanho and Sampaio 2005; Xu et al. 2017; Burgaud et al. 2010
Basidiomycota	*Ceratobasidium*	2770	South MAR	Xu et al. 2017
Ascomycota	*Chaetomium*	2770	South MAR	Xu et al. 2017
Chytridiomycota	*Chytridium*	1700; 860	Lucky Strike; Menez-Gwen	Le Calvez et al. 2009;
Ascomycota	*Cladosporium*	2770; 2770; 2300	South MAR; Rainbow (Rimicaris)	Xu et al. 2017; Burgaud et al. 2009
Ascomycota	*Clavispora*	881; 707	Mat from depression; Mat from Nafauna	Connell et al. 2009
Basidiomycota	*Cryptococcus*	1700; 2770; 881; 707; 860; 2300	Lucky Strike; South MAR; Mat from depression; Mat from Nafauna; Menez-Gwen; Rainbow	Le Calvez et al. 2009; Connell et al. 2009; Burgaud et al. 2010; Xu et al. 2017
Ascomycota	*Debaryomyces*	2620; 2300	Lau Basin; Rainbow	Burgaud et al. 2010
Basidiomycota	*Dioszegia*	707	Mat from Nafauna	Connell et al. 2009
Ascomycota	*Eupenicillium*	750	Lost City	Lopez-Garcia et al. 2007
Ascomycota	*Eutypa*	2630	Elsa (A. pompejana)	Burgaud et al. 2009
Ascomycota	*Exophiala*	2630; 800-3150; 2770; 860; 2300	Seawater; Elsa; MAR; South MAR; Menez-Gwen (B. azoricus); Rainbow (Rimicaris)	Gadanho and Sampaio 2005; Burgaud et al. 2009; Xu et al. 2017
Basidiomycota	*Filobasidium*	2630	Elsa	Le Calvez et al. 2009
Ascomycota	*Fusarium*	2770	South MAR	Xu et al. 2017
Ascomycota	Geomyces	881; 2300	Mat from depression; Rainbow (Rimicaris)	Connell et al. 2009; Burgaud et al. 2009
Ascomycota	Gleotinia	2770	South MAR	Xu et al. 2017
Ascomycota	Graphostroma	2770	South MAR	Xu et al. 2017
Ascomycota	Helicoon	2630	Elsa (A. pompejana)	Burgaud et al. 2009

Table 3. Continued.

Phylum	Closest relative	Depth (mbsl)	Location	Reference
Ascomycota	Helotiales	2630; 860; 2300; 2300; 2300	Elsa (A. pompejana); Menez-Gwen (B. azoricus); Rainbow (Rimicaris); Rainbow (B. azoricus commensal worm); Rainbow (B. azoricus)	Burgaud et al. 2009;
Ascomycota	Hexophiala	2630	Elsa	Le Calvez et al. 2009
Ascomycota	Hortaea	2300	Rainbow	Burgaud et al. 2010
Basidiomycota	Malassezia	750	Lost City	Lopez-Garcia et al. 2007
Ascomycota	Meyerozyma	2770	South MAR	Xu et al. 2017
Basidiomycota	Micropsalliota *	2770	South MAR	Xu et al. 2017
Ascomycota	*Penicillium*	2770	South MAR	Xu et al. 2017
Ascomycota	*Phaeotheca*	2300	Rainbow	Burgaud et al. 2010
Ascomycota	*Phialemonium*	2770	South MAR	Xu et al. 2017
Ascomycota	*Phialophora*	2770	South MAR	Xu et al. 2017
Ascomycota	*Phoma*	2770	South MAR	Xu et al. 2017
Ascomycota	*Pichia*	700; 800-3150; 881; 707	Lost City; MAR; Mat from depression; Mat from Nafauna	Burgaud et al. 2010; Gadanho and Sampaio 2005; Connell et al. 2009
Ascomycota	*Ramichloridium*	2770	South MAR	Xu et al. 2017
Basidiomycota	*Rhodosporidium*	800-3150; 881; 2300	MAR; Mat from depression; Rainbow	Gadanho and Sampaio 2005; Connell et al. 2009; Burgaud et al. 2010
Basidiomycota	*Rhodotorula*	1667; 800-3150; 2770; 2300	Exposure experiment; MAR; South MAR; Rainbow	Gadanho and Sampaio 2005; Connell et al. 2009; Burgaud et al. 2010; Xu et al. 2017
Ascomycota	*Scolecobasidium*	2300	Rainbow (Coral)	Burgaud et al. 2009
Basidiomycota	*Sporidiobolus*	707	Mat from Nafauna	Connell et al. 2009
Basidiomycota	*Sporobolomyces*	2770; 2300	South MAR; Rainbow	Burgaud et al. 2010 Xu et al. 2017;
Ascomycota	*Stachybotrys*	2770	South MAR	Xu et al. 2017
Basidiomycota	*Tilletiopsis*	2630; 2770	Elsa (Riftia); South MAR	Burgaud et al. 2009; Xu et al. 2017
Ascomycota	*Toxicocladosporium*	2770	South MAR	Xu et al. 2017
Basidiomycota	*Trichosporon*	800-3150; 2770	MAR; South MAR	Gadanho and Sampaio 2005; Xu et al. 2017
Basidiomycota	Unknown Agaricomycotina	1700; 860	Lucky Strike; Menez-Gwen	Le Calvez et al. 2009
Ascomycota	Unknown Chaetothyriales	1990	Mussel Hill hydrothermal vent	Van Dover et al. 2006
Ascomycota	Unknown Dothideomycetes	2630; 1700; 860	Elsa (A. pompejana); Lucky Strike; Menez-Gwen	Burgaud et al. 2009; Le Calvez et al. 2009
Ascomycota	Unknown Eurotiales	2300	Rainbow	Burgaud et al. 2009
Basidiomycota	*Tilletiopsis*	2630; 2770	Elsa (Riftia); South MAR	Burgaud et al. 2009; Xu et al. 2017
Ascomycota	*Toxicocladosporium*	2770	South MAR	Xu et al. 2017
Basidiomycota	*Trichosporon*	800-3150; 2770	MAR; South MAR	Gadanho and Sampaio 2005; Xu et al. 2017
Basidiomycota	Unknown Agaricomycotina	1700; 860	Lucky Strike; Menez-Gwen	Le Calvez et al. 2009
Ascomycota	Unknown Chaetothyriales	1990	Mussel Hill hydrothermal vent	Van Dover et al. 2006
Ascomycota	Unknown Dothideomycetes	2630; 1700; 860	Elsa (A. pompejana); Lucky Strike; Menez-Gwen	Burgaud et al. 2009; Le Calvez et al. 2009
Ascomycota	Unknown Eurotiales	2300	Rainbow	Burgaud et al. 2009
Ascomycota	Unknown Hypocreales	860; 3650	Menez-Gwen (B. azoricus); TAG (Rimicaris)	Burgaud et al. 2009
Basidiomycota	Unknown Sebacina *	2770	South MAR	Xu et al. 2017
Ascomycota	Unknown Sordariomycetes	2770	South MAR	Xu et al. 2017
Basidiomycota	Unknown Tomentella	2770	South MAR	Xu et al. 2017
Ascomycota	*Xylaria* *	2770	South MAR	Xu et al. 2017

Although we are getting a glimpse of the diversity of fungal communities at hydrothermal vents we still largely ignore their ecological relevance. Several yeast species retrieved from hydrothermal sites are capable of producing siderophores to acquire and utilise Fe (III), and *Rhodotorula graminis* is an Mn (II)-oxidizing fungus and might be involved in iron and manganese cycling. However, no study investigated such processes in deep-sea hydrothermal vents where fungi can scavenge iron and other metals by several pathways and may be functionally relevant in their biogeochemical cycling (Connell et al. 2009).

Fungi are known parasites of a multitude of organisms, and parasitic infections play significant roles in population dynamics. In hydrothermal vents, fungi have been found associated with benthic fauna. For example, at the Mussel Hill hydrothermal vent in Fiji Basin, a Capronia-like black yeast (order Chaetothyriales) was reported to induce a hemocytic immune response in the mussels *Bathymodiolus brevior* with tissue deterioration (Van Dover et al. 2007). Although it is not clear if the fungal infections were secondary or facilitated by concurrent infections of other pathogens, the identification of fungi in otherwise healthy individuals makes it clear that the fungus is not a strict saprophyte. The described infection and the high prevalence of the fungus within the population, progressive and pervasive connective tissue degradation, together with decreased volume of bacteriocytes and symbiotic bacteria associated with the black-body stage of the disease of the mussel, suggest that massive mussel mortality were imminent within the Mussel Hill vent field (Van Dover et al. 2007).

Furthermore, yeasts and filamentous fungi retrieved from hydrothermal vent fauna suggest that fungi can benefit from nutrients as in the case of those isolated from gill chambers of shrimps or have a role in the decomposition of organic material entrapped in mussel byssi (Burgaud et al. 2009, 2010). Supporting pathogenic associations, *Scolecobasidium* sp.– the causative agent of necrotic patches on different corals–was found associated to deep-sea corals, near Rainbow hydrothermal vent site, indicating that this halophilic fungus could be implicated in deep-sea coral diseases. Also, the pathogenic genus *Exophiala* sp. Was repeatedly reported from sediments (Gadanho and Sampaio 2006, Burgaud et al. 2009, Xu et al. 2017) and alongside nematodes (Bhadury et al. 2009).

4. Deep-sea sediments

Benthic deep-sea ecosystems are represented mainly by soft sediments characterised by the presence of highly diverse taxa belonging to prokaryotes and metazoans (i.e. meiofaunal and macrofaunal organisms; Corinaldesi 2015). Metazoan life is generally present only in the top 20-50 cm of the sediment, whereas prokaryotes are present even in the deep biosphere (i.e. several hundred meters below the sediment surface; Orcutt et al. 2013).

Figure 4. Abyssal plane, image credit BBC.

One of the first reports of fungi in deep-sea sediments is dated back to the '60s (Roth 1964). Since then, several known and new fungal taxa have been found in different deep-sea sedimentary habitats (Nagahama et al. 2003). However, despite these reports, the number of studies addressing fungal diversity and ecology in deep-sea sediments are still relatively scarce compared to other benthic components.

Concerning diversity, Ascomycota and Basidiomycota represent the most abundant groups (Singh et al. 2011, Zhang et al. 2016; Table 4). Although Basidiomycota has been claimed to account for a relatively low diversity, different methodological approaches have provided contrasting results. For example, multiple-primer amplification and sequencing of sediment samples of the Pacific Ocean retrieved seven basidiomycete classes and only five Ascomycete classes along with two uncultured fungal groups affiliating with uncultured soil fungal taxa and uncultured Zygomycetes (Xu et al. 2016). Moreover, a large number of sequences with low similarity to public databases even at high taxonomic levels has been reported (Xu et al. 2014).

Table 4. Fungal diversity from deep-sea sediments

Phylum	Closest relative	Depth (mbsl)	Location	Reference
Ascomycota	Acremonium	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Aspergillus	5000; 5017-6986; 1190-1589	CIB; Pacific; Okinawa	Singh et al. 2010; Xu et al. 2014; Zhang et al. 2016
Ascomycota	Aureobasidium	5017-6986	Pacific	Xu et al. 2014
Basidiomycota	Auricularia	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Bionectriaceae	5000	CIB	Singh et al. 2010
Ascomycota	Candida	1200-10000; 5000; 5017-6986	Pacific; CIB; Pacific	Nagano et al. 2010; Singh et al. 2010; Xu et al. 2014
Ascomycota	Capnodium coffeae	5000	CIB	Singh et al. 2010
Ascomycota	Chaetomium	5017-6986; 1190-1589	Pacific; Okinawa	Xu et al. 2014; Zhang et al. 2016
Ascomycota	Cladosporium	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Coniellab	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Corynespora	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Cryptococcus	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Debaryomyces yamadae	5000	CIB	Singh et al. 2010
Ascomycota	Emericella	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Exophiala	1190-1589; 5017-6986	Okinawa; Pacific	Xu et al. 2014; Zhang et al. 2016
Ascomycota	Faurelina	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Fusarium	5017-6986; 1190-1589	Pacific; Okinawa	Xu et al. 2014; Zhang et al. 2016
Ascomycota	Gloeotinia	1200-10000; 5017-6986	Pacific; Pacific	Nagano et al. 2010; Xu et al. 2014
Ascomycota	Hortaea	5000	CIB	Singh et al. 2010
Ascomycota	Lecanicillium	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Lecythophora	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Leptosphaeria	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Lophiostoma	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Malassezia	5000; 5017-6986	CIB; Pacific	Singh et al. 2010; Xu et al. 2014
Ascomycota	Meyerozyma	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Microdochium	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Minimidochium	1190-1589	Okinawa	Zhang et al. 2016
Zygomycota	Mortierella	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Myriogenospora	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Myrothecium	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Nectria	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Nodulisporium	5000	CIB	Singh et al. 2010
Ascomycota	Paecilomyces	5017-6986; 1190-1589	Pacific; Okinawa	Xu et al. 2014; Zhang et al. 2016
Ascomycota	Penicillium	1200-10000; 5017-6986; 1190-1589	Pacific; Pacific; Okinawa	Nagano et al. 2010; Xu et al. 2014; Zhang et al. 2016
Ascomycota	Periconia sp.	5017-6986	Pacific	Xu et al. 2014
Chytridiomycota	Phlyctochytrium	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Phoma	5000; 1190-1589	CIB; Okinawa	Singh et al. 2010; Zhang et al. 2016
Ascomycota	Pichia	5000	CIB	Singh et al. 2010
Ascomycota	Plectosphaerella	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Pleurotus *	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Podospora	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Preussia	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Pseudogymnoascus	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Ramichloridium	5017-6986	Pacific	Xu et al. 2014
Basidiomycota	Rhodosporidium	5000	CIB	Singh et al. 2010
Basidiomycota	Russula *	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Sagenomella	5000	CIB	Singh et al. 2010
Basidiomycota	Schizophyllum *	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Simplicillium	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Stemphylium	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Stenella musicola	5000	CIB	Singh et al. 2010
Ascomycota	Thelebolus	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Trametes *	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Trichocladium	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Trichosporon	1200-10000; 5000; 5017-6986	Pacific; CIB; Pacific	Nagano et al. 2010; Singh et al. 2010; Xu et al. 2014

Table 4. Continued

Phylum	Closest relative	Depth (mbsl)	Location	Reference
Ascomycota	Ulospora bilgramii	5000	CIB	Singh et al. 2010
Basidiomycota	Unknow Polyporales	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Unknown	5000	CIB	Singh et al. 2010
Basidiomycota	Unknown	5000	CIB	Singh et al. 2010
Ascomycota	Unknown	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Unknown Agaricomycetes	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Unknown Dothideomycetes	5000	CIB	Singh et al. 2010
Ascomycota	Unknown Dothideomycetes	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Unknown Eurotiomycetes	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Unknown Leotiomycetes	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Unknown Lycoperdaceae *	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Unknown Pezizomycetes	1190-1589	Okinawa	Zhang et al. 2016
Ascomycota	Unknown Pleosporales	5017-6986	Pacific	Xu et al. 2014
Ascomycota	Unknown Sordariomycetes	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Unknown Thelephoraceae *	5017-6986	Pacific	Xu et al. 2014
Basidiomycota	Unknown Trechisporales *	5017-6986	Pacific	Xu et al. 2014
Basidiomycota	Unknown Tremellomycetes	1190-1589	Okinawa	Zhang et al. 2016
Basidiomycota	Wallemia	5000	CIB	Singh et al. 2010
Ascomycota	Xylaria *	5017-6986	Pacific	Xu et al. 2014

Fungi in deep-sea sediments are thought to be involved in carbon cycling and symbioses. For instance, fungal hyphae have been observed to grow under high hydrostatic pressure forming various stages of accretion of particles around them, leading to the formation of aggregates withholding of humic material, carbohydrate, and proteins (Damare and Raghukumar 2008). Such evidence suggests that fungi in deep-sea sediments may be involved in humic aggregate formation as it happens in terrestrial habitats, indicating indeed that carbon sequestration process and benthic deep-sea food web needs to be further investigated. Furthermore, several genera including *Rhodotorula*, *Rhodosporidium*, *Malassezia*, *Trichosporon* and *Sterigmatomyces* comprehend several pathogens and parasites also of marine animals such as bivalves, nematodes and other organisms (Singh et al. 2011, Lin et al. 2012). For example, *Paecilomyces* and *Myrothecium* species were repeatedly found while surveying nematode molecular diversity, indicating a possible interaction between fungi and nematodes (Bhadury et al. 2009, 2011). Therefore, fungi can have a profound effect on biogeochemical processes and food web dynamics in sedimentary habitats as in soil.

Interestingly, fungal-nematodes relationships in benthic deep-sea ecosystems are neglected. However, because the critical ecological role of nematodes in these environments (Danovaro et al. 2008), the fungus-nematode interactions should be further investigated to understand the magnitude and direction of such relationship. For example, the co-amplified *Paecilomyces* and *Myrothecium* are known to include nematicidal species and often used as pest control.

5. Deep-sea sub-surface sediments

While debate continues whether Archaea or Bacteria predominate in sub-surface sediments (Schippers and Neretin 2006, Smith et al. 2011, Jorgensen 2011, Orcutt et al. 2013), fungi remain understudied despite they have been identified from a few centimetres below the seafloor (Damare et al. 2006a) down to 1740 m below the sediment surface (Ciobanu et al. 2014).

Fungi have been reported to dominate over other eukaryotic groups in sub-surface sediments collected in different oceanographic regions (Edgcomb et al. 2011a, Ciobanu et al. 2014; Table 5). Although some study did not specifically target fungal diversity, DNA- and RNA-based clone libraries suggest that Basidiomycetes and Ustilaginusmycets were the dominant fungal taxa and that fungal diversity decrease with increasing depth into the sediment. Tremellomycetes, Sordariomycetes and Eurotiomycetes were abundant at shallow depths while Saccharomycetes were detected even at depths between 630 and 1’365 m while deeper layers were dominated by Wallemiomycetes, Microbotryomycetes and Tremellomycetes (order Filobasidiales) (Ciobanu et al. 2014) indicating that depth within the sediment could be responsible for selection con community structuring processes. Similarly, DNA and RNA fungal signatures within subseafloor sediments of the Canterbury basin (New Zealand) by 454 pyrotag sequencing with fungal-specific primer set confirmed that a large number of OTUs belonging to Basidiomycota yeasts. This study also revealed low but unexpected fungal diversity along the sediment depth with some taxa occurring only at specific depths and others that were found in all samples. For example, *Leptosphaerulina* was found only at 346 mbsf while *Pleurostomophora and Exophiala* were only detected at 583 mbsf. These genera were correlated with high porosity and high organic carbon concentration occurring in the upper layers of the sediment. On the contrary, *M. guillermondii*, which also represented an abundant OTU, was detected throughout all the sediment layer using universal eukaryotic primer sets, but not using fungal-specific ITS primers. *Cryptococcus* also represented one of the most

abundant OTUs and its relative abundance has been found to be positively related with the concentration of methane supporting earlier evidence indicating *Cryptococcus* as the dominant taxon in methane seeps (Takishita et al. 2006).

Table 5. Fungal diversity in deep-sea subsurface sediments

Phylum	Closest relative	Depth (mbsl)	Depth (mbsf)	Location	Reference
Ascomycota	*Acremonium*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Aspergillus*	n.d.	4-1884; 12-31-40	Canterbury Basin; Suruga Bay	Redou et al. 2015; Nagano et al. 2016
Ascomycota	*Batcheloromyces*	344	346-1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Bjerkandera* *	344	4-1884	Canterbury Basin	Redou et al. 2015
Basidiomycota	*Bullera*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Cadophora*	n.d.	3	Suruga Bay	Nagano et al. 2016
Ascomycota	*Cladophialophora*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Cladosporium*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Cordyceps*	344	4-1884	Canterbury Basin	Redou et al. 2015
Basidiomycota	*Cryptococcus*	344	346–1740	Canterbury Basin	Redou et al. 2014
Ascomycota	*Cyberlindnera*	344	346–1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Elmerina* *	344	346–1740	Canterbury Basin	Redou et al. 2014
Ascomycota	*Eurotium*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Exophiala*	344	4-1884	Canterbury Basin	Redou et al. 2014; Redou et al. 2015
Basidiomycota	*Filobasidium*	344	346–1740	Canterbury Basin	Redou et al. 2014
Ascomycota	*Fusarium*	344	4-1884	Canterbury Basin	Redou et al. 2014; Redou et al. 2015
Ascomycota	*Galactomyces*	344	346–1740	Canterbury Basin	Redou et al. 2014
Ascomycota	*Leptosphaerulina*	344	346–1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Leucosporidiella*	344	346–1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Malassezia*	344	346–1740	Canterbury Basin	Redou et al. 2014
Ascomycota	*Meyerozyma*	344	4-1884	Canterbury Basin	Redou et al. 2014; Redou et al. 2015
Ascomycota	*Microascus*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Oidiodendron*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Paecilomyces*	344; n.d.	4-1884; 12	Suruga Bay	Redou et al. 2015; Nagano et al. 2016
Ascomycota	*Penicillium*	344	4-1884	Canterbury Basin	Redou et al. 2014; Redou et al. 2015
Ascomycota	*Phialophora*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Pichia*	n.d.	3-12	Suruga Bay	Nagano et al. 2016
Ascomycota	*Pleurostomophora*	344	346-1740	Canterbury Basin	Redou et al. 2014
Ascomycota	*Purpureocillium*	344	4-1884	Canterbury Basin	Redou et al. 2015
Ascomycota	*Rhinocladiella*	344	346-1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Rhodosporidium*	344	346-1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Rhodotorula*	344	4-1884	Canterbury Basin	Redou et al. 2014; Redou et al. 2015
Ascomycota	*Sarocladium*	344	4-1884	Canterbury Basin	Redou et al. 2015
Basidiomycota	*Sistotrema* *	344	4-1884	Canterbury Basin	Redou et al. 2015
Basidiomycota	*Trametes* *	344	4-1884	Canterbury Basin	Redou et al. 2015
Basidiomycota	*Tremella* *	344	346-1740	Canterbury Basin	Redou et al. 2014
Ascomycota	*Trichoderma*	344	346-1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Trichosporon*	344	346-1740	Canterbury Basin	Redou et al. 2014
Ascomycota	Unknown Chaetothyriales	344	346-1740	Canterbury Basin	Redou et al. 2014
Basidiomycota	*Wallemia muriae*	344	346-1740	Canterbury Basin	Redou et al. 2014

Information on diversity of fungi in the deep biosphere obtained through DNA analysis does not allow to infer on their ecological significance since fungal sequences may belong to extracellular DNA not associated with living biomass (Coolen et al. 2006, Boere et al. 2011, Kouduka et al. 2017) or to fungi in dormant stage. The majority of subsurface microbes are on

physiological standby, with only a small fraction actually active (Jorgensen 2011). However, meta-transcriptomic analyses instead suggest the presence of an active fungal community (Pachiadaki et al. 2016). Fungal-Associated transcripts for metabolic and cellular processes, cell and membrane functions, and catalytic activities have been observed in several sediment samples (Pachiadaki et al. 2016). Furthermore, fungal communities at comparable depths at the two geographically separated locations appear dominated by distinct taxa, and different gene expression. These results suggest that different organic content and quality and location might shape different fungal communities (Pachiadaki et al. 2016). Also, microscopic analysis of Canterbury Basin sediment samples from 4 and 403 mbsf revealed the presence of fungal structure and conidiogenesis (Pachiadaki et al. 2016).

