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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, ranging from 0.17 to 5.78 µgC g-1, and abundance increased with increasing carbohydrate concentrations in the sediments, suggesting that deep-sea fungi have a role in the utilisation of this component of the organic matter. 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 thermo-haline 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.

Keywords	Benthic deep-sea ecosystems; fungal abundance; fungal diversity; submarine canyons; Mediterranean Sea
Manuscript category	Biological Oceanography

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Dipartimento
di Scienze
della Vita
e dell'Ambiente
DISVA

Ancona, 08.05.2018

Dear Editor,

please find enclosed the main text and figures of the manuscript entitled: "*Benthic deep-sea fungi in submarine canyons of the Mediterranean Sea*" by Giulio Barone et alii, submitted for consideration to *Progress in Oceanopgraphy* within the special issue "**Ecology and functioning of Mediterranean submarine canyons**".

This is an original manuscript not submitted or presented elsewhere in which we investigated, for the first time, the quantitative relevance and diversity of fungi in deep-sea sediments of submarine canyons of the Mediterranean Sea. In this study, we show that fungi are an important component within the benthic deep-sea food webs and that they are highly diversified. Our results also indicate that different environmental characteristics encountered in the different canyons investigated can have a major role in influencing fungal diversity and assemblage composition. We think that this work expands our knowledge on the ecology and diversity of fungi inhabiting Mediterranean submarine canyons and we hope that it might be of interest for your journal.

For any requests, please do not hesitate to contact me at the address and numbers reported here below.

Looking forward to hearing from you soon, we remain.

Best regards

On behalf of all co-authors

Antonio Dell'Anno

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HIGHLIGHTS

- Submarine canyons host abundant and diverse fungal communities
- Fungal abundance, biomass and diversity are driven by carbohydrate concentrations
- Deep-sea sediments can harbour a high number of novel fungal taxa
- Thermohaline and trophic conditions may promote a high turnover diversity of fungi

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

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Running title: Fungal abundance and diversity in Mediterranean canyons

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, ranging from 0.17 to 5.78 µgC g^{-1}, and abundance increased with increasing carbohydrate concentrations in the sediments, suggesting that deep-sea fungi have a role in the utilisation of this component of the organic matter. 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 thermo-haline 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

1. 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 (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.

Fungi in terrestrial and freshwater ecosystems are among the main decomposers of organic matter and play a key 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). Since deep-sea ecosystems can contain relatively high amounts of organic carbon (Pusceddu et al., 2009), fungi might play a key role in C cycling also in these ecosystems (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). 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.

2. Materials and methods

2.1. 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 (Figure 1). One of the investigated canyon (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 a 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. Sediment samples were collected at each benthic site by independent multiple corer deployments.

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.

2.2. 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). 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 (Dell'Anno et al., 2002).

2.3. 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., 2016). The FISH reaction was performed using the Pan-Fungal probe PF2 (5'-CTC TGG CTT CAC CCT ATT C-3') Cy-3 labelled (Kempf et al., 2000). Briefly, about 1 g of sediment was first treated using 4 ml of a mix containing EDTA, Tween 80, sodium-pyrophosphate and 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, 0.112M NaCl) and incubated for 30 minutes 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) conditions. 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. Then, the average width and cumulative length 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).

2.4 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, 2009; Danovaro et al., 2016).

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

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 et al. (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′ (Prevost-Boure 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.

2.6 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/). Barcodes and ITS1 primer pairs were removed before demultiplexing. Paired-end sequences were then merged with FLASH (Magoč and Salzberg, 2011). Merged sequences were quality filtered using the SEARCH 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/). Then, 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 (Figure S1).

2.7 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 thermo-haline and trophic variables. All statistical analyses were performed using Primer 6+ software.

3. Results and discussion

The thermo-haline 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).

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.

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 no 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 abundance and biomass (Figure S2).

From the slope of this relationship, we estimated that 1 μg of fungal biomass could be equivalent to 7.8×10^6 fungal 18S rDNA copies. Although such relationship should be view with caution and needs to be better refined with a broader spatial scale investigation, it can provide useful information on the quantitative relevance of deep-sea fungi based on copy number determinations (Taylor and Cunliffe, 2016).

Significant positive relationships between carbohydrate concentrations and fungal abundance and biomass were found ($r=0.715$ and $r=0.893$, both $p<0.01$, for the abundance and biomass, respectively; Figure 3). Also, 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 (Table S2). Since fungi are osmotrophic (i.e. feed by secreting enzymes into the environment to degrade organic matter externally before taking the resulting metabolites into the cell; Richards and Talbot, 2013; Richards et al., 2015), our results suggest that they could be highly specialized in the utilisation of carbohydrates which are typically characterised by a highly recalcitrant fraction, especially in benthic deep-sea ecosystems (Dell'Anno et al., 2000; Dell'Anno et al., 2013).

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).

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.

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). 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) and temperature ($r= 0.980$) and salinity ($r= -0.560$; Figure 7). These results confirm that also in the deep-sea sediments investigated trophic availability and thermo-haline 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 thermo-haline 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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Author Contributions: R.D., C.C., and A.D. conceived the study. G.B. participated in the oceanographic cruise for collecting sediment samples and performed laboratory analyses. G.B., E.R., M.T. and A.D. contributed to data elaboration and interpretation. G.B., E.R., and A.D. wrote the first draft of the manuscript. All authors contributed to results discussion and finalization of the manuscript.

Conflict of interest: All the other authors declare no competing financial interests.

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Captions of figures

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.

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.

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

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

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.

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.

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 thermo-haline and trophic conditions.