Culture-dependent methods on sediment samples down to 344 mbsl in the Canterbury Basin (New Zealand) allowed the recovery of both filamentous and yeast forms, and molecular investigations have allowed identifying 21 genera belonging to Ascomycota and Basidiomycota and one uncultured Agaromycetes (Rédou et al. 2015). The majority of taxa affiliated with *Rhodotorula* and *Meyrozyma.* In particular, the red yeast *Rhodotorula mucilaginosa* is widely distributed, and it has also been isolated from deep-sea sediments and basaltic crust (Singh et al. 2010). This study also indicated a negative relationship between diversity and increasing depth with 25 different species within the uppermost samples (4 to 37 mbsf) and only eight different species between 137 and 1884 mbsf (Rédou et al. 2015). Similarly, culturable fungal communities were investigated in marine subsurface sediment from 3 to 40 mbsl in Suruga Bay (Nagano et al. 2016). Although occurrence and diversity of culturable fungi were extremely low, fungi were successfully cultured from 5 out of 15 samples, and *Aspergillus*, *Pichia* and *Cadophora* represented the most common genera.

Species found in these studies have a high potential as saprotrophs and can be competitors of prokaryotic communities for the limited resource available in this environment. For example, the red yeast *Rhodotorula mucilaginosa* and *Fusarium oxysporum*, reported as the most abundant taxon in the Canterbury Basin sediment, are widely distributed organisms, even in deep-sea sediments and basaltic crust (Singh et al. 2010, Smith et al. 2011, Rédou et al. 2015). In particular, Fusarium oxysporium has been shown to grow under deep-sea marine conditions Burgaud et al. (2013) and to have important denitrifying capabilities (Cathrine and Raghukumar 2009) thus representing a competitor of prokaryotes for nitrogen substrates. These

hypotheses are also supported by transcriptomic analyses which revealed that fungi although occurring in lower abundance than prokaryotes, possess the ability to degrade a variety of organic substrates in deep-subseafloor sediments (Orsi et al. 2013a).

Supporting the suggested metabolic activities of fungi in such harsh environments, fungal diversity has been related to organic matter availability (Ciobanu et al. 2014, Rédou et al. 2015). For example, *Leptosphaerulina chartarum*, *Fusarium solani*, *Trichoderma sp.*, *Cyberlindnera jadinii* and in particular *Cryptococcus curvatus* were found strictly related to methane contents. Consistently, *C. curvatus* is the dominant taxa in methane seeps systems (Takishita et al. 2006). *Trichosporon mucoides*, *Malassezia pachydermatis*, *Meyerozyma guilliermondii*, *Pleurostomophora richardsiae*, *Exophiala dermatitidis* were only found in sediment samples with a low organic carbon concentration (Rédou et al. 2014). These heterotrophic fungi have also been retrieved from other deep sediments in which they have been demonstrated to be active members of microbial communities (Orsi et al. 2013b, Pachiadaki et al. 2016).

Overall, studies on micro-eukaryotic communities in subsurface sediments indicate that fungi are not only diverse but also viable. Indeed, these results suggest that fungi represent a ubiquitous component of benthic deep-sea ecosystems likely with major implications in C and nutrient cycling (Ciobanu et al. 2014).

Conclusions and perspectives

Fungi have been retrieved from any benthic deep-sea ecosystem investigated so far, but their ecology and the drivers influencing their abundance and diversity are still far to be elucidated. From the available information, it appears that the overall fungal diversity in benthic deep-sea ecosystems is relatively low, but some studies pointed out that fungi might often be the dominant micro-eukaryotic component. Concerning fungal diversity, Ascomycota and Basidiomycota often account for the largest share of reads while other phyla are scarce or absent. However, results on fungal diversity are frequently inconsistent with the literature likely resulting from differences in DNA isolation, primer selection and sequencing techniques, highlighting the need to standardise assessment protocols to compare fungal diversity more effectively (Zhang et al. 2016). For instance, high-throughput sequencing approach, the most successful and most widely adopted sequencing platform, had been widely applied in

investigating fungal diversity of many environmental samples, such as soil, plants and marine corals, it was rarely applied in exploring the fungal diversity of deep-sea sediment samples (Zhang et al. 2016). Furthermore, despite the use of fungal-specific primers revealed the presence of diverse fungal phylotypes, the specific primers designed for amplification and sequencing of fungal 18S, ITS or 28S rDNA regions might be biased towards specific fungal taxonomic groups (Singh et al. 2012). For instance, deep-sea sediment samples from the Pacific Ocean provided different results when different primer sets were employed (Xu et al. 2016). At the OTU level, the ITS resulted in 18 OTUs while, the 18S rDNA primer pairs returned 44 different OTUs and the 28S rDNA primers pair 78 OTUs, including 41 OTUs. At the same time, community structure changed based on the primer set used. Moreover, all studies highlighted that a significant fraction of sequences fails to match public database even at a high taxonomic level, suggesting the presence of taxa to be identified yet. Primer set selection and limited match of the retrieved sequences to public databases can be one of the main reasons for the low diversity described.

There is evidence that environmental conditions may have a role in shaping fungal assemblage composition. For instance, patterns of fungal diversity in marine ecosystems suggest that fungal communities can depend on the amount and quality of organic carbon (Tisthammer et al. 2016). However, information on this issue is still too limited to conclude, especially for benthic deep-sea ecosystems. Moreover, the quantitative relevance and ecological role of fungi in benthic deep-sea ecosystems are still largely unknown. Also, several fungal signatures have been observed during nematodes DNA barcoding providing further evidence that some fungi can be involved in parasitic associations with nematodes (Troemel et al. 2008, Ardila-Garcia and Fast 2012). Such findings suggest that fungi might also have an essential role in controlling population and community dynamics of metazoans (i.e. nematodes) inhabiting benthic deep-sea ecosystems (Vanreusel et al. 2010), but evidence regarding the involvement of fungi in pathogenic or parasitic associations is rare or absent in benthic deep-sea ecosystems. Overall, there is an urgent need to improve our knowledge of factors influencing the abundance and diversity of fungi in different benthic deep-sea habitats and ecosystems for a better understanding of the functioning of the largest and less explore biome on Earth.

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Chapter 2

Benthic deep-sea fungi in submarine canyons of the Mediterranean Sea

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Abstract: Fungi are ubiquitous components of microbial assemblages in aquatic ecosystems, but their quantitative relevance, ecological role and diversity in benthic deep-sea ecosystems are still largely unknown. Here, we investigated patterns and drivers of benthic fungal abundance, biomass and diversity from 200 to 1000 m depth in three submarine canyons of the Mediterranean Sea (Tricase, Crotone and Squillace canyons). The Crotone and Squillace canyons, which are close to the coast and influenced by river inputs, showed significantly higher fungal abundance, biomass and diversity (as operational taxonomic units, OTUs) compared with the Tricase canyon that was far from the coast and without nearby estuaries. Fungal biomass, abundance and diversity increased with increasing concentrations of carbohydrates, which in deep-sea sediments include highly refractory organic compounds. Overall, a total of 1742 fungal OTUs, belonging to all fungal phyla known to date, were found and Ascomycota represented the dominant phylum. However, only 36% of the reads belonged to known genera. In particular, Tricase and Crotone canyons hosted the highest proportion of unknown fungal taxa, suggesting that deep-sea sediments can harbour a high number of novel fungal lineages. Our findings also reveal that fungal assemblage composition in the investigated canyons was influenced by trophic and thermohaline conditions, which may promote a high turnover diversity of benthic deep-sea fungal assemblages. Overall results reported here indicate that the submarine canyons of the Mediterranean Sea can represent hot-spots of abundant and highly diversified fungal assemblages and pave the way for a better understanding of the ecological role of fungi in the largest ecosystem on Earth.

Key Words: Benthic deep-sea ecosystems, fungal abundance, fungal diversity, submarine canyons, Mediterranean Sea

Introduction

Deep-sea ecosystems represent more than 65% of the world's surface and >95% of the global biosphere (Herring, 2002), and host yet undiscovered biodiversity and a significant portion of the world's genetic diversity (Danovaro et al., 2017). In benthic deep-sea ecosystems, biomass is dominated by bacteria and archaea, followed by unicellular eukaryotes and small metazoans (<0.5 mm in size, meiofauna). These organisms are essential for carbon cycling and nutrient regeneration, and thus vital for sustaining oceanic production (Dell'Anno and Danovaro, 2005, Sogin et al., 2006, Jørgensen and Boetius, 2007, Danovaro et al., 2015, Danovaro et al., 2017). Recent findings, based on culture-dependent and independent approaches, revealed that fungi are present in deep-sea environments across a variety of ecosystem types spanning from hypersaline anoxic basins (Bernhard et al., 2014, Edgcomb et al., 2017) to cold seeps (Nagahama et al., 2011, Thaler et al., 2012), from hydrothermal vents (Burgaud et al., 2009, Burgaud et al., 2010, Xu et al., 2017) to surface and subsurface sediments (Orsi et al., 2013, Pachiadaki et al., 2016). Fungi have also been reported as the dominant unicellular eukaryotic group in the marine snow in bathypelagic waters with biomass similar to that of prokaryotes (Pernice et al., 2015, Pernice et al., 2016, Bochdansky et al., 2017).

Theoretical estimates suggest that fungi can be the most diversified component of unicellular eukaryotes on Earth, with more than 5 million species of which only 5% have been described (Hawksworth, 1997, Blackwell, 2011). This gap applies in particular to open ocean ecosystems where a significant fraction of fungal diversity is still unknown (Jeffries et al., 2016). Recent studies suggest that a variety of environmental factors (e.g. temperature, salinity, nutrients) can influence the diversity and assemblage composition of fungi in marine ecosystems (Li et al., 2016, Tisthammer et al., 2016). However, drivers controlling the distribution and diversity of fungi in benthic deep-sea ecosystems remain to date largely unexplored.

In terrestrial and freshwater ecosystems, fungi are among the main decomposers of organic matter, and they play an important role in the processing of the most refractory fraction of organic carbon (Carlile et al., 2001, Clipson et al., 2006, Hwang et al., 2006, Dighton, 2007). In marine sediments, and especially in benthic deep-sea ecosystems, the sedimentary organic matter is typically composed of a large fraction of recalcitrant compounds, resistant to biological degradation (Pusceddu et al., 2009). Fungi are expected to be specialized in the decomposition of refractory organic compounds, yet their role in C cycling in benthic deep-sea

ecosystems remains poorly understood (Hyde et al., 1998, Burgaud et al., 2009, Cathrine and Raghukumar, 2009, Jebaraj et al., 2010).

In this study, we investigated the abundance, biomass and taxonomic composition of fungal assemblages along the continental margins of the Central Mediterranean Sea. Continental margins are characterised by open slopes and submarine canyons, which are essential for C cycling and nutrient regeneration processes at a global scale (Bousquet et al., 2000, Dickens, 2003, Fernandez-Arcaya et al., 2017). In particular, submarine canyons can channel large amounts of organic matter photosynthetically produced from the continental shelf down to deep-sea ecosystems (Monaco et al., 1999, Sànchez-Vidal et al., 2008, Allen and Durrieu de Madron, 2009, Puig et al., 2014). For this reason, we selected three submarine canyons characterised by different environmental conditions and investigated fungal abundance, biomass and diversity at depths ranging from 200 to 1000 m. To identify the factors potentially controlling their quantitative importance and diversity in deep-sea sediments, we explored the role of environmental conditions, including the organic matter quality and quantity.

Materials and methods

Study area and sampling design

Sediment sampling was carried out in the Ionian Sea (Central Mediterranean Sea) during the oceanographic cruise "SAND 2016" held on board of the research vessel R/V Minerva Uno in May 2016. Sediment samples were collected within the main axis of three canyons located along the SE Italian margin at 200, 500 and 1000 m depths (Fig. 1). One of the investigated canyons (hereafter defined "Tricase") located along the Apulian margin, is far from any continental freshwater inputs. The other two investigated canyons are located along the Calabrian margin and were close to river estuaries. The Northern canyon, extending for about 30 km, is located in front of the Crotone municipality (canyon "Crotone") and its head is close to Neto river mouth. The head of the canyon "Squillace" is close to the coastline in front of the Squillace municipality and is characterised by the presence of sporadic, but intense river inputs from Ghetterllo stream. Sediment samples were collected at each benthic site by independent multiple corer deployments.

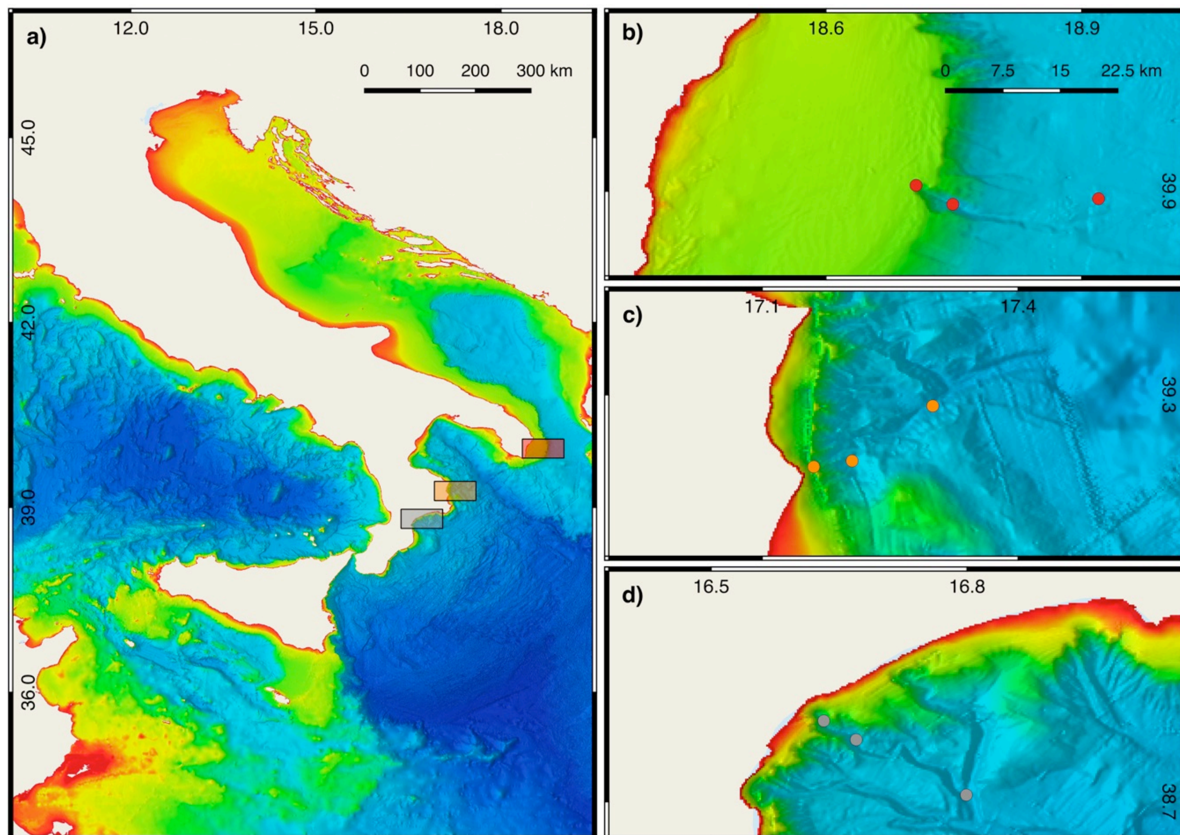

Figure 1. Study area and sampling location (a). Details of benthic sites investigated within Tricase (a), Crotone (b) and Squillace (c) canyons. Bathymetry has been obtained from EMODnet (http://portal.emodnet-bathymetry.eu). Maps elaborated with QGIS.

The top 1 cm of each sediment sample was used for the analysis of the quantity and biochemical composition of organic matter, fungal abundance (based on q-PCR analysis of 18S rRNA genes), biomass and diversity. At each station, temperature and salinity of bottom waters were measured using CTD casts.

Quantity and biochemical composition of organic matter

The three major biochemical classes of organic compounds (proteins, carbohydrates and lipids) in deep-sea sediments were determined according to previously described procedures (Danovaro, 2010). Briefly, about 0.5 g of wet sediment was used for each analysis. Protein concentration was obtained via colourimetric method, which allows the reaction of proteins with rameic tartrate and the Folin–Ciocalteau reactive in a basic environment (pH 10). The reaction provides a stable blue colouration whose intensity is proportional to the protein concentration in the reaction solution. Carbohydrate concentration was obtained via

colourimetric assay based on the reaction between sugars and phenol in the presence of concentrated sulfuric acid. Lipids were extracted by direct elution with chloroform and methanol followed by reaction with sulfuric acid. Protein, carbohydrate and lipid concentrations were determined spectrophotometrically and expressed as albumin, glucose and tripalmitin equivalents, respectively. All analyses were carried out in 3 replicates. Protein, carbohydrate and lipid concentrations were then converted to carbon equivalents (conversion factors: 0.49, 0.40 and 0.75 gC g^{-1}, respectively) to determine biopolymeric C content in the sediments (Dell'Anno et al., 2002).

Fungal biomass

To detect and quantify fungi in the sediment samples, fluorescence in-situ hybridisation (FISH) coupled with Calcofluor white staining (which targets chitin, cellulose and carboxylated polysaccharides) have been used following procedures previously described (Bochdansky et al., 2017). The FISH reaction was performed using the Pan-Fungal probe PF2 (5′-CTCTGGCTTCACCCTATTC-3′) Cy-3 labelled (Kempf et al., 2000). Briefly, about 1 g of sediment was fixed for 1 h in pre-filtered (with 0.2 µm pore size filters) buffered formaldehyde solution (2% vol/vol; Pernthaler et al., 2002). After fixation, samples were centrifuged twice to remove formaldehyde residues and resuspended in PBS. Then, samples were treated using 4 ml of a mix containing EDTA, Tween 80, sodium-pyrophosphate, methanol and ultrasounds treatment to separate fungi from the sediment matrix. After centrifugation, sediment samples were washed twice with PBS buffer and then treated with increasing concentrations of ethanol (50, 80 and 96%, for 3 min each). The sediment was then suspended in 500 µl hybridisation buffer containing 0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris–HCl pH 7.2, 30 %v/v formamide and 1 µM PF2 (Kempf et al., 2000), then incubated for 3 h at 46 °C in the dark. Samples were then transferred in sterile tubes containing pre-warmed washing buffer (20 mM Tris–HCl pH 8.0, 0.01% w/v SDS, 5 mM EDTA, 0.112 M NaCl) and incubated for 30 min at 48 °C. After centrifugation and resuspension of the sediment samples with 0.2 µm pre-filtered water, aliquots of the slurry (n = 3) were filtered on 0.2 µm polycarbonate filters (Millipore). Filters were then stained with 0.5 mM Calcofluor white and incubated in the dark for 5 min. Subsequently, slides were washed with 0.02 µm pre-filtered water and analysed under epifluorescence microscopy.

The whole filter was examined, and length and width measures were taken for each fungal-like structure. A positive FISH signal was detected in almost all (i.e., more than 90%) of the calcofluor-stained structures identified. However, consistently with previous findings (Gonçalves et al., 2006, Bochdansky et al., 2017), FISH signal was typically weaker and less homogeneously distributed over the fungal structures compared with the calcofluor signal. This could be due to the low and/or uneven distribution of nucleic acids in fungal hyphae (Teertstra et al., 2004), as well as to the low permeability of fungal cells to FISH probes (Bochdansky et al., 2017). Thus, for a more reliable assessment of fungal biomass the calcofluor signals were used (according to Damare and Raghukumar, 2008), while the FISH approach was used to exclude unspecific calcofluor signals (i.e., chitin-containing structures but not identified as fungi by FISH). The average width and cumulative length of each identified fungal structure were converted to a cylinder with half-spheres at ends, and the biovolume was converted into fungal biomass, assuming 1 μm3 of fungal biovolume equivalent to 1 pg C (Damare and Raghukumar, 2008).

DNA extraction and purification for molecular analysis

The DNA was extracted and purified from the sediment samples using the PowerSoil DNA isolation kit (QIAGEN) following the manufacturer's instruction with slight modifications to remove extracellular DNA (based on three subsequent washing steps) before DNA extraction (Danovaro, 2010; Danovaro et al., 2016).

Quantitative real-time PCR of fungal 18S rRNA gene sequences

The DNA extracted from two sediment samples collected at each study site by independent multiple corer deployments was used for quantitative real-time PCR (qPCR) analysis which was performed as described in Taylor and Cunliffe (2016) with slight modifications. Briefly, fungi-specific primers FR1 5′-AIC CAT TCA ATC GGT AIT-3′ and FF390 5′-CGA TAA CGA ACG AGA CCT-3′ (Prévost-Bouré et., 2011) were used with the Sensi-FAST SYBR Q-PCR kit (Bioline, London, UK). The 15 µl reactions contained 8 µl Sensi-FAST master mix, 1 µl of each primer (final concentration 1 µM), 1 µl of DNA template and 5 µl nuclease-free molecular-grade water (Taylor and Cunliffe, 2016). A Bio-Rad iQ5 was used to perform qPCR. The following qPCR thermal cycles were used: 94°C for 3 min, then 40 cycles of 94°C for 10s, annealing at 50°C for 15s, elongation at 72°C for 20s and acquisition of fluorescence data at

82°C. Standard curves were generated using known concentration of *Aspergillus niger* 18S rDNA.

Fungal diversity

DNA extracted from two sediment samples collected at each study site by independent multiple corer deployments was amplified using the primer set ITS1F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) which amplify the internal transcribed spacer-1 (ITS1) region of the fungal rRNA gene (Walters et al., 2015). Amplicons were sequenced on an Illumina MiSeq platform by LGC group (Berlin, Germany) following Earth Microbiome Project protocols (http://www.earthmicrobiome.org/emp-standard-protocols/). Raw sequences were demultiplexed and barcodes and ITS1 primer pairs were removed afterwards. Paired-end sequences were then merged with FLASH (Magoč and Salzberg, 2011). Merged sequences were quality filtered using the USEARCH tool (Edgar, 2010) to remove sequences with expected error >1.0 and analysed with the QIIME software package (Caporaso et al., 2010). Operational taxonomic units (OTUs) were assigned with a threshold of 98.5% pairwise identity as indicated by the UNITE fungal ITS database (http://unite.ut.ee/). Prior to taxonomic identification, OTUs were checked by means of the ChimeraSlayer tool within the QIIME software package against the UNITE ITS database to identify and remove potential chimeras. Then, non-chimeric OTUs were classified taxonomically against the UNITE database (http://unite.ut.ee/, Version 7.1, November 20, 2016). To allow a proper comparison among samples, we followed the approach by Gihring et al. (2012) with sample normalisation to 2500 randomly-selected sequences (corresponding to the lowest read count obtained in our samples). Rarefaction curves highlighted that 2500 sequences used for the comparison among all samples were generally sufficient to describe the fungal diversity in the different benthic deep-sea ecosystems investigated (Fig. S1).

Statistical analyses

Two-way analysis of variance (ANOVA) was performed to test for differences in organic matter content, fungal abundance, biomass and OTU richness among canyons and depths. When significant differences were encountered, post-hoc tests were also carried out. ANOSIM analysis was performed to test for the presence of statistical differences in the trophic

conditions at the seafloor between canyons. Permutational multivariate analysis of variance (PERMANOVA) was used based on Bray-Curtis similarity matrix and visualised using cluster analysis to test for differences in fungal community composition among canyons and depths. Distance-based multivariate analysis for a linear model (DistLM) forward (Anderson, 2008) was performed to identify potential factors influencing fungal abundance, biomass, OTU richness and assemblage composition. P values were obtained with 9,999 permutations of residuals under the reduced model (Anderson, 2008). Temperature, salinity and trophic resources (as protein, carbohydrate and lipid concentrations) were used as predictor variables. Distance-based redundancy analysis (dbRDA) was finally used to visualise the relationships between fungal assemblage composition of the different canyon systems and thermohaline and trophic variables. All statistical analyses were performed using Primer 6+ software.

Results and discussion

The thermohaline conditions of bottom waters of the benthic systems investigated in the present study changed across depths and canyons, with temperature values ranging from 13.77 to 15.20 °C, and salinity values ranging from 38.75 to 38.93 (Table 1). Lowest temperature and salinity values were generally observed at the greatest depth (i.e. 1000 m). Also, the analysis of organic matter quantity in the sediments revealed differences among the investigated canyons (Tables 1, TableS1), with concentrations of proteins and carbohydrates significantly higher in Crotone and Squillace canyons than in Tricase canyon ($p<0.05$ and $p<0.01$, for proteins and carbohydrates, respectively). The highest organic matter content in the sediments of Crotone and Squillace canyons is likely due to their proximity to the coast and the presence of nearby river inputs which amplify the magnitude of organic matter exported from the water column and settling on the seafloor (Lopez-Fernandez et al., 2013).

Table 1. Temperature, salinity and protein (PRT), carbohydrate (CHO), lipid (LIP) and biopolymeric C concentrations in the different sites of the Tricase, Crotone and Squillace canyons. Mean values and standard deviations (±) are reported.

Canyon	Water depth m	Temperature °C	Salinity	PRT mg g-1	CHO mg g-1	LIP mg g-1	Biopolymeric C mg g-1
Tricase	200 m	14.58±0.01	38.8±0.01	1.91±0.55	2.21±0.59	0.82±0.28	2.43±0.72
	500 m	14.23±0.05	38.75±0.01	2.42±0.73	2.37±0.29	1.22±0.4	3.05±0.78
	1000 m	13.85±0.01	38.8±0.01	0.77±0.52	2.1±0.25	1.72±0.68	2.51±0.87
Crotone	200 m	15.07±0.12	38.91±0.01	2.87±0.24	3.56±0.23	1.61±0.76	4.04±0.78
	500 m	14.4±0.03	38.88±0.01	2.09±0.48	2.44±0.23	0.48±0.19	2.36±0.47
	1000 m	13.77±0.02	38.76±0.01	2.22±0.29	2.18±0.11	0.3±0.1	2.19±0.26
Squillace	200 m	14.78±0.06	38.82±0.01	2.21±0.36	3.77±0.59	0.6±0.31	3.04±0.64
	500 m	14.64±0.05	38.92±0.01	3.5±0.78	3.08±0.22	0.28±0.05	3.16±0.5
	1000 m	13.78±0.01	38.76±0.01	2.96±0.34	2.61±0.21	0.66±0.58	2.99±0.68

The amount of organic matter in deep-sea sediments represents a significant factor influencing the abundance and distribution of benthic assemblages (Danovaro et al., 2014). Fungal abundance, expressed as number of fungal 18S rDNA copies ranged from 1.4×10^6 to 5.1×10^7 copies g^{-1} and was significantly lower in Tricase ($0.38 \pm 0.04 \times 10^7$ copies g^{-1}) than in Crotone and Squillace canyons (2.7 ± 0.5 and $1.3 \pm 0.4 \times 10^7$ copies g^{-1}, respectively; $p<0.01$; Figure 2a). Our results fall within previously reported ranges for deep-sea sediments of the Pacific Ocean (3.5×10^6 - 5.2×10^7 28S rDNA copies g^{-1}; Xu et al., 2014), providing the first evidence of the quantitative importance of fungi also in benthic deep-sea ecosystems of the Mediterranean Sea. In all canyons, the 18S rDNA copy number changed significantly with water depth, with highest values at the shallowest depth in Crotone and Squillace canyons and at 500 m depth in Tricase canyons.

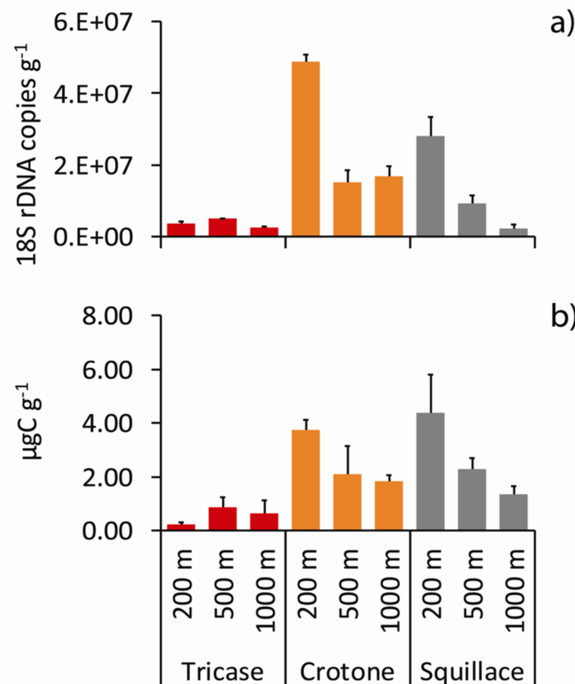

Figure 2. Fungal abundance, expressed as 18S rDNA copy number (a), and biomass (b) in the different benthic sites of the Tricase, Crotone and Squillace canyons. Mean values and standard deviations are reported.

Fungal biomass ranged from 0.17 to 5.78 µgC g^{-1}, with values significantly lower in the sediments of Tricase (0.63 ± 0.14 µgC g^{-1}) than in Crotone and Squillace canyons (2.40 ± 0.43 and 2.73 ± 0.49 µgC g^{-1}, respectively; $p<0.01$; Figure 2b). The distribution of fungal biomass along the bathymetric gradients within each canyon was similar to that of 18S rDNA copy number. Data on fungal biomass are practically non-existent for deep-sea surface sediments

(Damare and Raghukumar, 2008). However, the fungal biomass values reported here are similar to those of other benthic components reported at equal depths in the whole Mediterranean Sea (Gambi et al., 2017) suggesting that fungi can represent a significant component of benthic biomass in deep-sea sediments. We found a significant relationship between fungal biomass estimated based on microscopy and fungal abundance estimated based on the molecular quantification of 18S rDNA copies (Figure S2). To our knowledge, our study provides the first direct comparison between the two methods (i.e., the microscopy and the molecular approach) used to assess fungal quantitative relevance and distribution in contextual samples, providing a tentative conversion factor of average of 7.8×10^6 fungal 18S rDNA copies per μg of fungal biomass in deep-sea sediments. This value should be better constrained by future studies, as the two methods are prone to different biases, including cell extraction and staining efficiency or variable 18S rDNA copy numbers per cell in different fungal species. In our work, the fact that the two independent methods led to the same conclusions on the quantitative relevance and distribution of fungi in our samples, make our study particularly robust compared with other studies based only on microscopy or molecular approaches (Taylor and Cunliffe, 2016).

Fungi are heterotrophic organisms known to consume carbohydrates (Richards and Talbot, 2013; Richards et al., 2015; Couturier et al., 2016). In marine sediments, most of these compounds originate from sinking organic detritus of marine and terrestrial origin, and they are enriched in complex and refractory molecules, remnants of the degradation processes occurred in the upper water column (Dell'Anno et al., 2000; Dell'Anno et al., 2013). The multivariate multiple regression analysis provided evidence that carbohydrate concentration in the sediment was the primary factor explaining the distribution of the abundance and biomass of fungi in the benthic deep-sea ecosystems investigated ($r=0.715$ and $r=0.893$, both $p<0.01$, for abundance and biomass, respectively; Figure 3; Table S2). This result is consistent with the expectation that benthic deep-sea fungi can contribute to the degradation of organic detritus in deep-sea sediments, and that they are specialised in the utilisation of the most recalcitrant organic compounds, including complex carbohydrates which would otherwise be hardly decomposed by other organisms (Jones and Pang, 2012).

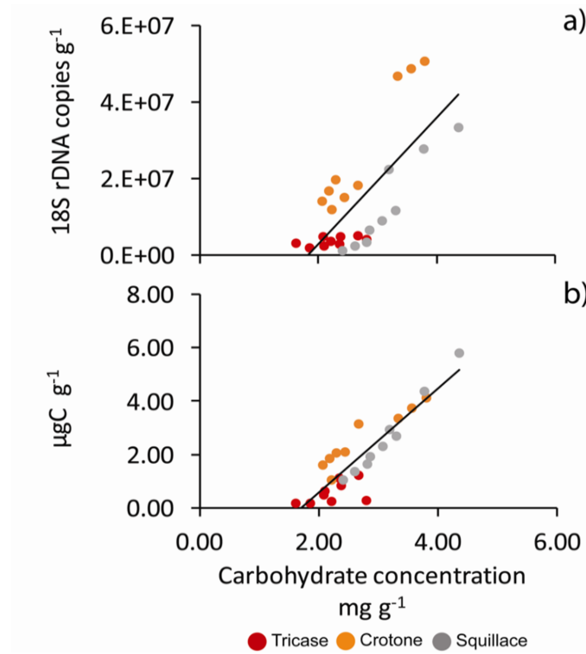

Figure 3. Relationships between carbohydrate concentrations in the sediments of the different canyons investigated and fungal abundance (a) and biomass (b).

Our results also show that the clustering of the 1203476 fungal ITS sequences (obtained after quality check) allowed us to identify a total of 1742 fungal OTUs, belonging to all fungal phyla known to date. Ascomycota represented the dominant phylum (accounting for 68% of the total reads), followed by Basidiomycota (10%) and Chytridiomycota (4%). The dominance of such phyla has been consistently reported in other benthic deep-sea ecosystems (Zhang et al., 2016).

The number of fungal OTUs we found in the sediments of the different canyons was similar compared with that reported in other deep-sea ecosystems (Zhang et al., 2016). The Tricase canyon displayed a significantly lower OTU number (range: 64-71 OTUs) compared to Crotone and Squillace canyons (range: 113-325 and 173-221 OTUs, respectively; $p<0.01$; Figure 4).

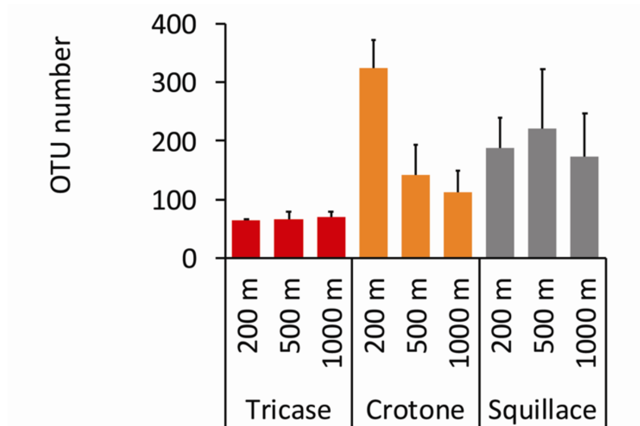

Figure 4. OTU number in the different benthic sites within Tricase, Crotone and Squillace canyons. Mean values and standard deviations are reported.

In our dataset, the OTUs affiliating to currently known fungal families were represented by only 19-38% of the total reads (Figure 5). The classified fungal OTUs affiliated to 206 genera belonging to 132 families, 66 orders and 27 classes.

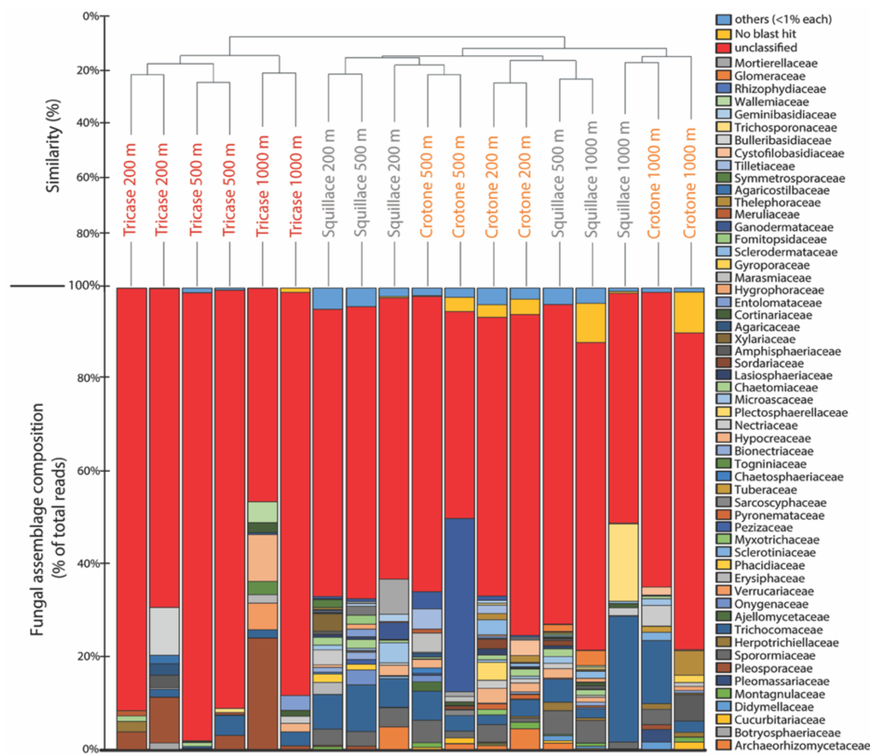

Figure 5. Taxonomic composition (at the family level on data normalized to 2500 sequences) of the benthic fungal assemblages in the different canyons investigated. To better visualise differences among the investigated sites the output of cluster analysis is also reported.

At all benthic sites, Pleosporales was the most represented fungal order (accounting for ca. 20% of the total reads in each sample). This group is commonly present in marine environment and can account for a relevant fraction of the fungal diversity (up to 18% of all OTUs and sequences) in benthic deep-sea ecosystems (Li et al., 2016). Moreover, members belonging to the Pleosporales order are known to be adapted to high hydrostatic pressure (Nagano and Nagahama, 2012), possibly contributing to the ecological success of such taxon in deep-sea ecosystems.

Most of the fungi that we successfully classified were affiliated to genera such as *Aspergillus*, *Penicillium*, *Epicoccum*, *Cryptococcus* and *Candida* previously encountered in other deep-sea environments (Nagahama et al., 2003; Edgcomb et al., 2011; Rédou et al., 2014). However, these genera represented overall only ca. 36% of the total reads, indicating that the majority of fungal taxa belonged to genera not represented in UNITE database (Kõljalg et al., 2013).

The majority of fungal OTUs were unclassified below the order level and overall represented up to 69% of the total sequences. The quantitative relevance of unclassified sequences in our study was much higher than that reported for coastal sediments (Picard, 2017), indicating that deep-sea ecosystems might harbour a higher richness of novel fungal lineages compared with shallow benthic ecosystems.

The composition of fungal assemblage in the sediments of the Tricase canyon was significantly different ($p<0.01$) from that of the other canyons, which otherwise showed no significant differences (Figure 5). These results suggest that submarine canyons far from the coastline and lacking river inputs can host distinct fungal assemblages from those close to river estuaries.

The analysis of the turnover (β-)diversity highlighted that the similarity of the fungal assemblage composition among different sites was very low (Table S3 and Figure 6). Indeed, the within-canyon similarity (i.e., the similarity of fungal assemblage composition among samples collected at a different depth within the same canyon) was on average 11%, while the inter-canyon comparisons resulted in an average similarity of 7% (Table S3). Moreover, the Tricase canyon showed the highest percentage of unique OTUs (i.e., OTUs found in Tricase but not in Squillace nor Crotone canyons; Table S4).

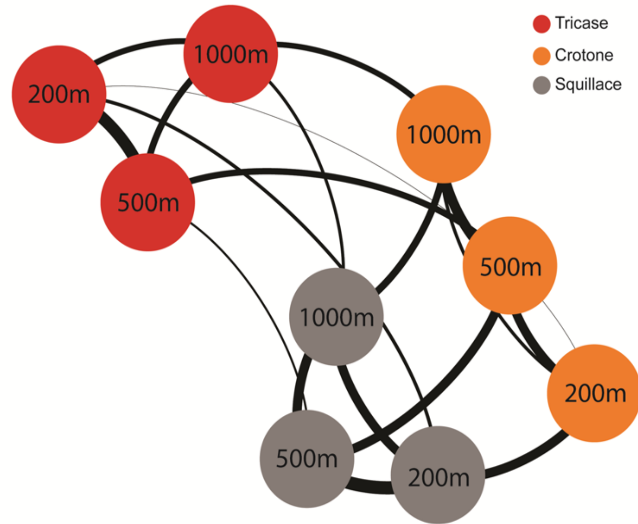

Figure 6. Network visualisation based on the output of SIMPER analysis carried out on fungal community composition among the nine sites investigated. Line width is proportional to similarity values.

Overall, the three canyons shared only 46 out of 1742 OTUs, that cumulatively accounted for only 22% of the total sequences. Twenty-seven of these 46 shared OTUs (overall accounting for 14% of the total sequences) were not classified, while the others shared OTUs (each of them contributing for ≤0.45% of the total sequences) included taxa belonging to *Epicoccum nigrum*, *Illyonectria robusta*, *Trichoderma bissettii*, *Cryptococcus victoriae*, *Aspergillus sydowii*, *Fusarium sp*, *Penicillum halotolearns* and *Thermomyces lanuginosus*.

Distance-based redundancy analysis highlighted that the fungal assemblage composition in the sediments of the different canyons was related to an array of factors including organic matter content (as carbohydrates and lipid concentrations, r=0.624 and r=0.434, respectively), temperature (r=0.980) and salinity (r=−0.560; Fig. 7). These results confirm that also in the deep-sea sediments investigated trophic availability and thermohaline conditions are important drivers of fungal assemblage composition (Hanson et al., 2008, McGuire et al., 2010, Li et al., 2016, Taylor and Cunliffe, 2016, Tisthammer et al., 2016). Our findings also suggest that changes in the thermohaline and trophic conditions among submarine canyons may promote a high turnover diversity of benthic deep-sea fungal assemblages.

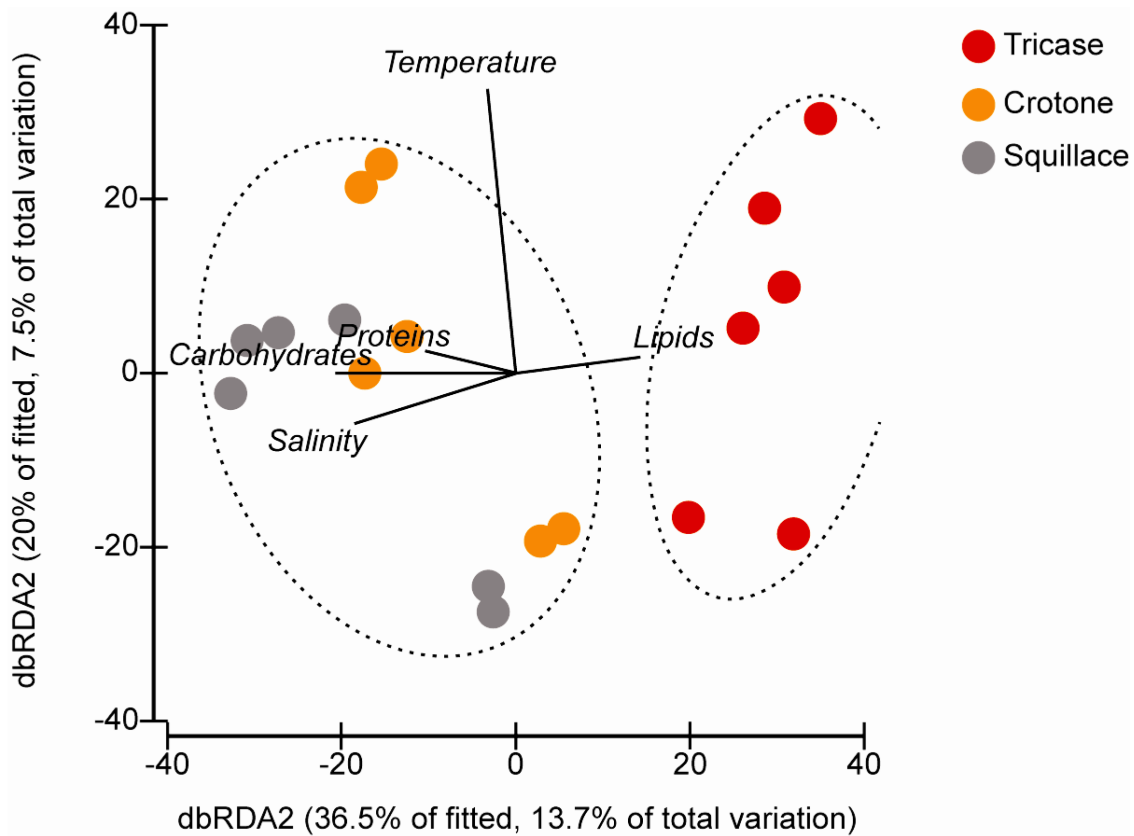

Figure 7. Output of the distance-based redundancy analysis (dbRDA) carried out on fungal community composition in the different benthic deep-sea sites in relation with thermohaline and trophic conditions.

These results confirm that also in the deep-sea sediments investigated trophic availability and thermohaline conditions are important drivers of fungal assemblage composition (Hanson et al., 2008; McGuire et al., 2010; Li et al., 2016; Taylor and Cunliffe, 2016; Tisthammer et al., 2016). Our findings also suggest that changes in the thermohaline and trophic conditions among submarine canyons may promote a high turnover diversity of benthic deep-sea fungal assemblages. Overall results of the present study indicate that the submarine canyons of the Mediterranean Sea host abundant and highly diversified fungal assemblages most of which still unidentified and pave the way for a better understanding of the ecological role of fungi in the largest ecosystem on Earth.

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Supplementary materials—Benthic deep-sea fungi in submarine canyons of the Mediterranean Sea

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Table S1. ANOSIM and SIMPER outputs testing for differences and dissimilarity in sediment organic matter contents between the different canyons investigated and the variables responsible for the estimated differences. R is the sample statistic (global R) and P the probability level (**=P<0.01; ns=P>0.05).

	ANOSIM		SIMPER			
	R	**P**	**Dissimilarity**	**Explanatory variable**	**Explained variance (%)**	**Cumulative explained variance (%)**
Tricase vs. Crotone	0.153	**	22.35	**Proteins**	40.78	40.78
				Lipids	33.48	74.26
				Carbohydrates	25.74	100
Tricase vs. Squillace	0.449	**	27.02	**Proteins**	43.85	43.85
				Carbohydrates	30.74	74.59
				Lipids	25.41	100
Crotone vs. Squillace	0.12	n.s.	16.82	**Proteins**	n.s	n.s
				Lipids	n.s	n.s
				Carbohydrates	n.s	n.s

Table S2. Multivariate multiple regression analysis outputs testing the effects of organic matter content (proteins, carbohydrates and lipids), temperature and salinity on fungal abundance (as 18S rDNA copies) and biomass. Pseudo-F and P values (*=P<0.05; **=P <0.01; ***=P <0.001; ns=P >0.05) are reported as well as the cumulative variance explained by the significant variables.

Variable	**Pseudo-F**	**P**	**Cumulative variance %**
Fungal abundance (18S rDNA copies)			
Carbohydrates	11.556	***	31.6
Lipids	3.814	*	41.0
Proteins	1.771	ns	-
Salinity	1.654	ns	-
Temperature	0.667	ns	-
Fungal biomass			
Carbohydrates	98.421	***	79.7
Lipids	4.249	ns	-
Proteins	2.275	ns	-
Temperature	0.82	ns	-
Salinity	2.196	ns	-

Table S3. Output of SIMPER analysis showing the dissimilarity (turnover diversity) of fungal assemblage composition within the canyon and between the canyons investigated.

Type of comparison			**Turnover diversity (% Bray-Curtis dissimilarity)**
within canyon	**Tricase**	**200 m vs. 500 m**	86.19
		200 m vs. 1000 m	91.97
		500 m vs. 1000 m	91.12
	Crotone	**200 m vs. 500 m**	89.03
		200 m vs. 1000 m	94.3
		500 m vs. 1000 m	87.88
	Squillace	**200 m vs. 500 m**	85.22
		200 m vs. 1000 m	88.92
		500 m vs. 1000 m	88.45
between canyons	**200 m**	**Tricase vs. Crotone**	97.01
		Tricase vs. Squillace	94.23
		Crotone vs. Squillace	88.7
	500 m	**Tricase vs. Crotone**	91.5
		Tricase vs. Squillace	95.42
		Crotone vs. Squillace	88.89
	1000 m	**Tricase vs. Crotone**	92.2
		Tricase vs. Squillace	94.52
		Crotone vs. Squillace	90.65

Table S4. Percentage of unique and shared OTUs between replicates of the same site, within the canyon and between the canyons.