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

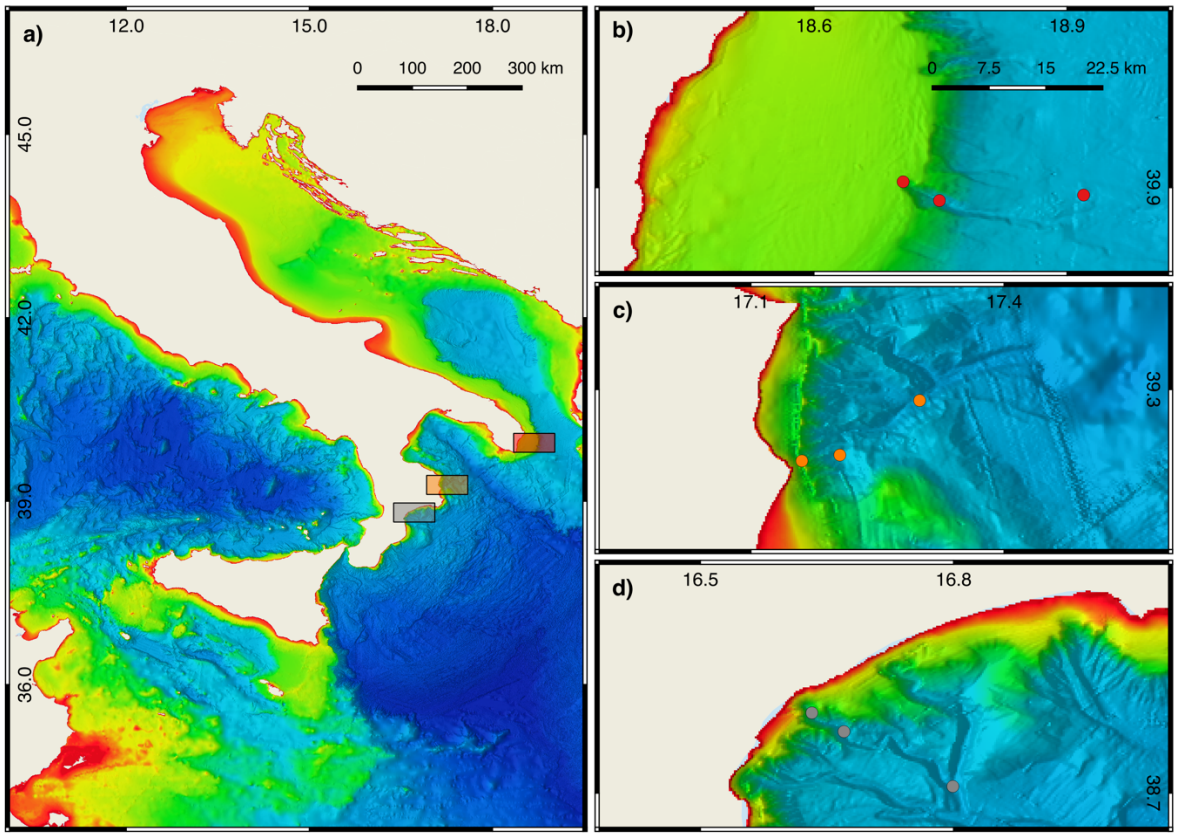

a)
12.0
15.0
18.0
0 100 200 300 km
45.0
42.0
39.0
36.0
b)
18.6
18.9
0 7.5 15 22.5 km
39.9
c)
17.1
17.4
39.3
d)
16.5
16.8
38.7