Type of comparison			**Shared** %	**Unique** %
between replicates of the same site	**Tricase**	200 m	9.4	90.6
		500 m	15.7	84.3
		1000 m	10.2	89.8
	Crotone	200 m	12.5	87.5
		500 m	14.6	85.4
		1000 m	7.6	92.4
	Squillace	200 m	12.2	87.8
		500 m	7.0	93.0
		1000 m	6.8	93.2
	Average		10.7	89.3
within canyon	**Tricase**	200 vs. 500 m	18.8	90.0
		200 vs. 1000 m	14.0	93.9
		500 vs. 1000 m	13.8	92.5
	Crotone	200 vs. 500 m	12.2	91.1
		200 vs. 1000 m	19.8	94.8
		500 vs. 1000 m	19.9	91.2
	Squillace	200 vs. 500 m	29.3	86.3
		200 vs. 1000 m	18.6	89.2
		500 vs 1000 m	26.9	88.0
	Average		19.2	90.8
between canyons	**Tricase *vs.* Crotone**	200 m	3.8	96.2
		500 m	7.9	92.1
		1000 m	7.6	92.4
	Tricase *vs.* Squillace	200 m	6.6	93.4
		500 m	5.6	94.4
		1000 m	5.9	94.1
	Crotone *vs.* Squillace	200 m	10.3	89.7
		500 m	10.9	89.1
		1000 m	8.1	91.9
	Average		7.4	92.6

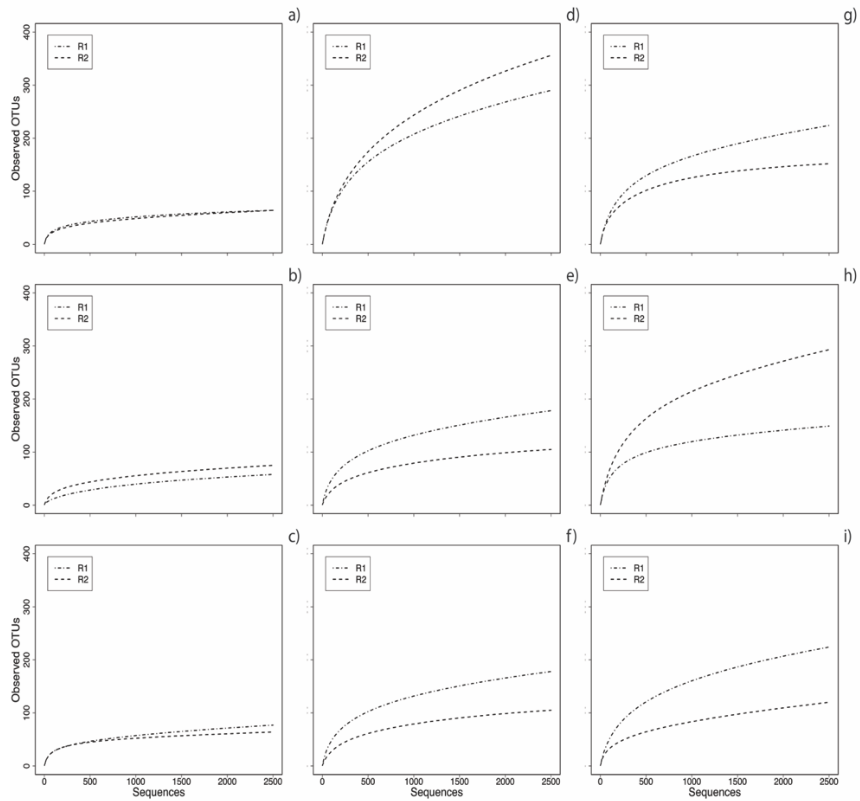

Figure S1. Rarefaction curves calculated for each of the two independent replicates (dashed lines, 2500 sequences each) analysed in all benthic deep-sea sites of the canyons investigated.

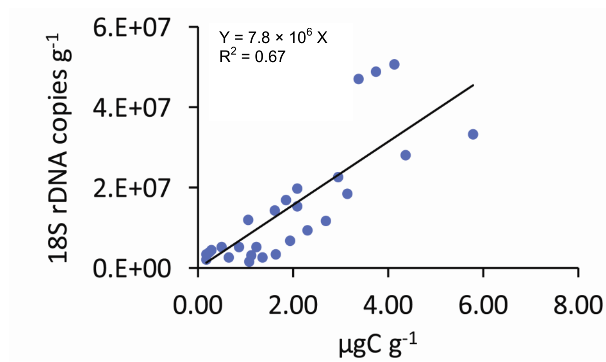

Figure S2. Relationship between benthic fungal abundance (as 18S rDNA copies) and biomass in the sediments of the three canyons.

Chapter 3

Fungal abundance and diversity in the sediments of the deepest ecosystem on Earth

Abstract: Hadal trenches can be a depocenter of organic matter cycling and can host benthic microbial biomasses much higher than in the adjacent abyssal sediments. However, the microbial assemblages in these unique ecosystems remain mostly unexplored, especially for fungi. In this study, we investigated the distribution of benthic fungal abundance and diversity in relation to changes in environmental and trophic conditions along a transect crossing the Mariana trench down to the Challenger Deep. At the Challenger Deep, we found that the benthic fungal abundance was significantly higher than in the Mariana flank and in adjacent abyssal sediments. Here, the fungal diversity was otherwise lower, and fungal assemblages were composed of unique and undescribed fungal taxa, never reported to date. Fungal abundance was mainly driven by the concentration of biopolymeric carbon and phytopigments, suggesting that higher food availability and sedimentation rates might sustain larger fungal standing stocks at the bottom of the trench. Fungal assemblage composition was mainly influenced by thermo-haline conditions and oxygen concentrations of the bottom waters. Overall results of this study provide new insights on the factors shaping fungal distribution and diversity in benthic deep-sea ecosystems, highlighting hadal trenches as a potential hot spot of highly specialised and locally highly abundant novel fungal taxa, that might play a key role in C and nutrient cycling in these isolated and extreme habitats.

Introduction

Fungi are ubiquitous organisms on Earth where they comprise millions of species (Hawksworth and Lücking 2017) and have a key ecological role in the decomposition of organic matter (Hyde et al. 1998, Tedersoo et al. 2014, Peay et al. 2016). However, the fungal ecology and diversity have only recently started to be investigated in benthic deep-sea ecosystems. Over the last decade, the marine fungal diversity has been investigated in the water column (Gutiérrez et al. 2011, Pernice et al. 2015, Bochdansky et al. 2016, Taylor and Cunliffe 2016) and in sediments (Damare and Raghukumar 2008, Singh et al. 2010, Edgcomb et al. 2011, Picard 2017), of different ecosystems including submarine canyons (see Chapter 2), hydrothermal vents (Burgaud et al. 2009, 2010, Xu et al. 2017), hypersaline anoxic basins (Bernhard et al. 2014, Edgcomb et al. 2016), and methane seeps (Thaler et al. 2012). These studies provide evidence that marine fungi are a diversified component of the microbial food webs. Nevertheless, only a few studies explored fungal diversity in the hadal zone (Takami et al. 1997, Raghukumar et al. 2004, Nagano et al. 2010).

Hadal zones (6000–11,000 m water depth) account for only 1-2% of the entire seafloor area but represent the deepest 45% of the oceanic depth range (Jamieson 2015). Hadal zones, mainly represented by trenches, are the most remote and least explored habitat of our planet (Luo et al. 2017), and compared with abyssal plains, exhibit greater hydrostatic pressure and relatively isolated hydro-topography (Taira et al. 2004, 2005). Food availability in hadal trenches is influenced by topographic and geomorphological factors, including the distance from the land (Ichino et al. 2015, Leduc et al. 2016), and physical processes such as lateral downslope transport of organic matter in addition to the vertical fluxes from the overlying water column (Turnewitsch et al. 2014).

Some trenches can be a depocenter of organic matter accumulation which can sustain higher benthic microbial biomass and metabolic activities compared to abyssal plains (Glud et al. 2013, Danovaro et al. 2014, Xu et al. 2014, Luo et al. 2017). However, while technological and methodological advances allowed to identify distinct active prokaryotic communities even in the sediments of the deepest trench on Earth (i.e. the Mariana trench), information on fungal diversity and ecology in such ecosystems is still limited.

Available information of the diversity of fungi in trench systems, obtained from culture-dependent and independent approaches, suggests that their diversity can be much lower when compared to other environmental settings (Nagano et al. 2010; Nagahama et al. 2011, Picard 2017, Xu et al. 2017). However, factors influencing their abundance and diversity in trench systems remain mostly unknown. In this study, we analysed the distribution of abundance and diversity of benthic fungi along a transect crossing the Mariana trench down to the Challenger Deep in order to provide new insights on their quantitative relevance and diversity and factors shaping their assemblage composition.

Material and methods

Study site and sampling

The Mariana Trench is located under oligotrophic waters (Wenzhöfer et al., 2016) off Guam. It belongs to the Izu-Bonin-Mariana subduction system and extends for 2550 km reaching a maximum depth of ca. 10,960 m at the Challenger Deep. Sediment samples (n=3) were collected from 4 sites along a bathymetric transect across the Mariana Trench intersecting the Challenger Deep of the Mariana Trench (Figure 1) by ABISMO during the KR14-01 cruise on board of the research vessel Kairei in collaboration with the JAMSTEC Institute (January 2014). Aliquots of the sediment surface were used to carry out the analysis of the biochemical composition of the organic matter and fungal abundance and diversity. Sediment samples were frozen and stored at -20°C until laboratory analyses.

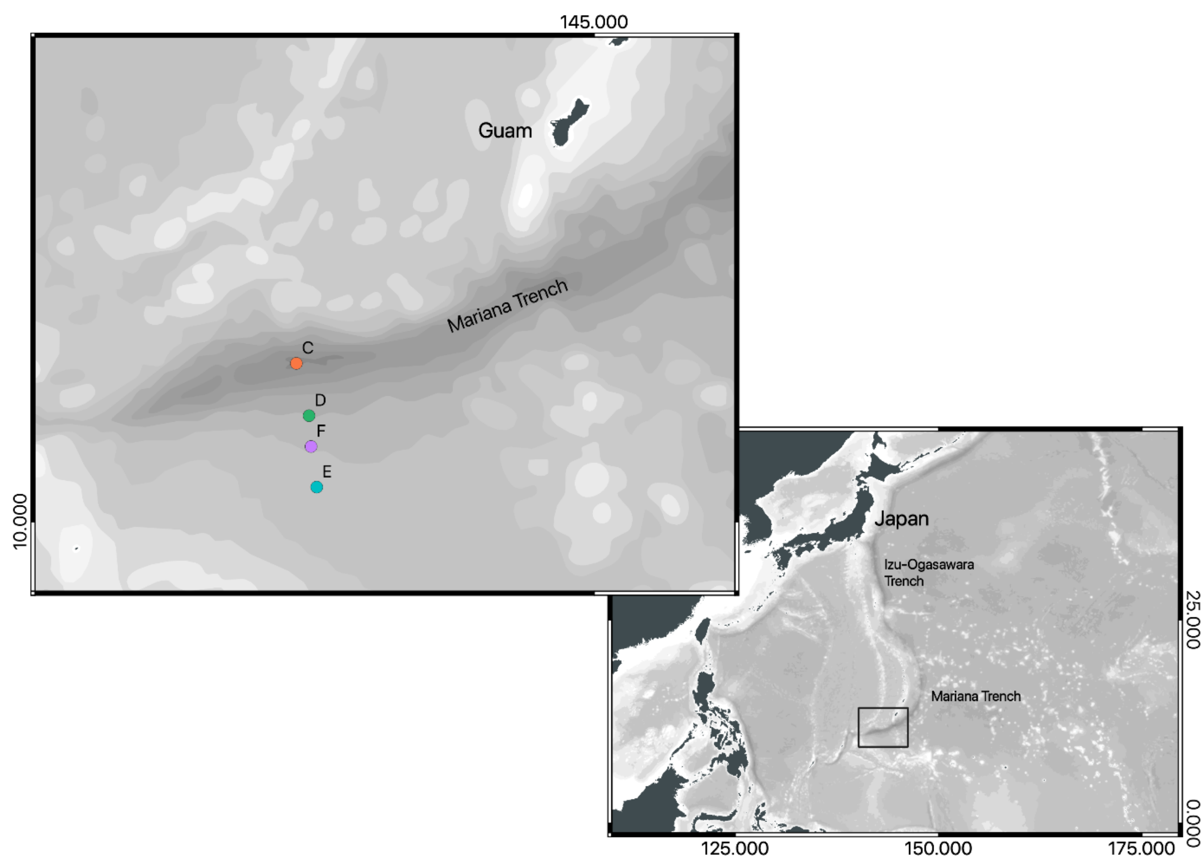

Figure 1. Geographical location of the study area and site location across the Mariana trench. Land maps used in these figures are from the public domain Natural Earth and freely available for personal, educational, and commercial purposes (http://www.naturalearthdata.com/about/ terms-of-use/).

Environmental and trophic conditions

Temperature, salinity and oxygen concentrations of bottom waters in the Mariana trench were obtained from the literature Nunoura et al. (2015) and Xu et al. (2018).

Trophic conditions of the benthic systems were assessed on the basis of the quantity and biochemical composition of organic matter (Danovaro 2010). In particular, the three major biochemical classes of organic compounds (proteins, carbohydrates and lipids) in deep-sea sediments were determined. Briefly, about 0.5 g of wet sediment was collected and used for the quantification of proteins (PRT), carbohydrates (CHO) and lipids (LIP). Protein concentration was assessed via colorimetric assay, allowing the reaction of proteins with rameic tartrate and the Folin-Ciocalteau in a basic environment (pH 10) which provides a stable blue colouration with an intensity proportional to protein concentration. Carbohydrate concentration was determined via colorimetric assay based on the reaction between

carbohydrates and phenol in the presence of concentrated sulfuric acid which provides a colouration whose intensity is proportional to carbohydrate concentration. Lipids were extracted by direct elution with chloroform and methanol followed by reaction with sulfuric acid for colorimetric assay. Protein, carbohydrate and lipid concentrations were determined spectrophotometrically and expressed as albumin, glucose and tripalmitin equivalents, respectively. All analyses were carried out in 3 replicates. Protein, carbohydrate and lipid concentrations were then converted to carbon equivalents (conversion factors: 0.49, 0.40 and 0.75 gC g-1, respectively) to determine biopolymeric C content (BPC) in the sediments (Dell'Anno et al. 2002). Total phytopigment concentrations (CPE) was determined as the sum of chlorophyll-a and phaeopigment concentrations determined fluorometrically.

DNA extraction and purification for molecular analysis

Before DNA extraction, sediment samples were washed to remove extracellular DNA and the presence of inhibitors (Danovaro 2010). Then, DNA was isolated from the sediment samples using the PowerSoil DNA isolation kit (QIAGEN) accordingly to manufacture procedure. Aliquots of DNA were then used for quantitative real-time PCR and high-throughput sequencing.

Fungal abundance

For estimating fungal abundance, DNA was amplified by quantitative real-time PCR (qPCR) targeting the 18S rRNA gene of fungi (Taylor and Cunliffe 2016). Briefly, fungi-specific primers FR1 (5′-AIC CAT TCA ATC GGT AIT-3′) and FF390 (5′-CGA TAA CGA ACG AGA CCT-3′) which amplify an 18S rRNA gene fragment of about 350bp (Chemidlin Prévost-Bouré et al. 2011) were used with the Sensi-FAST SYBR Q-PCR kit (Bioline, London, UK). The 15 µl reactions contained 8 µl Sensi-FAST master mix, 1 µl of each primer (final concentration 1 µM), 1µl of DNA template and 5 µl nuclease-free molecular-grade water (Taylor and Cunliffe 2016). A Bio-Rad iQ5 was used to perform qPCR. The following qPCR thermal cycles were used: 94°C for 3min, then 40 cycles of 94 °C for 10 s, annealing at 50 °C for 15 s, elongation at 72°C for 20 s and acquisition of fluorescence data at 82°C. Standard curves were generated using known concentrations of Aspergillus niger 18S rDNA. 18S rRNA gene copies were then standardised per gram of dry sediment.

ITS1 sequencing

For the analysis of fungal diversity, the ribosomal internal transcribed spacer 1 (ITS) was amplified using the primer set ITS1F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′- GCTGCGTTCTTCATCGATGC-3′) (Schoch et al. 2012, Walters et al. 2015) and the amplicons sequenced on a Illumina MiSeq platform by LGC group (Berlin, Germany) following Earth Microbiome Project protocols (http://www.earthmicrobiome.org/emp-standard-protocols/). The sequences were then analysed by using QIIME2 (https://qiime2.org). Briefly, pair-ends reads were merged using VSEARCH (Edgar 2010). Then, sequence quality control and feature table construction were performed using dada2 which infers sample sequences exactly and resolves differences of as little as one nucleotide (Callahan et al. 2016). Representative sequences were then confronted against UNITE database for taxonomic affiliation (Version:7.2; Last updated: 2017-12-01). OTU table was then rarefied to the least number of sequences reaching saturation to allow comparison among samples with different reads abundance (Hughes and Hellmann 2005).

Statistical analyses

Permutational analysis of variance was used to test for differences in fungal abundance between locations. Spearman correlation analysis was used to investigate relationships between environmental and trophic variables and fungal abundance. (Dis-)similarity matrix between samples was generated following Bray Curtis's method from the OTU table. SIMPER analysis was conducted to explore dissimilarity in fungal assemblage composition among sampling sites, and to identify the most important OTUs contributing to the observed differences (Clarke 1993). Distance-based linear regression and distance-based redundancy analysis (db-RDA) were also carried out to identify potential drivers of fungal abundance and assemblage composition (Legendre and Legendre 2012). Finally, non-metric Multidimensional Scaling (nMDS) analysis was carried out to represent the pairwise (dis-)similarity based on Bray Curtis distance matrix between sites in a low-dimensional space and to visualise patterns of environmental and trophic variables (Li et al. 2016, Oksanen et al. 2018). Statistical analyses were performed and graph generated with R3.50 availing of vegan 2.5-2 (Oksanen et al. 2018), ggplot2 (Wickham 2016), and iNEXT (Chao et al. 2014, Hsieh et al. 2016). Network plots were generated via Gephi 0.9.2. DistLM, RDA and PERMANOVA were performed using

Primer+PERMANOVA (ver:6; Legendre and Anderson 1999, Clarke and Gorley 2006)). Site location and bathymetry were generated using QGIS3.

Results and discussion

Fungal relative abundance

In this study, we provide the first evidence that the bottom of the Mariana Trench can be a hot spot of fungal abundance. Indeed, fungal abundance at the bottom of the Mariana Trench (22.1 1.5 × 107 18S rRNA copies/g) was about 22-folds higher than fungal abundance reported in the flank of the Mariana Trench (0.98 0.02 × 107 18S rRNA copies/g) and about 7-folds higher than in abyssal sediments (3.25 2.3 × 107 18S rRNA copies/g; Figure 2). No significant differences were observed between the Mariana flank and abyssal sediments. The number of fungal 18S rRNA copies reported for the bottom sediments of the Mariana Trench is comparable to the number of prokaryotic 16S rRNA genes (used as a proxy of prokaryotic abundances) previously reported (Nunoura et al. 2018). However, this comparison should be view with caution since the number of the fungal rRNA gene copies in fungal genome is highly variable and higher than that of prokaryotic rRNA copies (Simon and Weiss 2008). Nevertheless, even assuming 200 copies per fungal cell (Simon and Weiss 2008), our estimates suggest that fungi are not a negligible component of the benthic microbial food web of Earth deepest ecosystem.

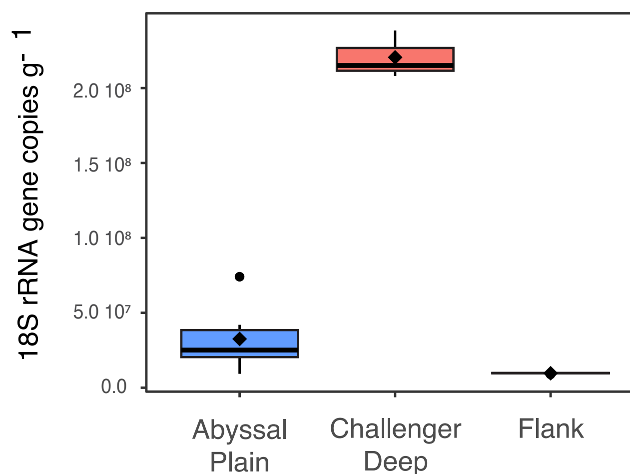

Figure 2. Boxplot representation of log-transformed 18S rRNA gene copy/g over the 4 stations (E and F, Abyssal plain; C, Challenger Deep; D, southern flank). Diamonds indicate arithmetic means.

Fungal abundance in the sediment investigated was significantly and positively related with the temperature of the bottom waters and the amount of total phytopigments (CPE; as a proxy of the input of organic matter produced by photosynthesis) and BPC and inversely related with oxygen concentrations of the bottom waters (Figure 3). Consistently, multivariate multiple regression analysis (distLM) revealed that oxygen concentrations of the bottom waters and CPE and BPC concentrations in the sediments explained cumulatively ca. 95% of the total variance of fungal abundance (Table 1). The high organic matter concentrations in the bottom sediments of the Mariana trench agree with previous findings highlighting accumulation of organic matter able to sustain high benthic prokaryotic standing stocks and oxygen consumption (Glud et al. 2013, Ichino et al. 2015). Therefore, the high fungal abundance we found could represent an additional component, besides prokaryotes, relevant in C cycling and biogeochemical processes occurring in the deepest ecosystem on Earth.

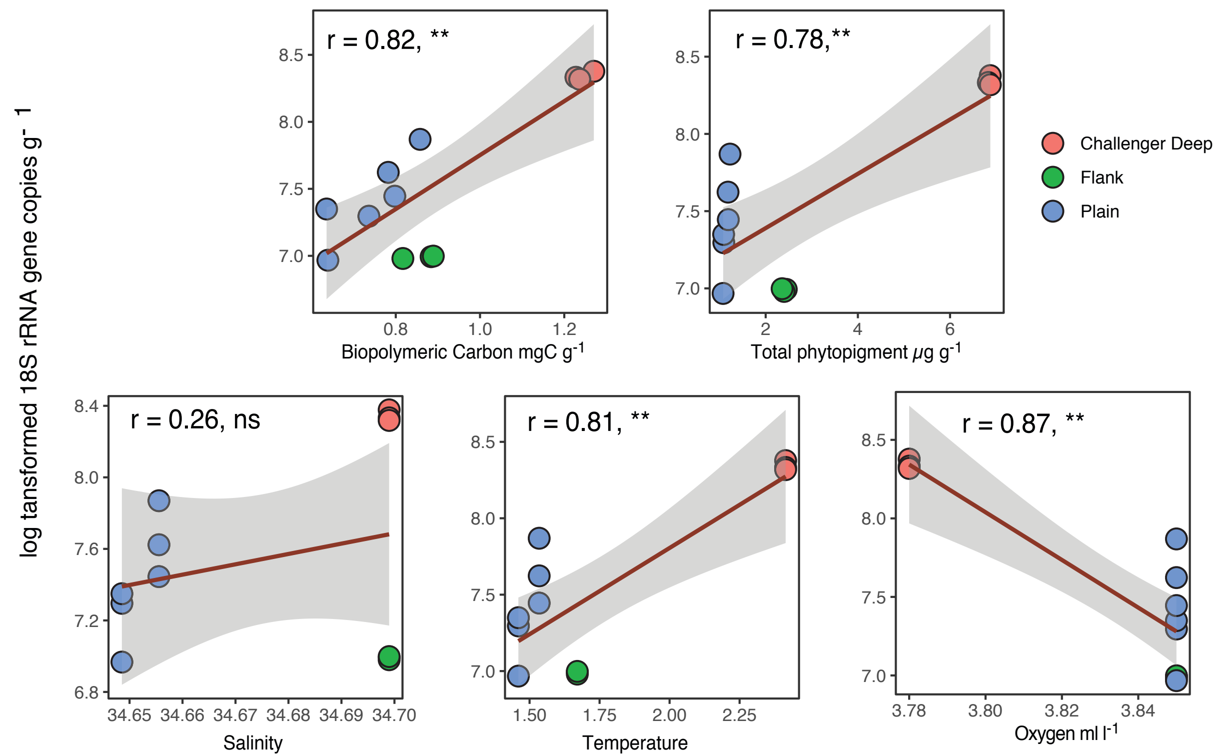

Figure 3. Spearman correlation of log-transformed 18S rRNA gene copies against environmental variables for the four stations (E and F, Abyssal plain; C, Challenger Deep; D, southern flank). The goodness of fit (r) and significance (p) of each test are reported for each scatterplot.

Table 1. Output of the distLM analysis (forward selection) carried out to test the effect of environmental and trophic conditions on the distribution of fungal abundance in the sediments.

Variable	SS	Pseudo-F	P	R^2	Cumulative
SEQUENTIAL TESTS					
Oxygen	13.469	29.941	0.005	74.96	74.96
Total phytopigment	1.678	5.354	0.047	9.34	84.30
Biopolymeric Carbon	1.887	16.156	0.003	10.50	94.80

Fungal diversity and assemblage composition

Reads varied from 3133 to 26446 (abyssal plain and sediments of the bottom of the Mariana trench, respectively; Table 2) reaching saturation around 3000 sequences at which we rarefied our dataset (Hughes and Hellmann 2005). Consistently to fungal abundance, reads abundance indicated that high fungal sequences characterised the sediments of the bottom of the Mariana trench compared to flank and abyssal plain sediments (Table 2). The sequencing of the fungal ITS1 resulted in the identification of only 26 OTUs of which, after removing OTUs with no match on public database and Agaricomycetes whose presence is unlikely to be relevant for this analysis, only 18 OTUs were retained as fungi. Our results indicate that fungal OTU richness was overall low and negatively correlated with depth. In particular, only 3 OTUs were identified in the sediments of the bottom of the Mariana trench (Figure 4), while the sediment of the flank and the abyssal plain accounted for 5 and 12 OTUs, respectively. The very low OTU richness observed in the sediment of the Challenger Deep is consistent with previous findings (Nagano et al. 2010, Xu et al. 2018), suggesting that peculiar environmental conditions characterising the Challenger Deep can determine a selection of a few and highly adapted taxa.

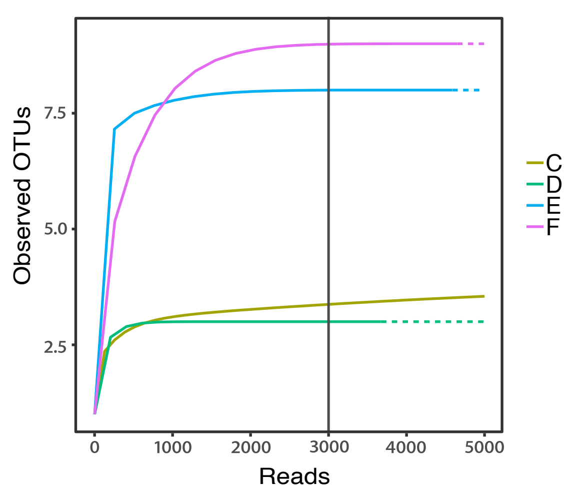

Figure4. OTUs accumulation curves

Table 2. Diversity index of fungal communities in Mariana trench sediemnts. H' is the Shannon diversity index, d is Simpson's index and J' is Pielous evenness index.

	Total Reads	H'	d	J's	OTUs richness
E	3133.00	0.952	1.748	1.532	5
F	3781.00	0.930	2.906	1.935	8
D	8533.00	0.993	1.547	1.599	5
C	26446.00	0.783	1.0197	0.809	3

All fungal OTUs affiliated to unknown Ascomycota. This pattern agrees with evidence previously reported indicating ascomycetes as the major if not the only phylum present in the water column overlying the Mariana trench (Xu et al. 2018). Consistently, Ascomycota is also the dominant taxon identified from hadal sediments of the Izu-Bonin-Mariana subduction system (Nagano et al. 2010). Moreover, the limited match against international public databases can indicate the presence of possibly new lineages as previously reported from similar locations, suggesting that the deepest ecosystem on Earth can host novel fungal taxa yet to be identified.

Network analysis based on OTU table indicated that no OTU was shared among benthic sites except one which was shared between the two abyssal sites and the Challenger deep (Figure 5). These results suggest that the different environmental and ecological conditions characterising the Mariana trench can select distinct fungal communities. Similarly, major differences of eukaryotic and prokaryotic assemblages have been reported along the vertical profile of the water masses overlying the Mariana Trench (Nunoura et al. 2015, Peoples et al. 2018, Xu et al. 2018).

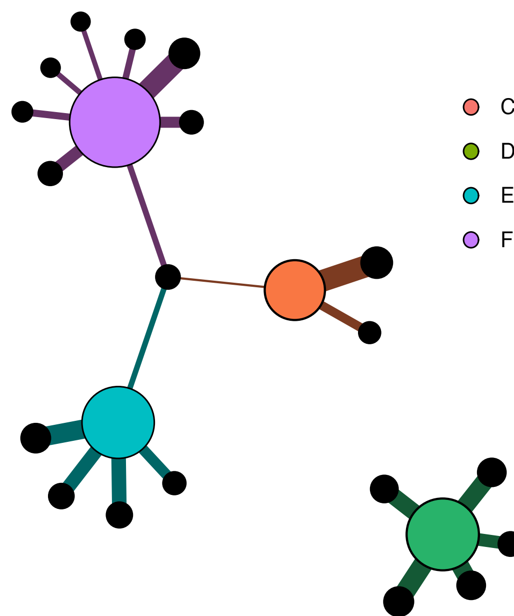

Figure 5. Bipartite Network plot of community composition for investigated sediments (E and F, Abyssal plain; C, Challenger Deep; D, southern flank). Edges width represent relative reads per sample. Black nodes represent OTU identity (diameter is proportional to the total reads abundance per OTU). Coloured nodes represent sites (diameter is proportional to OTU richness).

The high dissimilarity of fungal assemblage composition among the different benthic deep-sea sites (> 97% assessed by SIMPER analysis) was further highlighted by nMDS outputs which also revealed a segregation pattern associated with changes in environmental and trophic conditions (Figure 6). The high CPE and BPC concentrations observed in the sediments of the Challenger Deep are consistent with the hypothesis of major lateral transport of organic matter down to the greatest depths of the Mariana Trench (Ichino et al. 2015).

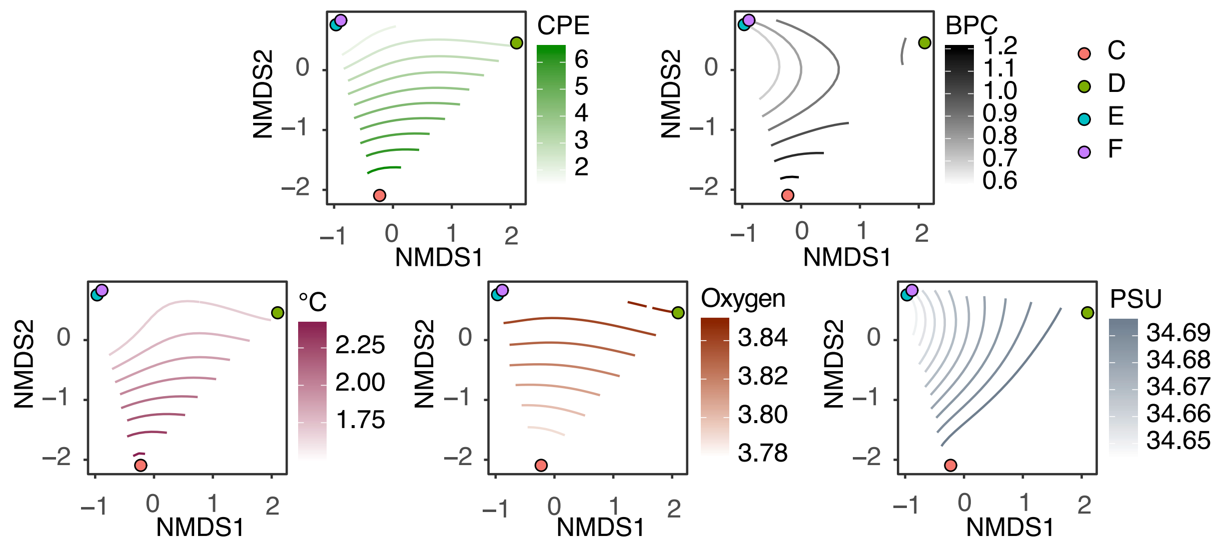

Figure 6. NMDS ordination maps (Stress < 0.01) based on Bray-Curtis's (dis-)similarity matrix with environmental surface fitting of environmental variables (CPE, BPC, Temperature, Oxygen and Salinity) for investigated sediments (E and F, Abyssal plain; C, Challenger Deep; D, southern flank).

The dbDRA analysis revealed that the assemblage composition of fungi in the different benthic sites was related to different physical-chemical conditions (i.e. salinity, temperature and oxygen concentrations; Figure 7) which altogether significantly explained almost their entire variance (Table 3). These results suggest that changes in physical and chemical characteristics of bottom waters can profoundly influence the assemblage composition of benthic deep-sea fungi even in hadal ecosystems (Tisthammer et al. 2016, Li et al. 2016). This result is also relevant on the light of the present global climate change which is expected to significantly alter current physical-chemical characteristics even of the deep-water masses significantly.

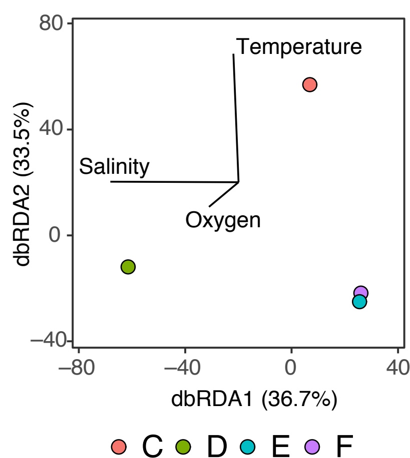

Figure 7. Multivariate relationship between Bray-Curtis (dis-)similarity matrix based on OTUs abundance patterns and environmental variables (only significant variables), visualised by dbRDA. Vectors show the relative contribution of each environmental variable on the ordination.

Table 3. Distance-based linear model results of Bray Curtis (dis-)similarity matrix.

	SS	Pseudo-F	P	R^2	Cumulative
SEQUENTIAL TESTS					
Salinity	15086	5.415	***	35.13	35.13
Temperature	14988	10.480	***	34.90	70.03
Oxygen	12848	4210.800	***	29.92	99.94

Conclusions

Despite recent methodological and technological advances, only 5% of the deep oceans have so far been explored and that less than 0.001% has been sampled and described concerning biodiversity (Danovaro et al. 2014, Corinaldesi 2015). Results presented in this study indicate that fungal assemblages in the bottom sediments of the Mariana trench were unexpectedly abundant and likely characterised by novel taxa. The peculiar environmental conditions of the Mariana trench appear to have an essential role in shaping assemblage composition by selecting fungal taxa largely different from those inhabiting abyssal plains. Overall findings reported in the present study provide new elements for a better understanding of the diversity and ecology of fungi in the deepest ecosystem on Earth.

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Chapter 4

Patterns and drivers of benthic deep-sea fungal abundance and diversity across oceans and eco-regions

Abstract: Fungi account for a significant fraction of Earth genetic diversity. However, their distribution and ecological role in benthic deep-sea sediments are yet largely unknown. In this study, we investigated the abundance, diversity and assemblage composition of fungi in a variety of benthic deep-sea ecosystems of the Pacific, Atlantic and Arctic Oceans. Our results indicate that fungal abundance, diversity and assemblage composition vary among and within oceans and eco-regions considered. Overall, high abundance and low diversity were encountered in deep-sea sediments of the Pacific Ocean and *vice-versa* in the Atlantic Ocean sediments, suggesting that fungal dynamics are controlled by differences in environmental and ecological factors between Oceans. Fungal abundance was largely dependent by the availability of trophic resources in the sediments, whereas assemblage composition by environmental conditions (e.g. temperature, salinity and dissolved oxygen concentrations of the bottom waters). Results presented in this study provide new insights on biogeographical patterns of benthic deep-sea fungi and factors influencing their distribution and pave the way for a better understanding of their ecological role in the functioning of the largest ecosystem on Earth.

Introduction

Fungi account for a significant fraction of Earth genetic diversity, and fungal activity influences plant and animal community structures, as well as the rates of ecosystem processes (Peay et al. 2016). In terrestrial and freshwater ecosystems, fungi are significant plant degraders detritus, thus significantly contributing to carbon cycling and nutrient regeneration processes (Dighton 2003). However, their diversity and ecological relevance in the functioning of marine ecosystems are less known when compared with terrestrial environments (Hyde et al. 1998, Carlile et al. 2001, Dighton 2003, Clipson et al. 2009).

Deep-sea sediments play a crucial role in biogeochemical cycles and host a significant fraction of Earth biodiversity (Danovaro et al. 2014, Corinaldesi 2015). Conversely to what previously hypothesised, several studies revealed that fungi are widespread across deep-sea habitats and ecosystems (Raghukumar et al. 2010, Richards et al. 2012), including hypersaline anoxic basins (Bernhard et al. 2014), methane hydrate-bearing and methane seeps (Lai et al. 2007, Wang et al. 2014), oxygen minimum zones (Jebaraj et al. 2010), hydrothermal vents (Le Calvez et al. 2009, Xu et al. 2017) and surface and subsurface sediments (Damare and Raghukumar 2008, Raghukumar et al. 2010, Edgcomb et al. 2011).

Experimental studies indicate that fungi isolated from deep-sea sediments can grow under high hydrostatic pressure and can efficiently exploit a wide array of organic compounds (Damare and Raghukumar 2008). Thus, fungi might have the potential to decompose also refractory organic compounds which represent the largest fraction of organic matter in benthic deep-sea ecosystems (Pusceddu et al. 2009), yet their role in carbon and nutrient cycling in benthic deep-sea ecosystems remains poorly understood. Moreover, recent studies suggest that a variety of environmental factors such as temperature, salinity and nutrients can influence deep-sea fungal assemblages (Tisthammer et al. 2016, Li et al. 2016, Barone et al. 2018). However, drivers controlling the distribution and diversity of fungi in benthic deep-sea ecosystems remain to date mostly unexplored.

In this study, we carried out a broad spatial scale investigation of benthic fungal abundance and diversity across different deep-sea habitats and ecosystems of the Pacific, Atlantic and Arctic oceans to provide new insights on biogeographical patterns and factors influencing their distribution.

Material and methods

Study areas and sampling strategy

Deep-sea sediments were collected in different sectors of the Pacific, Atlantic and Arctic Oceans (Figure 1). In the Pacific Ocean, samples were collected at several sites of the Clarion-Clipperton Fracture Zones (CCFZ) located in the Eastern Pacific and along a latitudinal transect in the Western Pacific. Sediment samples from the CCFZ were collected on board of the R/V Sonne (March-April 2015) in the French, German, Belgian, Interoceanmetal Joint Organization (IOM) areas (licensed for future activities of mineral exploitation by the International Seabed Authority) and from the designated Area of Particular Environmental Interest (APEI), within the framework of the European Project MIDAS (Managing Impacts of Deep Sea Resources Exploitation). Samples from the Western Pacific were collected during cruises YK1312 (01N and 12N) and YK1412 (39N) within a scientific collaboration with JAMSTEC (Japan). In the Atlantic Ocean, samples were collected within the EU HERMES and HERMIONE projects along bathymetric gradients of the Iberian (64PE252), Irish (64PE269) and Rockall (64PE249) margins. In the Arctic Ocean, sediment samples were collected off Svalbard during the cruise ARKXXI/1b. In all of the cruises, sediment samples were collected through multiple-corer deployments, and the top 1 cm was stored at -20°C until laboratory analyses. Sediment samples were used to analyse fungal abundance and diversity along with quantity and biochemical composition of organic matter.

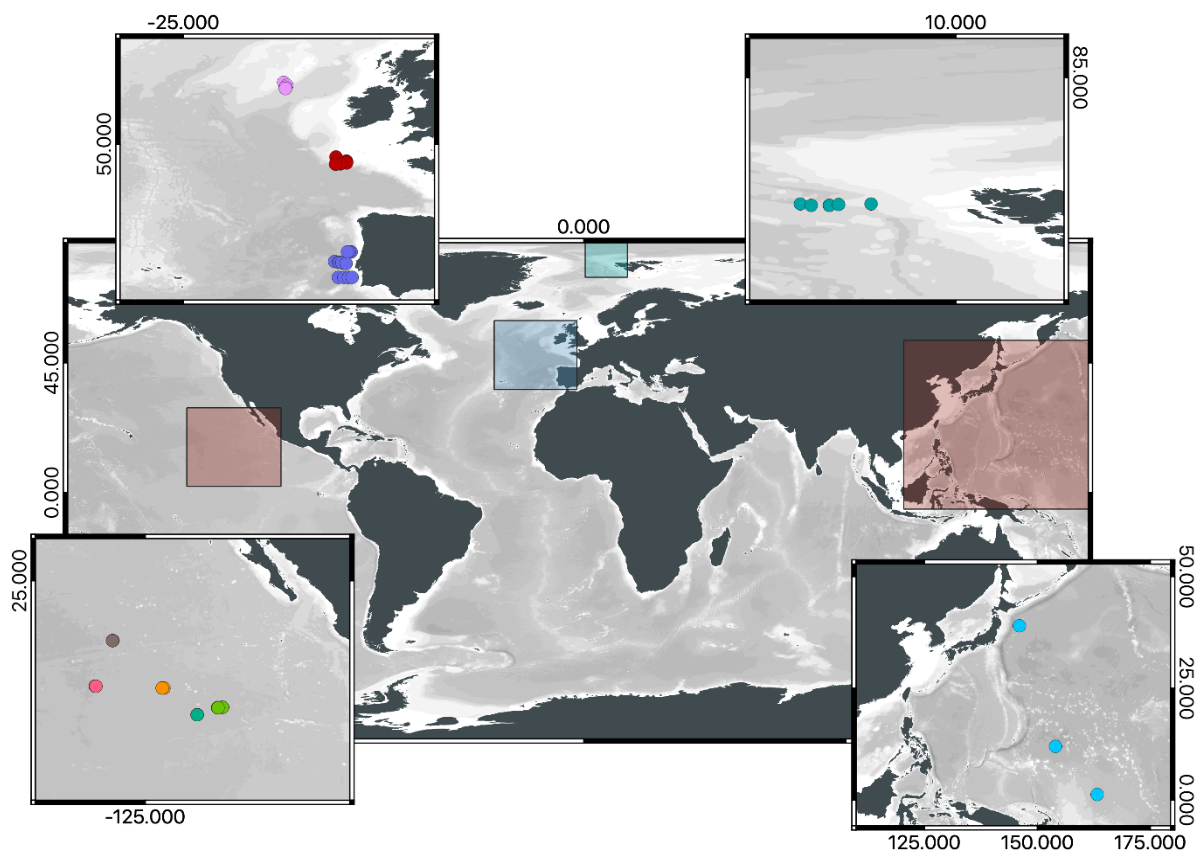

Figure 1. The geographic location of the study sites (44). Land maps used in these figures are from the public domain Natural Earth and freely available for personal, educational, and commercial purposes (http://www.naturalearthdata.com/about/ terms-of-use/). Red boxes represent Pacific Ocean stations while the blue and green highlight the Atantic and Arctic Oceans stations, respectively

Environmental and trophic conditions

Thermohaline characteristics and oxygen concentrations of the bottom water of the different investigated sites were extracted from Ocean Data View database as well as obtained from *in situ* measurements from CTD casts.

Trophic conditions of the benthic deep-sea ecosystems were assessed through the analysis of proteins, carbohydrates and lipids (Pusceddu et al., 2009), which represent the three major biochemical classes of organic compounds in deep-sea sediments (Danovaro 2010). Briefly, about 0.5 g of wet sediment was used for each analysis. Protein concentration was obtained via colourimetric method, which allows the reaction of proteins with rameic tartrate and the Folin–Ciocalteau reactive in a basic environment (pH 10). The reaction provides a stable blue colouration whose intensity is proportional to the protein concentration in the reaction solution.

Carbohydrate concentration was obtained via colourimetric assay based on the reaction between sugars and phenol in the presence of concentrated sulfuric acid. Lipids were extracted by direct elution with chloroform and methanol followed by reaction with sulfuric acid. Protein, carbohydrate and lipid concentrations were determined spectrophotometrically and expressed as albumin, glucose and tripalmitin equivalents, respectively. All analyses were carried out in 3 replicates. Protein, carbohydrate and lipid concentrations were then converted to carbon equivalents (conversion factors: 0.49, 0.40 and 0.75 gC g^{-1}, respectively) to determine biopolymeric C content in the sediments (Dell'Anno et al. 2002).

DNA extraction and purification for molecular analysis

The DNA was extracted and purified from the sediment samples using the PowerSoil DNA isolation kit (QIAGEN) with slight modifications to remove extracellular DNA (based on three subsequent washing steps) before DNA extraction accordingly to manufacturer instructions (Danovaro 2010). Aliquots of DNA were then used for quantitative real-time PCR and next-generation sequencing.

Quantitative real-time PCR of fungal 18S rRNA gene

Extracted DNA was used for quantitative real-time PCR (qPCR) analysis targeting the fungal-specific 18S rRNA gene (Taylor and Cunliffe 2016). Briefly, the fungi-specific primer sets FR1 (5′-AIC CAT TCA ATC GGT AIT-3′) and FF390 (5′-CGA TAA CGA ACG AGA CCT-3′) (Prévost-Bouré et al., 2011) were used with the Sensi-FAST SYBR Q-PCR kit (Bioline, London, UK). The 15 µl reactions contained 8 µl Sensi-FAST master mix, 1 µl of each primer (final concentration 1 µM), 1µl of DNA template and 5 µl nuclease-free molecular-grade water (Taylor and Cunliffe 2016). A Bio-Rad iQ5 was used to perform qPCR. The following qPCR thermal cycles were used: 94°C for 3min, then 40 cycles of 94 °C for 10 s, annealing at 50 °C for 15 s, elongation at 72°C for 20 s and acquisition of fluorescence data at 82°C. Standard curves were generated using known concentration of *Aspergillus niger* 18S rDNA. 18S rRNA gene copies were then standardised per gram of dry sediment.

Fungal diversity and assemblage composition

The ribosomal internal transcribed spacer 1 (ITS) was amplified and sequenced for the identification of fungal diversity using the primer set ITS1F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′- GCTGCGTTCTTCATCGATGC-3′) (Schoch et al. 2012, Walters et al. 2015). Sequencing was performed on Illumina MiSeq platform by LGC group (Berlin, Germany) following Earth Microbiome Project protocols (http://www.earthmicrobiome.org/emp-standard-protocols/). Bioinformatic analyses were performed using QIIME2 (https://qiime2.org). Briefly, pair-ends reads were merged using VSEARCH (Edgar 2010). Then, sequence quality control and feature table construction were performed using dada2 which infers sample sequences exactly and resolves differences of as little as one nucleotide (Callahan et al. 2016). Representative sequences were then matched against UNITE database for taxonomic affiliation (Version:7.2; Last updated: 2017-12-01). OTU table with taxonomy was then used for diversity analyses. To compare diversity among sample sites, we rarefied our dataset down to the lowest read abundance obtained using QIIME2. This method allows comparing samples with a different number of reads, thus minimising artefacts due to sequencing depth (McMurdie and Holmes 2014). Alpha-diversity analyses were carried out using vegan 2.5-2 (Oksanen et al. 2018). Networks diagrams were generated as SIMPER graphical output, and bipartite networks were made upon OTUs reads abundance to decipher the structure of complex fungal communities using Gephi 0.9.2 (Barberán et al. 2012).