Figure 1

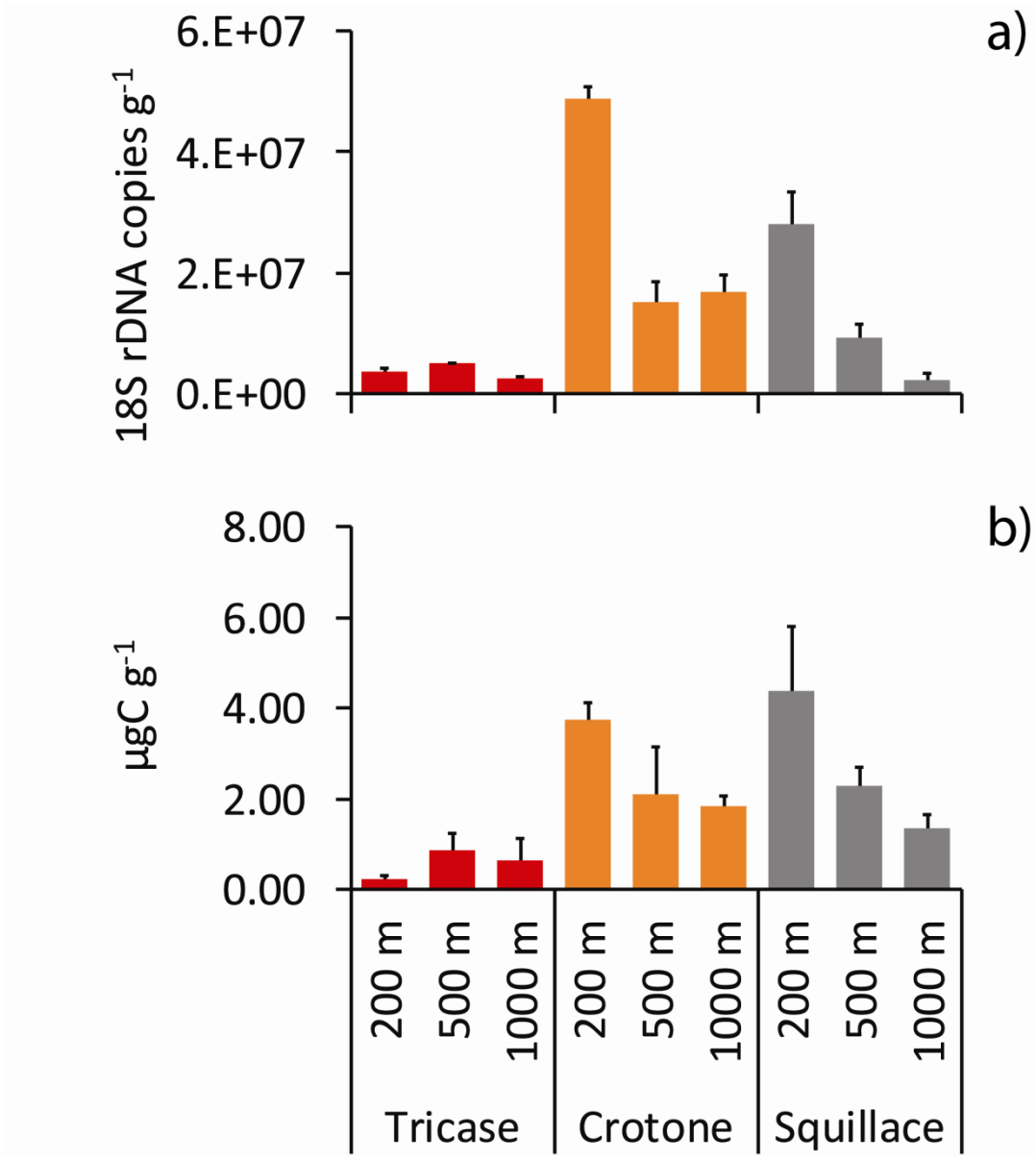

a)
18S rDNA copies g-1
6.E+07
4.E+07
2.E+07
0.E+00
b)
µgC g-1
8.00
6.00
4.00
2.00
0.00
200 m
500 m
1000 m
200 m
500 m
1000 m
200 m
500 m
1000 m
Tricase
Crotone
Squillace