Statistical analyses

Difference between sites, areas and oceans, in term of trophic characteristics and fungal abundance, were tested via permutational analysis of variance (PERMANOVA) as it allows to compare variances even in univariate environment in non-parametric conditions using PRIMER+PERMANOVA v6 (Anderson 2014). Distance matrixes were calculated using Euclidean distance. Non-parametric Spearman correlation analysis was used to identify relationships between environmental and trophic variables and the number of fungal 18S rRNA gene copies. Forward distance-based multivariate linear regression analysis was carried out to identify potential drivers influencing fungal abundance (Legendre and Legendre 2012). To examine the relationships between fungal abundance and depth, we performed partial regression analysis (Rex et al. 2006), which considers the residuals of the multiple regression

between abundance and geographical coordinates to standardise abundance across locations which is then tested against depth. SIMPER was conducted to explore (dis-)similarity between fungal assemblage composition and to identify the most important OTUs contributing to the observed differences using PRIMER+PERMANOVA v6 (Clarke 1993). Fungal communities were converted in (dis-)similarity matrices following Jaccard's method, and then PERMANOVA was used to test for significant differences. Forward distance-based multivariate linear regression was also carried out to identify relationships between the environmental variables and fungal assemblage composition in multidimensional environment. Non-parametric multivariate regression tree analysis supported by Similarity Profile (SIMPROF) routine was carried out employing PRIMER PERMANOVA v6 to describe how best the assemblage samples are split into groups, by successive binary division (in the full high-d space) with the endpoint to produce a divisive clustering of the biotic samples where each cluster has an interpretation in terms of a sequence of inequalities on the environmental variables (De'ath 2002, Clarke and Gorley 2006).

Results and discussion

Patterns and drivers of fungal abundance

The abundance of fungal 18S rRNA gene copies (as a proxy of fungal abundance) was almost one order of magnitude higher in the Pacific Ocean (23.7×10^6 18SrRNA gene copy g^{-1}; $p > 0.0008$) compared to the Atlantic Ocean (0.65×10^6 18S rRNA gene copy g^{-1}; Figure 2c). The Arctic Ocean displayed abundance values lower than the Pacific Ocean (3.9×10^6 18SrRNA gene copy g^{-1}), although not statistically different, while it was significantly higher than that of the Atlantic Ocean ($p > 0.002$). The highest values of fungal 18S rRNA gene copies in the Pacific Ocean where found in the German, IOM and Belgian area (5.51, 5.59, 5.29×10^7 18S rRNA gene copy g^{-1} respectively; Figure 2b). Overall, Pacific sediments were characterised by a low variability of fungal 18S rRNA gene copies when compared to that of Atlantic Ocean (Figure 2a), likely related to the less variable environmental conditions occurring in abyssal plains, where Pacific samples were collected.

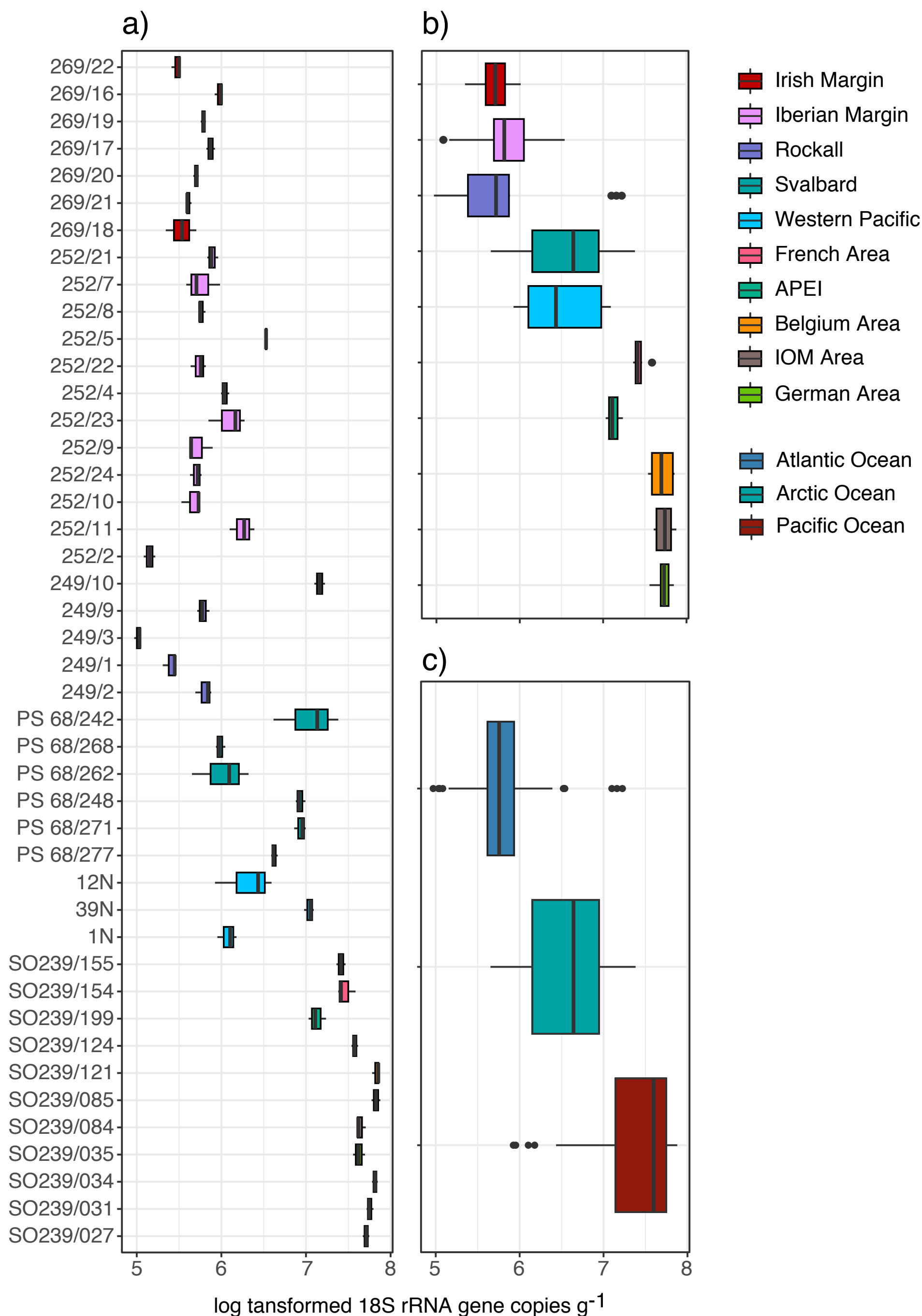

Figure 2. Box-plot representation of log(x+1) transformed fungal 18S rRNA gene copies g^{-1} for a) sites, b) areas and c) ocean. Error bars display 95% confidence interval; the black line within the box indicates median.

Interestingly, fungal abundance was overall significantly and positively related to water depth (Figure 3a). So far, a meta-analysis of the distribution of benthic deep-sea assemblages carried out on a global scale indicates that all the multicellular organisms (meio-, macro- and megafauna) significantly decrease with increasing water depth and only prokaryotes exhibit no relationship (Rex et al. 2006). The positive relationship observed in this study between fungal

abundance and depth suggest that this component can be more adapted, that any other eukaryotic component investigated so far, to the progressive food limitation both in terms of quantity and bioavailability with increasing water column depth. Within the different Oceans and the different geographic areas, fungal abundance remains quite constant with increasing water depth (Figure 3b-c), suggesting that environmental and ecological conditions acting at smaller spatial scale, besides water depth may exert an additional role in influencing patterns of benthic deep-sea fungal standing stocks (Figure 3c).

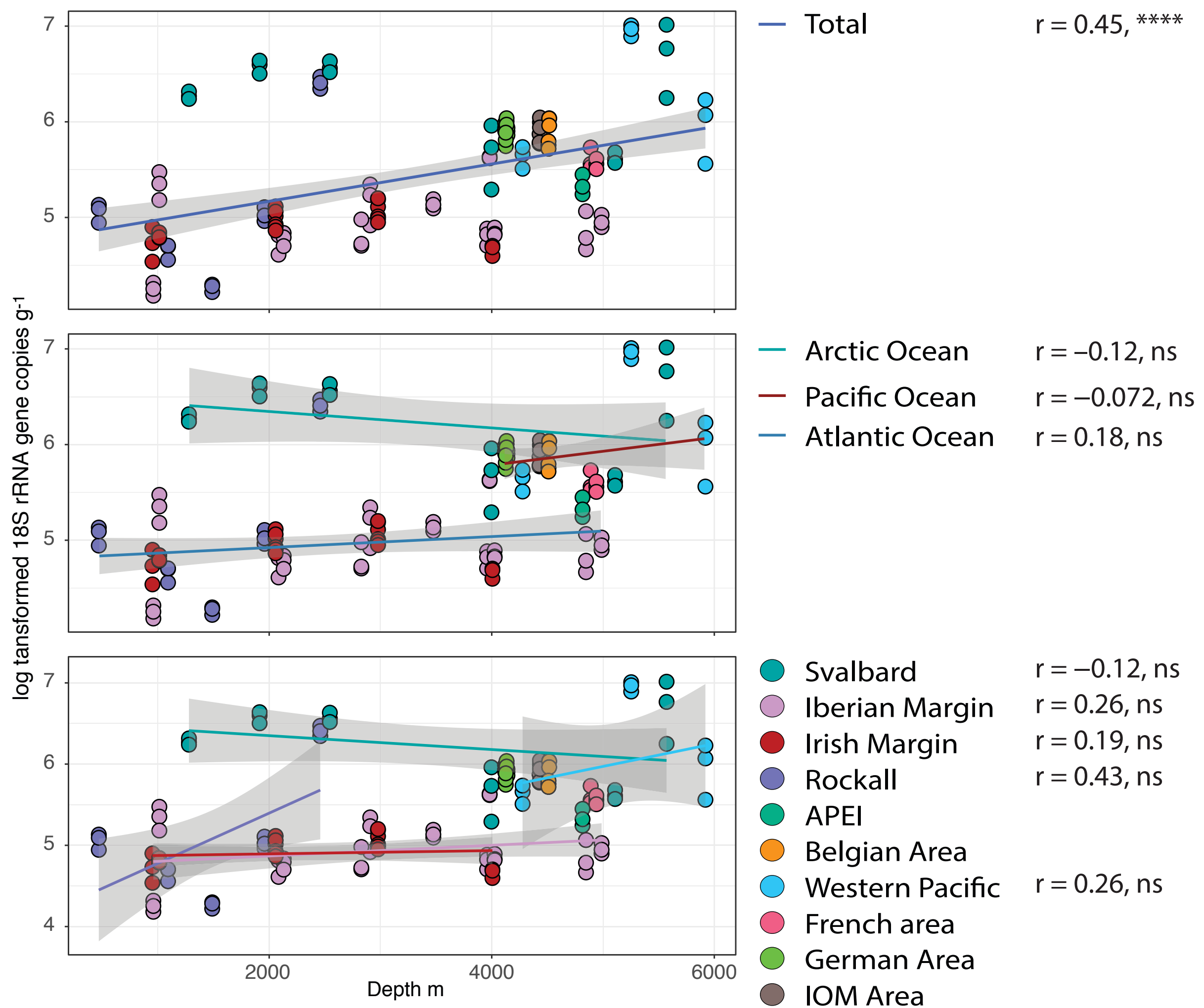

Figure 3. Outputs of the partial regression analysis after removing the effect of longitude and latitude between the number of 18S rRNA gene copies as proxy of fungal abundance and water depth. Correlation coefficient and significance values ($p < 0.05$ = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****) are provided.

Spearman's correlation analyses revealed significant relationships between fungal abundance and trophic resource availability ($p > 0.0001$, Figure 4). In particular, stronger positive

relationships were found between fungal abundance and carbohydrate as well as biopolymeric carbon concentrations (r = 0.82 and r = 0.87 respectively). These results obtained from an extensive spatial scale investigation reinforce previous findings obtained on a local scale (Barone et al. 2018) on the role of food availability in controlling the abundance of benthic deep-sea fungi.

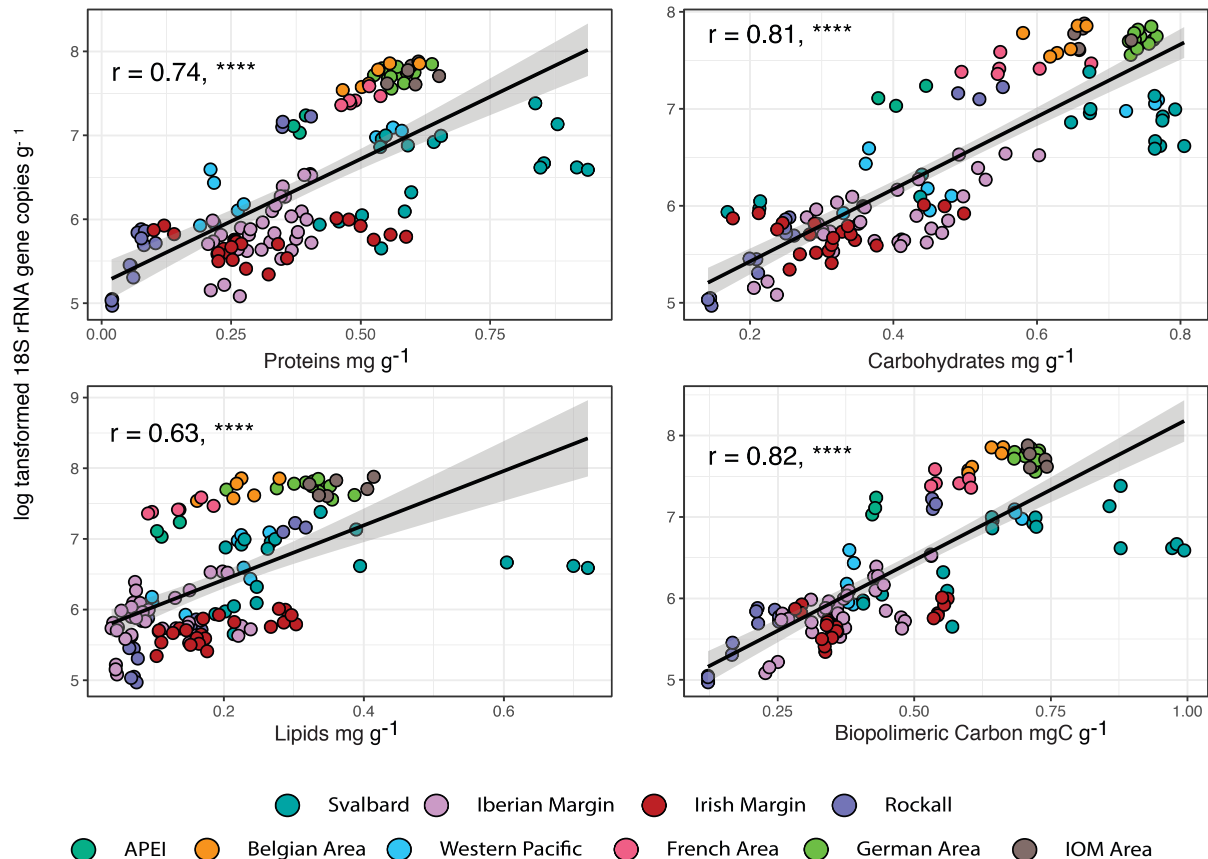

Figure 4. Scatterplots showing the relationship between log-transformed fungal relative abundances and trophic resource availability. Spearman rank (r) and significance (p) values are reported for each plot.

Multivariate multiple regression analysis (distLM forward) indicates that carbohydrate concentrations alone explained ca. 70% of the variance of the distribution of fungal abundance on a global scale (Table 1). These results provide support to the expectation that fungi are involved in the degradation of organic detritus not only in the water column (Gutiérrez et al. 2011) but also in benthic deep-sea ecosystems, and suggest that they can be highly specialised of the most recalcitrant organic compounds, including complex carbohydrates (Pusceddu et al. 2009).

Table 1. Distance-based multivariate linear model (DistLM) for relative fungal abundance displaying the % of variation explained by descriptor variables, and the % of variation explained in multivariate environment (Forward method). P indicates the significance of the relationship ($p < 0.05$ = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****).

Variable	SS (trace)	Pseudo-F	P	Explained %	Cumulative %
SEQUENTIAL TESTS					
Carbohydrates	64.59	282.20	****	69.47	69.47
Oxygen	8.94	56.58	****	9.62	79.09
Salinity	4.28	34.44	****	4.60	83.69
Temperature	0.06	0.45	Ns		
Depth	0.04	0.35	Ns		
Lipids	0.03	0.26	Ns		
Proteins	0.12	0.96	Ns		

Fungal diversity and assemblage composition

The number of reads obtained in the present study by the high-throughput sequencing of the fungal ITS1 region varied considerably amongst sites. Therefore, to compare the different sites, the dataset was randomly normalised at the minimum number of reads which correspond to 3000 sequences (Figure 5). Except for one sample, 3000 sequences were sufficient to reach saturation. Overall, a total of 2648 OTUs was observed. Of these, 1807 belonged to fungi, 520 had no match and 161 affiliated to Rhizaria. We excluded from the dataset all the sequences not belonging to fungi corresponding to 681 OTUs (37% reads). Also, Agaricomycetes (159 OTUs) were excluded, despite having been repeatedly found in marine and deep-sea ecosystems (Singh et al. 2011, Richards et al. 2015). The presence of Agaricomycetes is unlikely, as most species are typically terrestrial, wood decomposing with a large fruiting body and there is no evidence they live in ocean sediments (Li et al. 2016). Therefore, these signatures are assumed to be dead cells or dormant spores that reached deep-sea sediments by passive transportation via rivers, terrestrial runoff or aerial transportation. To this regard, the number of OTUs belonging to Agaricomycetes was significantly more abundant in Atlantic samples (141 OTUs, $p < 0.0001$), where benthic sites where located closer to the coastline, than in the Pacific (22 OTUs) suggesting that the proximity to the land may represent a source of these taxa.

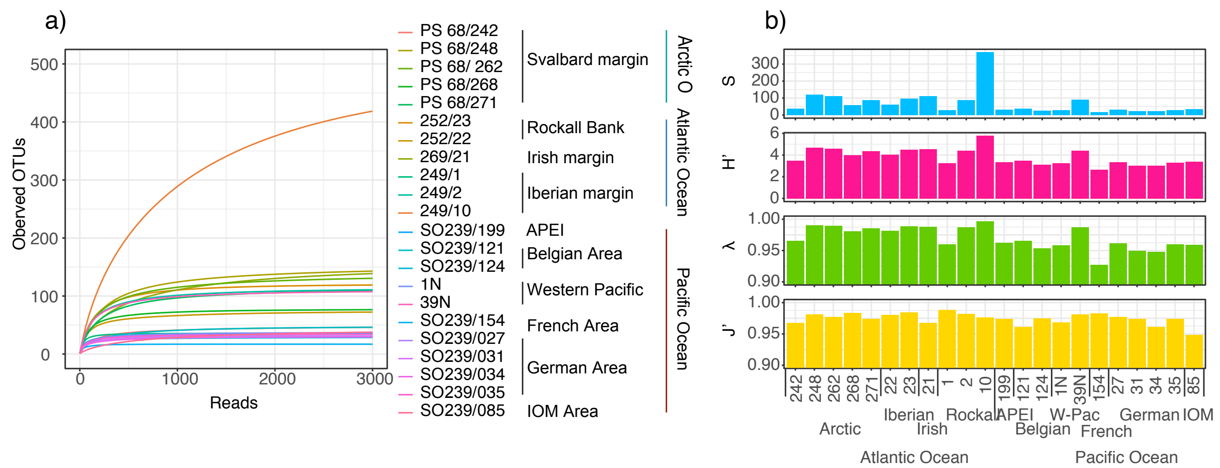

Figure 5. Rarefaction curves of the retained samples (22). Alpha diversity indexes (S indicates OTU richness, H' Shannon diversity index, λ Simpson's index and J' Pielou's evenness index).

Fungal OTU richness was higher in the Atlantic sediments (range: 27-369 OTUs) than in Pacific sediments (range: 15-90 OTUs; Figure 5b). The higher fungal diversity of the Atlantic sediments was also confirmed by both the Shannon and Simpson diversity indices. At the same time higher fungal OTU richness was observed at high latitudes compared to low latitude sites ($p < 0.0001$). These results suggest that high latitude ecosystems close to continental margins can host a higher fungal richness when compared to tropical/equatorial systems as previously reported for the diversity of large deep-sea metazoans (i.e. holothurians, Woolley et al. 2016). In the present study, the value of Pielou's index (J') was consistently high in all benthic deep-sea habitats and ecosystems investigated. These findings suggest that benthic deep-sea fungal assemblages are composed of evenly distributed taxa (characterised by OTUs with considerably low reads abundance), independently from habitat, ecosystem and biogeographic region.

Our results indicate that although the OTU number of fungi in the Pacific sediments was lower than in the Atlantic Ocean, their abundance (as 18S rRNA gene copy number) was significantly higher. Different factors can be invoked to explain low abundance and high diversity patterns and vice-versa we observed. For instance, species dispersal can be more relevant in dynamic ecosystems (Magurran and Hendriks 2003), such as the continental margins of the Atlantic Ocean when compared to the abyssal plain of the Pacific Ocean and this could favour the "immigration" of a higher number of fungal taxa exported from the continental shelf and/or terrestrial run-off (Picard 2017, Serván et al. 2018). At the same time, the wider environmental

fluctuations and the higher bottom heterogeneity characterising the benthic ecosystems of the Atlantic Ocean can allow the coexistence of more diverse but less abundant fungal assemblages by reducing competitive exclusion and promoting niche partitioning (Hutchinson 1961; Whittaker et al., 2001).

In the present study, we found that benthic deep-sea fungi were largely represented by Ascomycota, which alone accounted for 1389 OTUs (40843 reads). This observation is consistent with previous findings in which Ascomycota dominated over others phyla (Xu et al. 2014). We also found 386 OTUs (5402 reads) failing to match any known phylum, but retained as Fungi with confidence limit greater than 0.9. The remaining fraction of diversity was due to fungi affiliating with Chytridiomycota (73 reads and 8 OTUs), Mucromycotina (30 reads and 9 OTUs), Neocallimastigomycota (17 reads and 1 OTUs), Basidiomycota (13 reads and 3 OTUs), Monoblepharomycota (6 reads and 1 OTUs) and Rozellomycota (5 reads and 1 OTUs). We also found that the number of reads belonging to Basidiomycota, after the removal of those affiliated with Agaricomycetes, was very low, expanding previous findings based on a limited dataset (Li et al. 2016, Picard 2017) on the negligible relevance of this group to the fungal diversity in the deep sea. However, biases related to the limited amplification of Basidiomycota using ITSF-ITS2 primers cannot be ruled out (Stoeck et al. 2006, Singh et al. 2011, Xu et al. 2014).

In this study, we found that an important fraction of reads affiliated with taxa involved in parasitic or pathogenic associations. Among these, we identified the genus *Knufia*, which include black yeasts belonging to the family Chaetothyriaceae (Chaetothyriales, Ascomycota), and from the same family the black yeast genus *Exophiala*, which has been suggested as the causative agent of epizootic events of mussels inhabithing hydrothermal vent systems (Van Dover et al. 2007). We also identified several OTUs affiliating to *Malassezia* which represent another widespread taxon reported in several habitats and locations, from polar regions to deep-sea vents (Amend 2014). Moreover, the presence of Rhizopus, a genus including saprophytic fungi and specialised parasites was also detected. Although only a few studies have investigated the role of fungal pathogens in deep-sea ecosystems (Van Dover et al. 2007, Sapir et al. 2014), the widespread presence of sequences affiliating to black yeasts we observed suggest that pathogenic fungi may have a not negligible role on metazoan dynamics across different benthic deep-sea habitas and ecosystems.

Our study also reveals the presence of several saprophytic fungal taxa including *Aspergillus* and *Penicillium* genera. Members of these genera are widespread especially in nutrient-depleted environments, such as the deep-sea ecosystems, as well as in extreme environments including hypersaline anoxic basins (Alexander et al. 2009, Stock et al. 2012, Edgcomb et al. 2016). We also identified several OTUs affiliating to Saccharomycetales and Spizellomycetales chytrids, which include either members with important implications in nutrient cycling or parasites of metazoans such as nematodes. In our dataset, one abundant OTU affiliated with Neocallimastigaceae family in the Neocallimastigomycota phylum. This group contains fungi capable to hydrolyse the most recalcitrant plant polymers and degrade entirely non-lignified plant cell walls. Overall, the presence of many saprotrophic fungal taxa let us hypothesise an active role in the degradation processes of the most recalcitrant fraction of organic matter in benthic deep-sea sediments worldwide.

Overall, only a small fraction of the sequences was successfully affiliated at a taxonomic level lower than phylum, indicating that deep-sea sediment can host a large number of new taxa with no relatives described yet. These results are also consistent with previous findings which reported low taxonomic resolution for most of the microbial taxa inhabiting the deep sea (Zinger et al. 2011, Zhang et al. 2014, Barone et al. 2018). Thus, the current fungal databases as well as algorithms used for bioinformatic analysis (http://qiime2.org) still need implementation to allow a deeper understanding of fungal diversity patterns. At the same time, estimates of species richness of fungi based on OTU number should be view with caution due to intraspecific variation of fungal ITS (Schoch et al. 2012, Kiss 2012), which can lead to an overestimation of their actual diversity.

Fungal assemblage composition significantly changed among oceans ($p = 0.0001$, PERMANOVA). In particular, fungal assemblage composition of the Arctic and Atlantic Oceans was significantly different from that of the the Pacific Ocean ($p = 0.0018$ and 0.0004, respectively), which clustered apart (Figure 6).

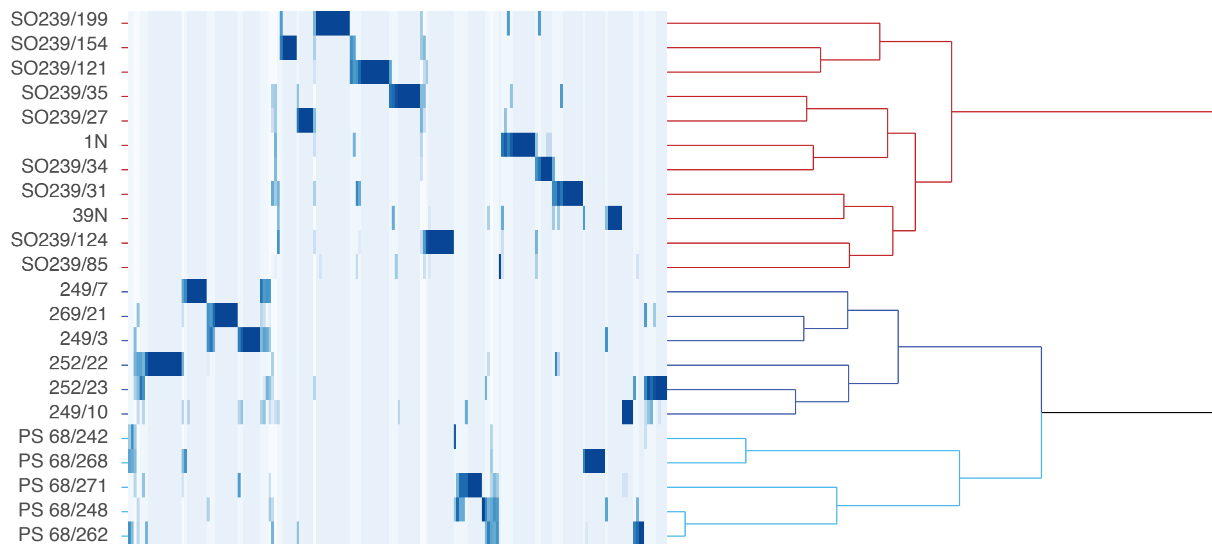

Figure 6. Cladogram calculated on Jaccard's (dis-)similarity matrix and heat map showing read abundance for the most abundant (99.9%) OTUs for each sample.

Fungal assemblage composition was highly variable from small to inter-ocean scales. Network analysis, indeed, revealed that overall only 12 fungal OTUs were found in all oceans and that no OTUs (core OTUs) were shared among all benthic deep-sea sites investigated (Figure 7). We also found that many fungal OTUs were shared between a few benthic deep-sea sites and that the majority of OTUs were unique of each site.

These results indicate that differences in environmental and ecological conditions acting at the local scale can promote a high fungal turnover diversity which, in turn, is responsible for the increase of benthic deep-sea fungal diversity at increasing spatial scales (from γ-diversity of each deep-sea habitat/ecosystem to the overall oceanic ε-diversity).

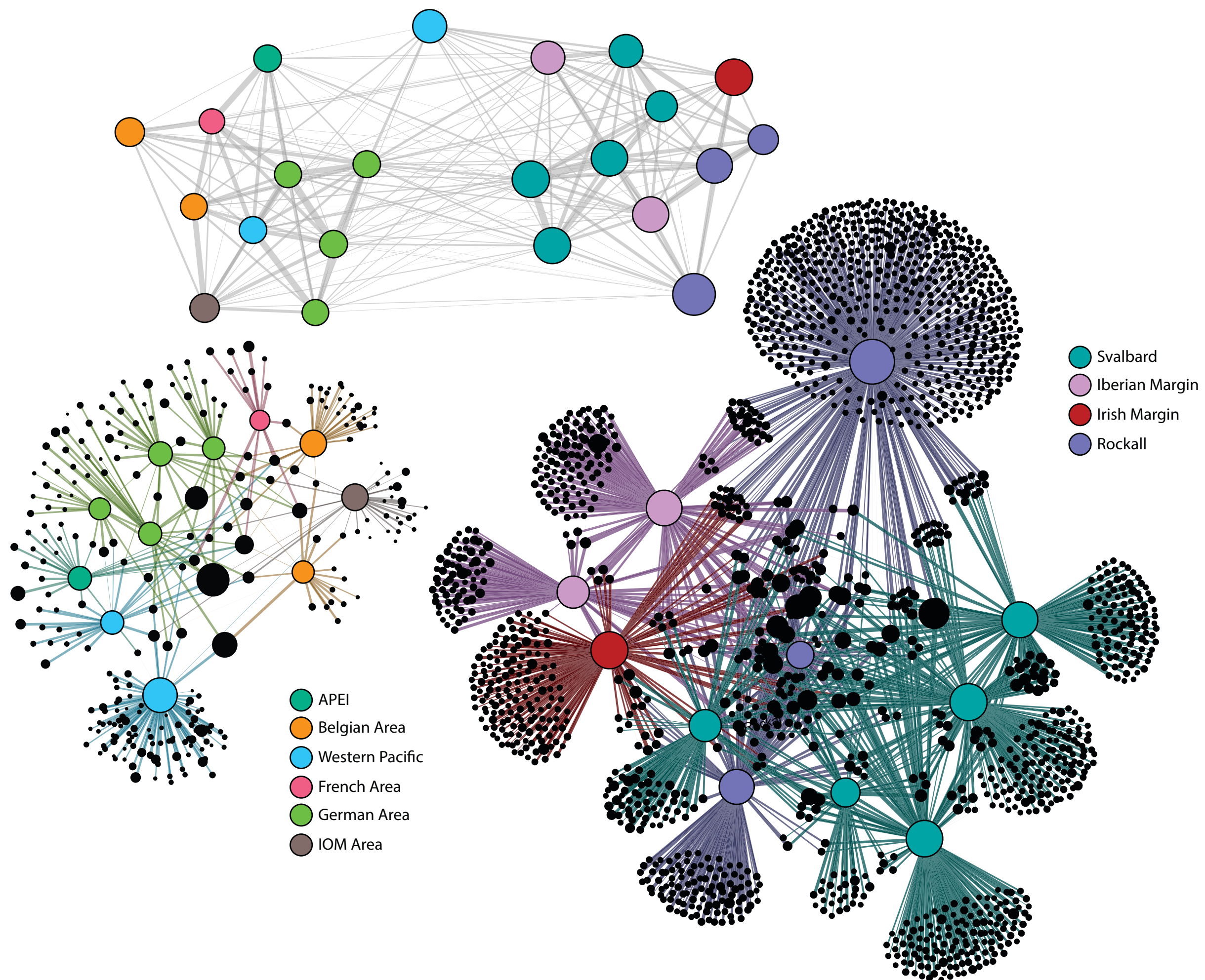

Figure 7. Network diagrams. A) SIMPER representation of sites in which edge width is proportional to SIMPER scores for each site, the wider the edge, the higher is the similarity score. B, C) Bipartite diagrams based on OTU table in which edge width is proportional to reads abundance of each OTU (black nodes; site node size is proportional to OTU richness while OTU node size is proportional to overall reads abundance).

Db-RDA analysis revealed that differences in fungal assemblage composition were related to different environmental settings (Figure 8). In particular, temperature and oxygen cumulatively explained about 14% of the variability of fungal assemblage composition (Table 2). These results reinforce previous findings on the importance of environmental factors in influencing the fungal assemblage composition in marine ecosystems (Shearer et al. 2006, Zhang et al. 2015, Li et al. 2016, Tisthammer et al. 2016) and indicate that other factors, which need to be identified yet, have to be considered for a better understanding of drivers shaping fungal assemblages in benthic deep-sea ecosystems worldwide.

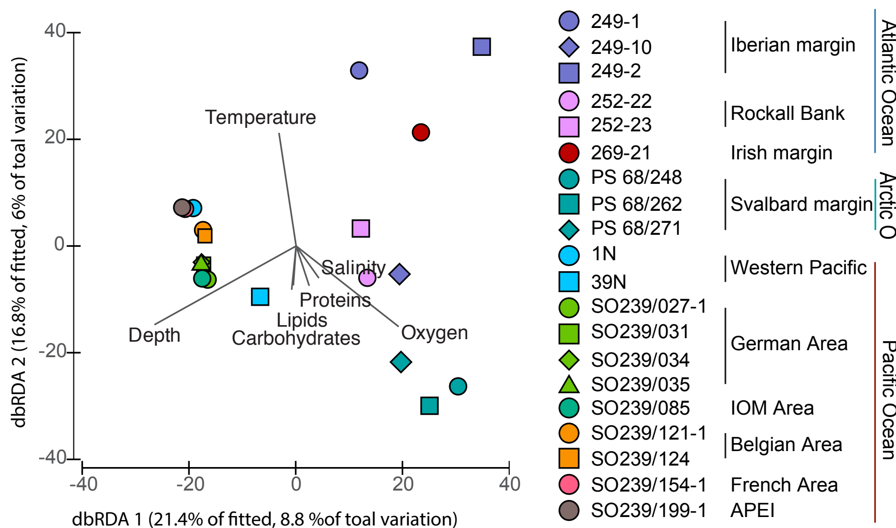

Figure 8. Multivariate relationship between Jaccard's (dis-)similarity matrix based on OTUs abundance patterns and environmental all environmental variables visualised by db-RDA. Vectors show the relative contribution of each environmental variable on the ordination.

Table 2. Distance-based multivariate linear model (DistLM) for fungal assemblage composition % of variation explained by each descriptor variables, and the % of variation explained in multivariate environment (Forward method). P indicates the significance of the relationship (p < 0.05 = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****).

Variable	SS (trace)	Pseudo-F	P	Explained %	Cumulative %
SEQUENTIAL TESTS					
Oxygen	7494	1.6221	****	8.27	8.27
Temperature	5683.6	1.2471	***	6.27	14.536
Lipids	5028.3	1.1105	ns	5.55	
Depth	4909.4	1.0904	ns	5.42	
Salinity	4638.4	1.0324	ns	5.12	
Carbohydrates	4605.8	1.0272	ns	5.08	
Proteins	4635.4	1.0367	ns	5.11	

Multivariate non-parametric regression tree analysis showed that fungal assemblage composition between oceans was significantly explained by changes of salinity, oxygen and depth (Figure 9). Fungal assemblage composition among areas of the Pacific Ocean was mostly dependent on thermohaline conditions, whereas in the Atlantic Ocean also from the availability of trophic resources.

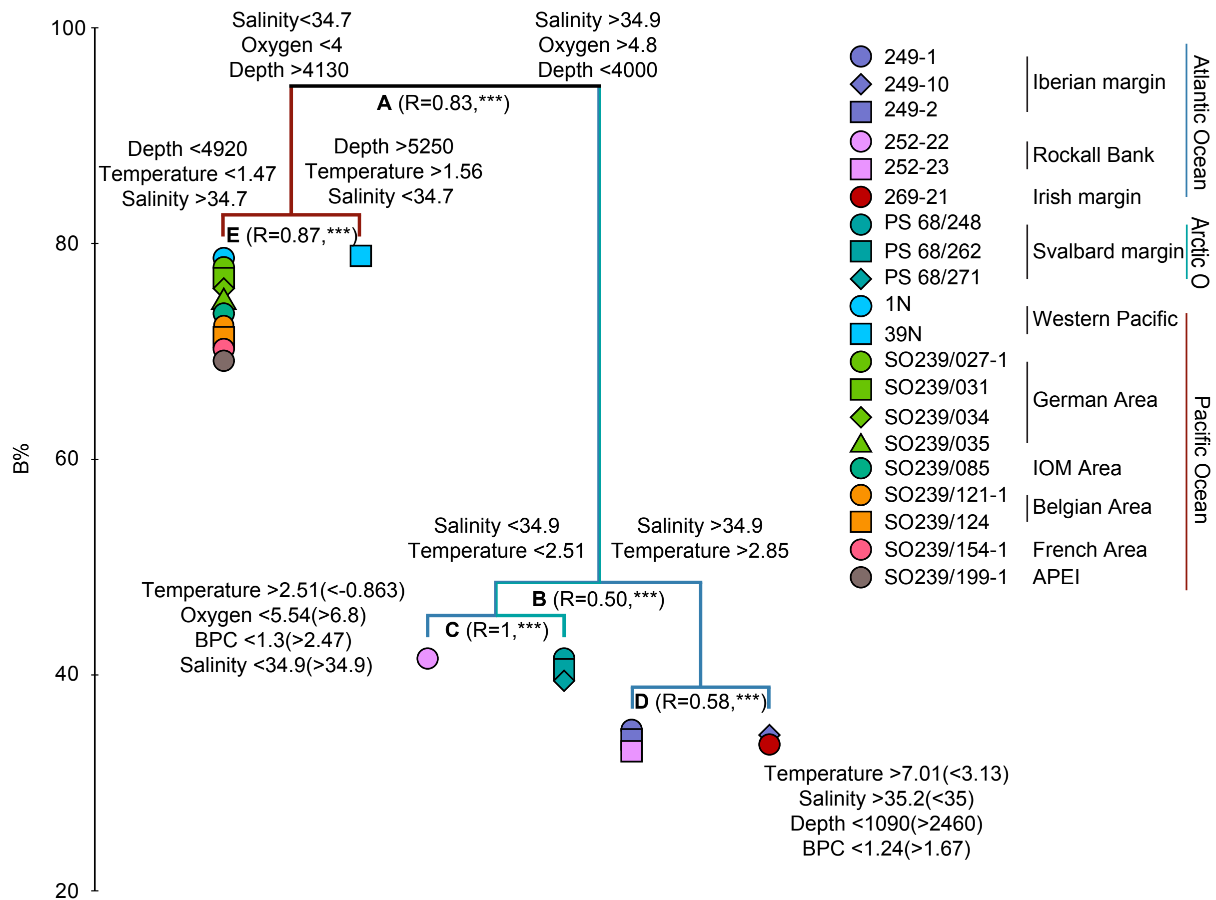

Figure 9. Multivariate non-parametric regression tree. Above each branch are indicated the variable responsible for partitioning. Where not possible right branch values are reported within brackets. Every node is named from A to D with the goodness of fit (R) and SIMPROF significance (*≤5%, **≤1%, ***≤0.1%).

Conclusions

Despite the growing interest on marine fungi, only a limited number of studies attempted to shed light on their biogeographical patterns (Zhang et al. 2015, Tisthammer et al. 2016, Li et al. 2016, Tian et al. 2018). Here we showed that the abundance of benthic fungi, contrarily to any other eukaryote previously investigated on a large spatial scale, does not decline with increasing water depth while, on the other hand, trophic availability has great influence on their abundance. Environmental factors such as oxygen, salinity, and water depth explained only a minor fraction of the variance of the fungal assemblage composition in benthic deep-sea ecosystems, indicating that other factors, including biological interactions, have the major role. Results presented in this study provide new insights on biogeographical patterns of benthic deep-sea fungi and factors influencing their distribution and pave the way for a better understanding of their ecological role in the functioning of the largest ecosystem on Earth.

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Chapter 5

Fungal abundance and diversity in deep-sea sediments of the Ross Sea (Southern Ocean)

Abstract: Fungi are present in any marine ecosystem investigated so far, but information on their quantitative relevance and diversity in polar ecosystems is scant. In this study, we explored for the first time the abundance and diversity of fungi in deep-sea sediments of three different areas of the Ross Sea (Southern Ocean), characterised by different environmental and sedimentary features, to shed light on their ecology and factors shaping their distribution. The fungal abundance (as the number of 18S rRNA gene copies) in the different sampling areas varied of almost two orders of magnitude consistently with changes of sediment features and availability of trophic resources. Similarly, higher fungal OTU richness was encountered in sediments characterised by higher organic matter content. The assemblage composition of fungi displayed major changes among the benthic deep-sea ecosystems investigated, suggesting that the variability of environmental conditions even at spatial scales of few kilometres may promote a high turnover diversity. Overall, results reported in this study provide new insights on factors influencing the abundance and diversity of benthic deep-sea fungi inhabiting the Southern Ocean and can be useful for a better comprehension of the potential responses of the benthic assemblages inhabiting polar ecosystems on the light of future climate change scenarios.

Introduction

In any marine ecosystem, microbial assemblages have a crucial role in primary production processes, through photosynthesis and chemosynthesis, and in C and nutrient cycling through degradation and utilisation of organic matter (Arrigo et al. 1998, Azam and Malfatti 2007, Falkowski et al. 2008, Edwards et al. 2012). Life in benthic deep-sea ecosystems is mostly dominated in terms of abundance and biomass by prokaryotes which fundamentally influence biogeochemical processes (Jørgensen and Boetius 2007, Mayor et al. 2012). Fungi are a diverse and ubiquitous component of the marine food webs and being capable of also degrading the highly refractory organic matter that other microorganisms cannot, are assumed to contribute to C and nutrient cycling (Tisthammer et al. 2016, Li et al. 2016, Barone et al. 2018). However, the influence of fungi on nutrient and carbon cycling in benthic deep-sea sediments is yet to be defined.

Polar marine ecosystems are highly dynamic and despite their harsh environment conditions host diverse planktonic and benthic assemblages, including fungi (López-García et al. 2001, Zhang et al. 2015, Bochdansky et al. 2016). Here, fungi can have significant effects on primary production dynamics and carbon fluxes within the marine food webs (Hassett and Gradinger 2016, Hassett et al. 2017). At the same time, their diversity and assemblage composition can significantly change in relation with changes in environmental conditions (Bubnova 2010) as also reported for other planktonic and benthic microbial assemblages (Carr et al. 2013, Wilkins et al. 2013, Emil Ruff et al. 2014, Signori et al. 2014). Global climate changes are significantly altering marine biodiversity and food web dynamics, and such effects are more pronounced at higher latitudes rather than at lower latitudes. Thus, changes in environmental conditions due to the present climate change in polar regions can induce a domino effect that could impact ecosystems from continental ice sheets to the seafloor (Learman et al. 2016).

In this study, we explored for the first time the abundance and diversity of fungi in the deep-sea sediments of the Ross Sea (Southern Ocean) to shed light on their ecology and the factors shaping their distribution. Such information is crucial for a better comprehension of the potential responses of the benthic assemblages inhabiting polar ecosystems on the light of the future climate change scenarios.

Material and methods

Study site and sampling strategy

Sediment samples were collected in the Ross Sea (Antarctica) (72 to 74°S, 174 to 175°E; Figure 1) during the austral summer of 2017 on board of *R/V ITALICA* in the framework of the XXXII Italian Antarctic Expedition. Samples were collected in three areas named B, C and D. Area B (average depth of 567 m) is located in the centre of the northern part of the Joides basin and is characterised by bio-siliceous olive-grey mud sediments. Area C is located at 439 m depth close to the shelf break in the northern flank of the Mawson Bank and is characterised by sand, gravel, pebbles, and coarse biogenic carbonate debris with high near-bottom current velocities (up to 20 cm s^{-1}; Fabiano and Danovaro 1998). In both areas B and C, two benthic sites located few km each other were selected (hereafter defined B1 and B2 and C1 and C2), whereas in area D only one site was selected. At each benthic deep-sea site, undisturbed sediment samples were collected using a multiple corer and, once retrieved, the first 1 cm of the sediment was immediately frozen and kept at -27 °C until processing for organic matter and fungal DNA analyses.

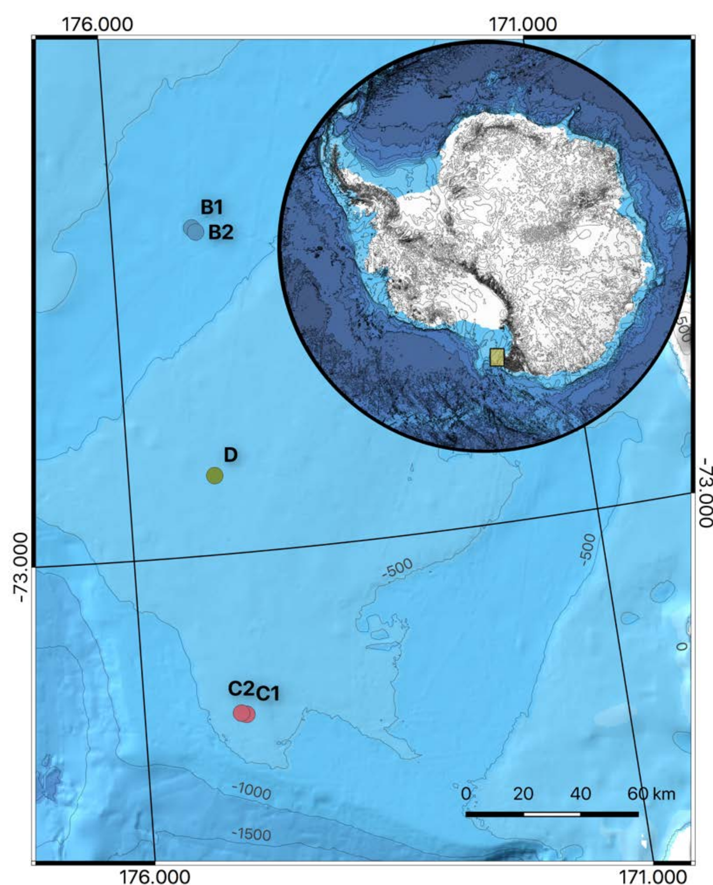

Figure 1. Graphical representation of study site locations where B1, B2, C1, C2 and D represent the three station at the three sites investigated which are located at an average depth of 587, 433 and 372 respectively for B, C and D. The map was generated upon freely available layers (Arndt et al. 2013, https://www.scar.org/science/ibcso/resources/)

Sedimentary organic matter

Trophic conditions of benthic systems were assessed on the basis of quantity and biochemical composition of organic matter (Danovaro 2010). In particular, the three major biochemical classes of organic compounds (proteins, carbohydrates and lipids) in deep-sea sediments were determined. Briefly, about 0.5 g of wet sediment was collected and used for the quantification of proteins (PRT), carbohydrates (CHO) and lipids (LIP). Protein concentration was assessed via colourimetric assay, allowing the reaction of proteins with rameic tartrate and the Folin-Ciocalteau in a basic environment (pH 10) which provides a stable blue colouration with an intensity proportional to protein concentration. Carbohydrate concentration was determined via a colourimetric assay based on the reaction between carbohydrates and phenol in the presence of strong sulfuric acid which provides a colouration whose intensity is proportional to carbohydrate concentration. Lipids were extracted by direct elution with chloroform and methanol followed by reaction with sulfuric acid for colourimetric assay. Protein, carbohydrate and lipid concentrations were determined spectrophotometrically and expressed as albumin, glucose and tripalmitin equivalents, respectively. All analyses were carried out in 3 replicates. Protein, carbohydrate and lipid concentrations were then converted to carbon equivalents (conversion factors: 0.49, 0.40 and 0.75 gC g^{-1}, respectively) to determine biopolymeric C content (BPC) in the sediments (Dell'Anno et al. 2002). Total phytopigment concentrations (CPE) were obtained as the sum of chlophyll-a and phaeopigment concentrations obtained by extraction with 90% acetone and analysed fluorometrically.

DNA extraction and purification for molecular analysis

The DNA was extracted and purified from the sediment samples using the PowerSoil DNA isolation kit (QIAGEN) following the manufacturer's instruction with slight modifications to remove extracellular DNA (based on three subsequent washing steps) before DNA extraction (Danovaro 2010).

Fungal abundance

DNA aliquots were used for quantitative real-time PCR (qPCR) analysis of fungal-specific 18S rRNA gene (Taylor and Cunliffe 2016). Briefly, fungi-specific primers (FR1 5′-AIC CAT TCA ATC GGT AIT-3′) and FF390 (5′-CGA TAA CGA ACG AGA CCT-3′) which amplify an 18S rRNA gene fragment of about 350bp (Chemidlin Prévost-Bouré et al. 2011) were used with

the Sensi-FAST SYBR Q-PCR kit (Bioline, London, UK). The 15 µl reactions contained 8 µl Sensi-FAST master mix, 1 µl of each primer (final concentration 1 µM), 1µl of DNA template and 5 µl nuclease-free molecular-grade water (Taylor and Cunliffe 2016). A Bio-Rad iQ5 was used to perform qPCR. The following qPCR thermal cycles were used: 94°C for 3min, then 40 cycles of 94 °C for 10 s, annealing at 50 °C for 15 s, elongation at 72°C for 20 s and acquisition of fluorescence data at 82°C. Standard curves were generated using known concentrations of Aspergillus niger 18S rDNA. The 18S rRNA gene copies were then standardised per gram of dry sediment.

Fungal diversity based on ITS1 sequencing

The ribosomal internal transcribed spacer 1 (ITS) was amplified and sequenced for the analysis of fungal diversity using the primer set ITS1F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′- GCTGCGTTCTTCATCGATGC-3′) (Schoch et al. 2012, Walters et al. 2015). Sequencing the ITS1 was performed on Illumina MiSeq platform by LGC group (Berlin, Germany) following Earth Microbiome Project protocols (http://www.earthmicrobiome.org/emp-standard-protocols/). Bioinformatic analyses were performed using QIIME2 (https://qiime2.org). Briefly, pair-ends reads were merged using VSEARCH (Edgar 2010). Then, sequence quality control and feature table construction were performed using dada2 which infers sample sequences exactly and resolves differences of as little as one nucleotide (Callahan et al. 2016). Representative sequences were then confronted against UNITE database for taxonomic affiliation (Version:7.2; Last updated: 2017-12-01). OTU table was then rarefied to the least number of sequences to allow comparison among samples with different reads abundance (Hughes and Hellmann 2005).

Statistical analyses

Permutational analysis of variance was used to test for differences in fungal abundance between locations. Because only one station at site D was available, a nested design in which stations were nested into sites (Anderson 2014) was used. Data were log(x+1) transformed and Euclidean distance was used to generate the resemblance matrix required for the test. Spearman rank correlation analysis was used to investigate relationships between trophic variables and fungal abundance (Dodge 2008). Rarefaction curves were generated using iNEXT R package (Hsieh et al. 2016), and alpha diversity measures were obtained from the rarefied abundance

table using vegan R package (Oksanen et al. 2018). SIMPER analysis was conducted with PRIMER+ PERMANOVA to explore dissimilarity in fungal assemblage composition among sampling sites, and to identify the OTUs contributing to the observed differences (Clarke 1993). Network plot was created to visualise OTUs shared across sites. Non-metric Multidimensional Scaling (nMDS) was used to represent the pairwise (dis-)similarity based on Bray-Curtis (dis-)similarity matrix between samples in a low-dimensional space and to visualise patterns of trophic variables associated to assemblage composition (Li et al. 2016, Oksanen et al. 2018). Distance-based linear model and distance-based redundancy analysis (db-RDA) were also carried out to identify drivers of fungal abundance and assemblage composition (Legendre and Legendre 2012). Statistical analyses were performed and graph generated with R3.50 availing of vegan 2.5-2 (Oksanen et al. 2018), ggplot2 (Wickham 2016), and iNEXT (Chao et al. 2014, Hsieh et al. 2016). Network plots were generated via Gephi 0.9.2. PERMANOVA, SIMPER, cluster, DistLM, db-RDA and multivariate regression tree analyses were performed using Primer+PERMANOVA (Clarke and Gorley 2006).

Results and discussion

The benthic trophic status of the deep-sea sediments investigated changed significantly among areas, with values ca. 10-folds higher of biopolymeric concentrations in sediments of area B (on average 2.78 ± 0.73 mgC g^{-1}) than in areas C and D (on average 0.25 ± 0.07 mgC g^{-1} and 0.25 ± 0.08 mgC g^{-1}, respectively). Similarly, total phytopigment concentrations in the sediment of area B (as a proxy of organic material produced by photosynthesis in surface waters and settiling on the seafloor) were significantly higher than in areas C and D. Significant changes of fungal abundance were also observed among areas. Indeed, the fungal abundance (as the number of 18S rRNA gene copies) varied of almost two orders of magnitude (Figure 2a) with values much higher at area B (15.9 ± 5.3 $\times 10^6$ copies g^{-1}), than C and D areas (1.3 ± 0.7 $\times 10^6$ copies g^{-1} and 3.5 ± 0.4 $\times 10^6$ copies g^{-1}; respectively). Such differences of fungal abundance between areas were consistent with the different sediment features and dynamics previously reported (Fabiano and Danovaro 1998). Fungal abundances were significantly related with the concentrations of the different biochemical classes of organic compounds (i.e. proteins, carbohydrates and lipids) as well as with the biopolymeric C and phytopigment concentrations (Figure 2b). Although correlation analysis does not allow to infer cause-effect relationships, these findings suggest that the distribution of fungal standing stock can be

influenced by the availability of trophic resources in the sediment. This conclusion is also supported by multivariate multiple regression analysis highlighting that the major fraction of the total variance of the distribution of fungal abundance (ca. 88 %) is explained by biopolymeric carbon and total phytopigment concentrations (Table 1). Previous studies carried out in the same areas of the Ross Sea reported higher meiofaunal abundance and lower prokaryotic standing stock at area B when compared to area C (Fabiano and Danovaro 1998; Fabiano and Danovaro 1999). The low prokaryotic abundance, despite the high food availability at area B, has let to hypothesise a major top-down control on the microbial component exerted by meiofaunal assemblages. However, this is not the case of fungi which at area B are more bottom-up controlled rather than by predatory pressure exerted by deposit feeders.

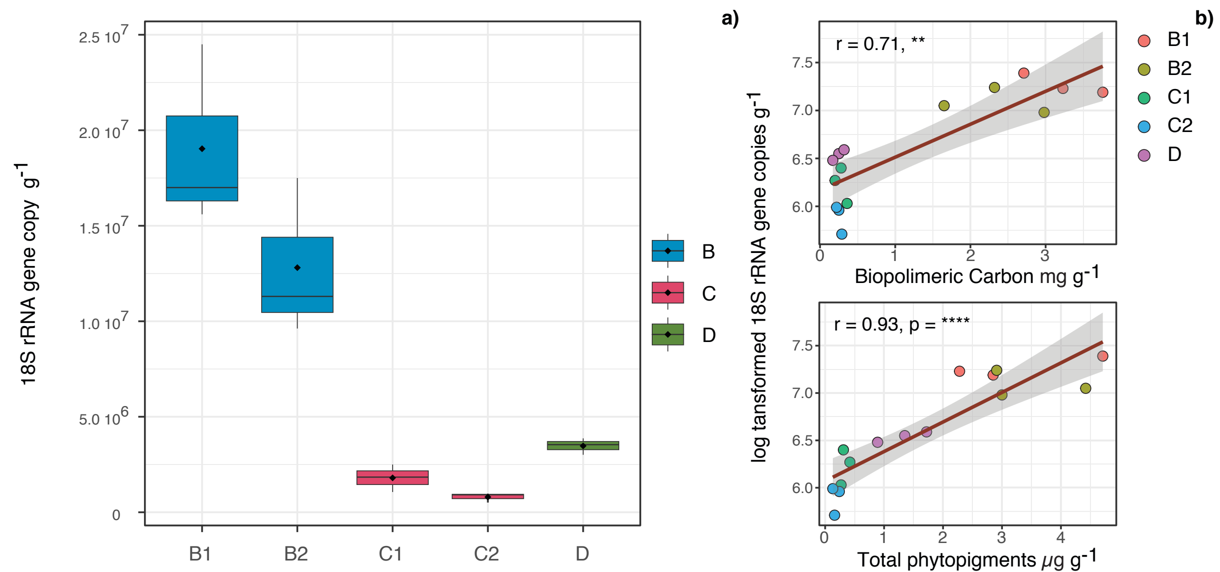

Figure 2. Copy number of fungal 18S rDNA of the different benthic deep-sea sites investigated (a), and relationships between log-transformed fungal 18S rRNA gene copies and trophic resource availability in the sediment (b).

Table 1. Results of distance based linear models with forward selection (DistLM). Response variable is fungal 18S rRNA gene copies and predictor variables are trophic resources. P indicates the significance of the relationship ($p < 0.05$ = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****), Prop is the % explained by the single predictive variable and Cumul is the cumulative % of variation explained in multivariate environment.

	R^2	SS(trace)	Pseudo-F	P	Prop.	Cumul.
SEQUENTIAL TESTS						
Total phytopigments	0.812	17.1	56.28	****	81.2	81.2
Biopolymeric Carbon	0.883	1.5	7.17	*	7.0	88.3

In the present study, 3293 reads per sample, which correspond to the lowest number of sequences obtained in the sediment of the site C2, were randomly selected (Figure 3a). Overall a total of 1443 OTUs of which 852 belonging to fungal taxa, 393 to unassigned OTUs, 190 OTUs affiliating to Rhizaria and 8 Agaricomycetes were obtained. From the 852 fungal OTUs, 660 belonging to Ascomycota, 39 to Mucromycota, 3 to Chytridiomycota, 23 to Basidiomycota, while the remaining 127 OTUs were affiliated to unknown fungi. With few exceptions, the taxonomic assignement of the OTUs was restricted to phylum level. We found OTUs belonged to *Malassezia* taxa which in deep-sea ecosystems are thought to be involved in both degradation processes of organic matter and parasitic interactions (Amend 2014). However, *Malassezia* reads were very low in the entire dataset which, instead, was characterised by a high number of reads belonging to *Mucor* and *Phycomyces* genera within Mucormycetes.

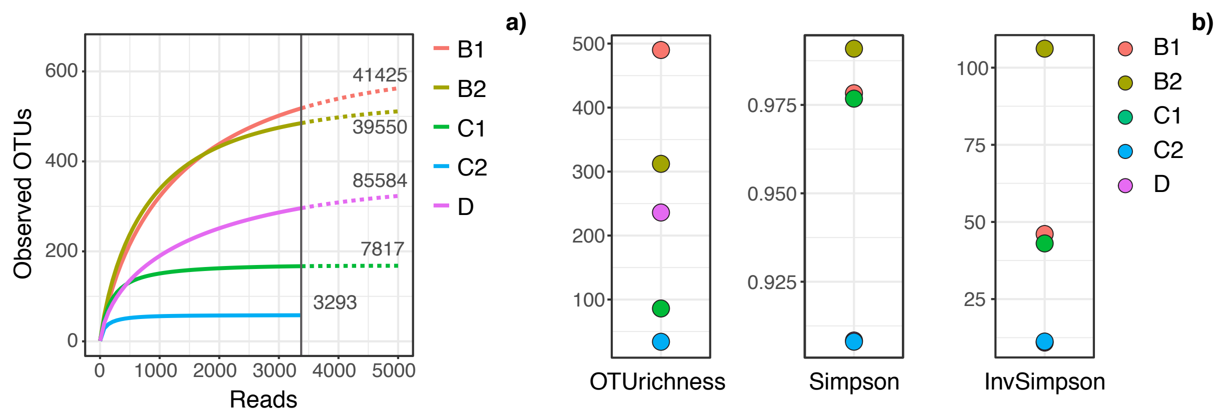

Figure 3. Rarefaction curves (a) and alpha diversity measures (b) constructed on the basis of fungal ITS1 sequencing for the investigated samples (B1, B2, C1, C2 and D),

Patterns of fungal richness was consistent with that of fungal abundance with high values at area B, followed by area D and C (Figure 3b), leading to hyphotesise that trophic availability has an important role driving also fungal diversity patterns in the Ross Sea sediments. Network analysis allowed to identify 4 OTUs shared among all of the benthic deep-sea sites investigated (i.e. core OTUs; Figure 4) which accounted for 7.2% of total fungal reads.

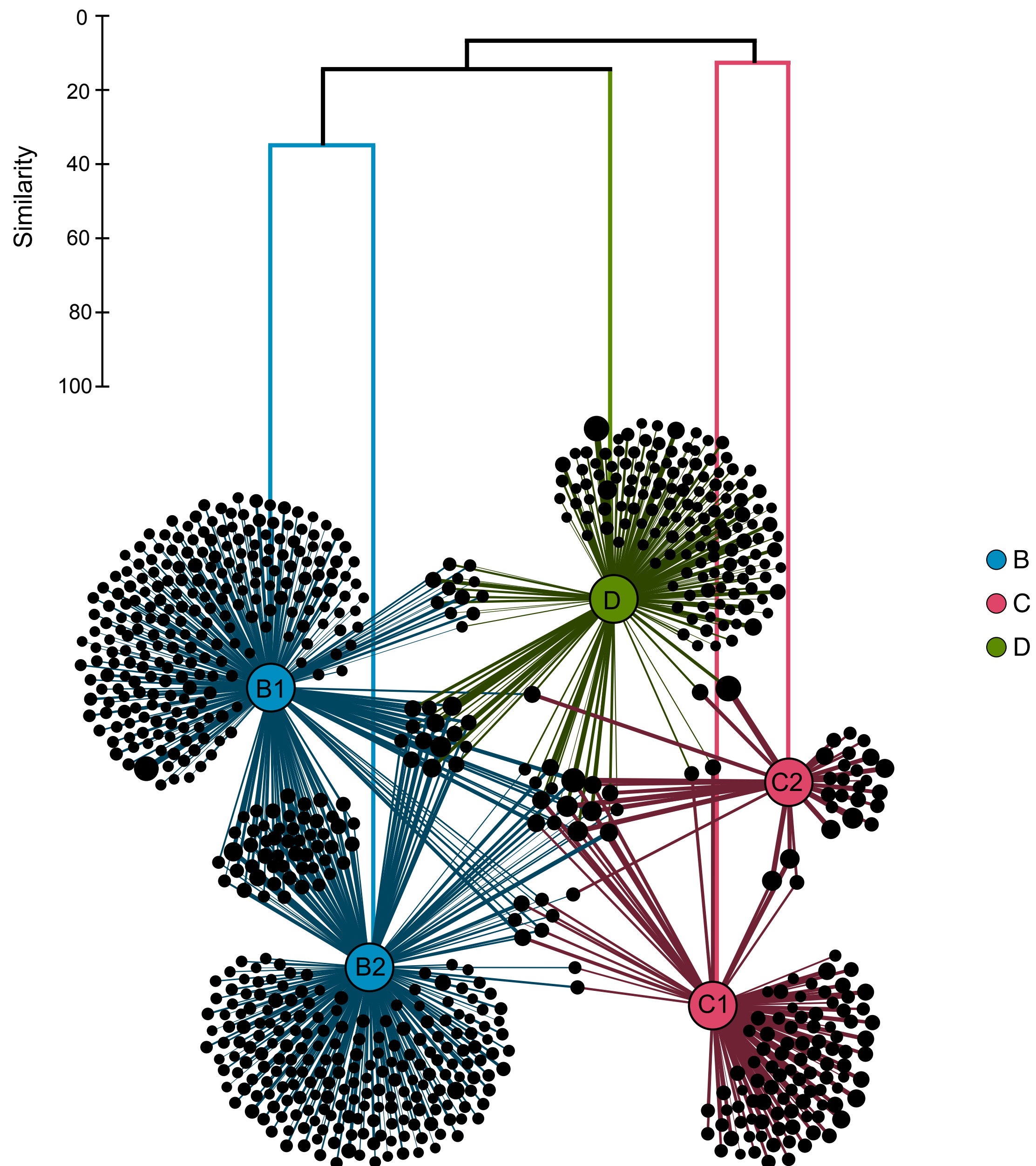

Figure 4. Bray-Curtis cluster analysis and network plot displaying similarity between samples and number of OTUs shared across the investigated stations. Black dots indicate OTUs and the size is the relative read abundance.

A A large fraction of OTUs (overall 586) was exclusive of each benthic site. SIMPER analysis indicates that the similarity of fungal assemblage composition was low not only among the different areas but also between the different benthic sites of the same areas. Indeed, the fungal assemblage composition of benthic sites of area B was similar for 35%, and even much lower similarity value was observed between benthic sites of the area C (13%). In the present study, we found that only total phytopigment concentrations explained a certain fraction of the total variance of fungal assemblage composition (37%; Table 2) significantly.

Table 2. Results of distance based linear models with forward selection (DistLM). Response variable is fungal assemblace composition and predictive variables are trophic resources. P indicates the significance of the relationship ($p < 0.05$ = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****), Prop is the % explained by the single predictive variable and Cumul is the cumulative % of variation explained in multivariate environment.

	R^2	SS(trace)	Pseudo-F	P	Prop.	Cumul.
SEQUENTIAL TESTS						
Total phytopigments	0.371	5853.6	1.77	*	37.1	37.1
Protein to Carbohydrate ratio	0.582	3321.2	1.01	ns		
Biopolymeric Carbon	0.774	3020.0	0.85	ns		

However, besides changes observed in the distribution of food availability in the sediment of the different areas which can explain to a certain extent changes of fungal assemblage composition among areas (Figure 5), other abiotic (e.g. habitat heterogeneity and physical factors related to variations in current regimes; Fabiano and Danovaro 1999) and biotic factors (e.g. competion, predation) acting at smaller spatial scales (between sites of the same area) can promote high turnover diversity of deep-sea fungal assemblages in the Ross Sea.

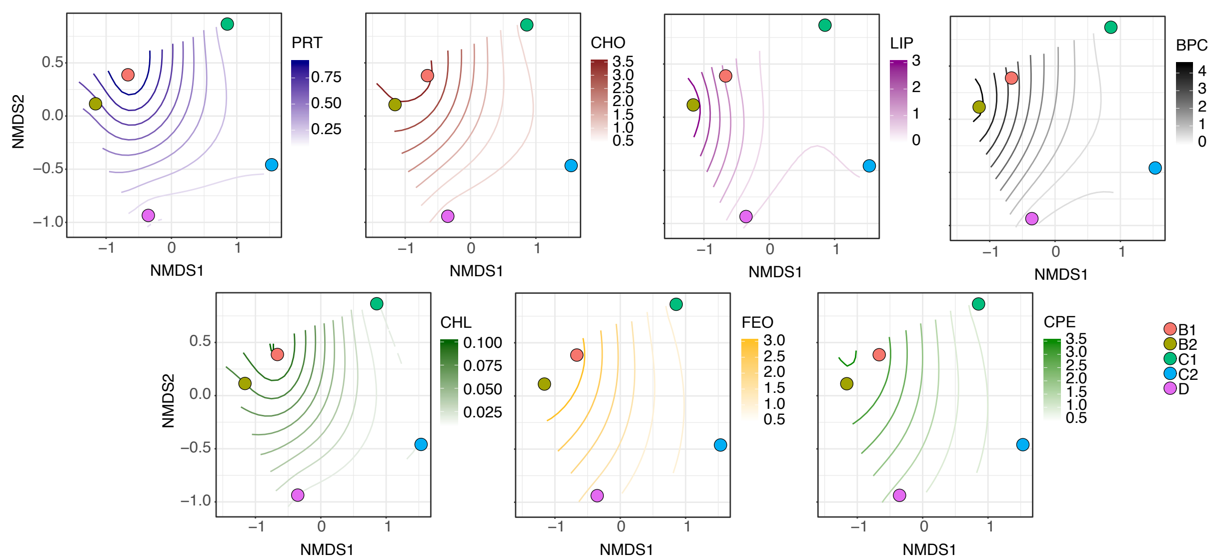

Figure 5. Non-metric Multidimensional Scalings (nMDS) based on Bray-Curtis (dis-)similarity matrix calculated. Resource concentrations were fitted as smooth surfaces to the ordination structure using a generalised additive model (Oksanen et al. 2018)

Conclusions

This study provides the first insight on the quantitative relevance and diversity of benthic deep-sea fungi in the Ross Sea. Our findings reveal that the distribution of fungal abundance and

richness is primarily driven by trophic availability, whereas food resources only partially explained changes in fungal assemblage composition. Our findings also suggest that the variability even at small spatial scale can favour high turnover diversity of deep-sea fungal assemblages, thus allowing the maintenance of an overall high fungal diversity. Results reported in this study could be relevant for a better understanding of the impact of the current climate changes in Antarctic ecosystems. Changing climate in Antarctica has, indeed, the potential to initiate a domino effect that could impact ecosystems from continental ice sheets to the seafloor (Learman et al. 2016). Alteration of ice coverage, temperature and salinity regimes can alter the planktonic food web structure which could profoundly influence organic carbon export to the seafloor, thus influencing the benthic community structure and functions. Variation in primary production and sedimentation due to altered freezing/melting cycles of Antarctica ice pack can drastically change ecosystem functioning (Lohrer et al. 2012), and we still have a limited knowledge of biogeochemical cycles and ecosystem process in which fungi are involved (Worden et al. 2015). Therefore, our results highlight the need to understand the role of benthic deep-sea fungi better and to include them into models which may allow to better forecast responses in ecosystem functioning due to climate-induced changes.

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Chapter 6

General conclusions

Fungi are among the most widespread group of organisms. On land, fungi are a crucial component of food webs and are actively involved in biogeochemical cycles, but the information regarding their distribution in marine ecosystems, and especially in the deep sea, is scant. In the first chapter, I revised the available literature for information on fungal diversity across several benthic deep-sea ecosystems. Overall, although a large number of studies investigated fungi in such habitats and ecosystems, information regarding factors driving their abundance and diversity is scarce. Therefore, in the present study, I explored for the first time patterns of fungal abundance and diversity, as well as the environmental factors influencing them, across oceans and eco-regions, including the most remote and deepest benthic deep-sea habitats on Earth.

Overall, our results indicate the fungal abundance strongly depend on trophic resource availability. In particular, carbohydrate concentrations, which in deep-sea sediments include the less bioavailable fraction of organic matter pools, were always related with fungal abundance. Thus, we concluded that fungi in benthic deep-sea sediments might be responsible for the degradation of the most refractory fraction of organic matter which is inaccessible to other taxonomic groups.

We also found that benthic deep-sea ecosystems host a relatively high fungal OTU richness with many taxa possibly novel to science. Overall, fungal diversity considerably varies across benthic deep-sea ecosystems suggesting that a complex array of biological and environmental factors can influence diversity patterns. Our analyses reveal that temperature, salinity, oxygen and trophic resources considerably influence fungal assemblage composition and that, among the observed variables, thermohaline conditions and dissolved oxygen are major drivers of patterns of fungal assemblage composition at wide spatial scale.

Therefore, we conclude that fungi are a significant, although largely overlooked, component of the benthic assemblages inhabiting the deep-sea floor. Also, these findings indicate that fungi should be included in food-web and biogeochemical cycle models.