Figure 2

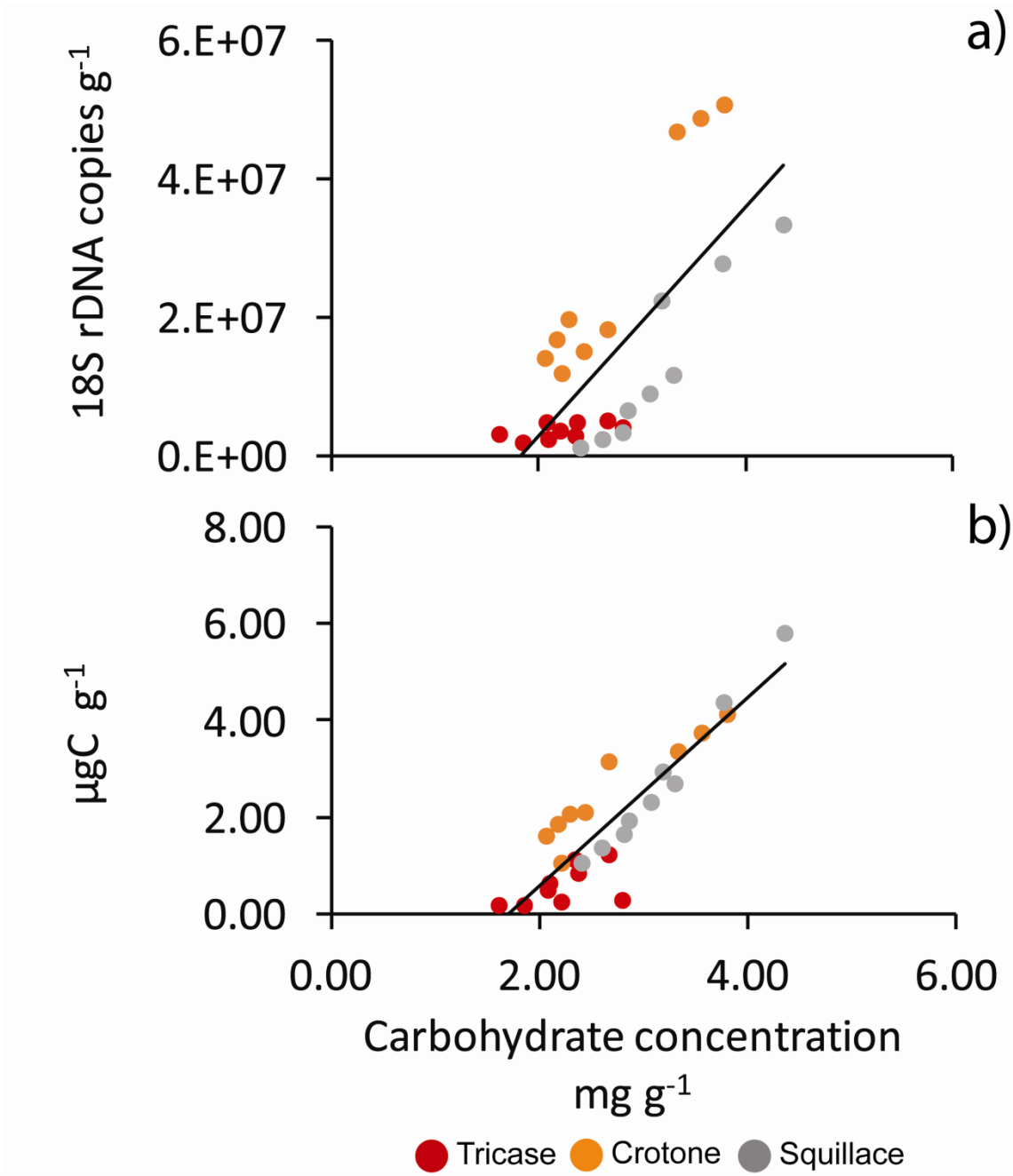

a)
18S rDNA copies g-1
6.E+07
4.E+07
2.E+07
0.E+00
b)
µgC g-1
8.00
6.00
4.00
2.00
0.00
0.00
2.00
4.00
6.00
Carbohydrate concentration
mg g-1
Tricase
Crotone
Squillace

Figure 3

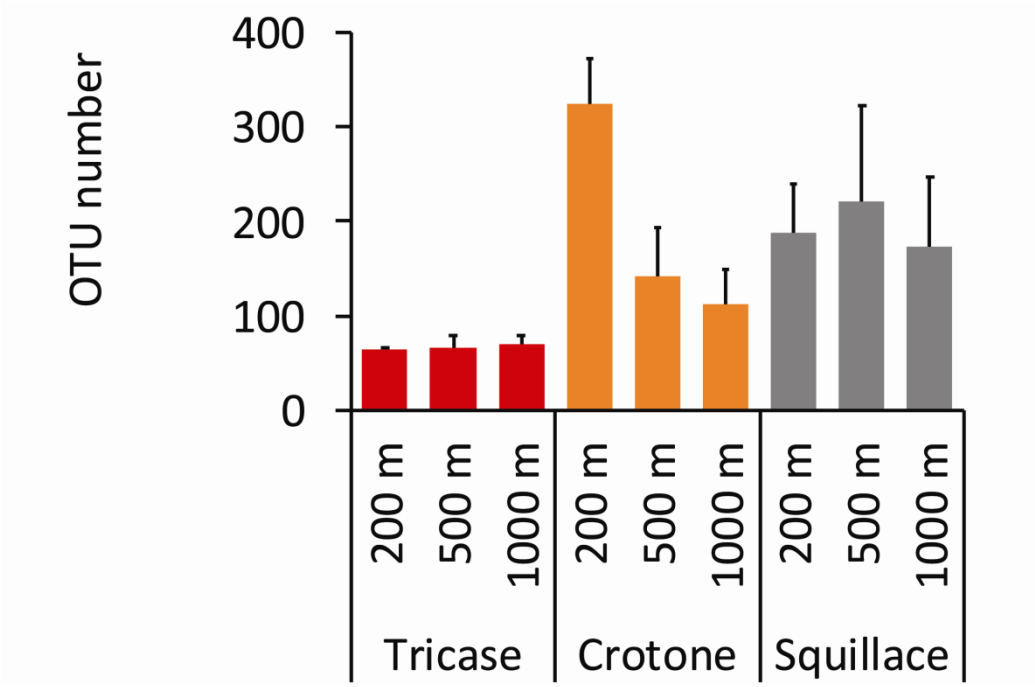
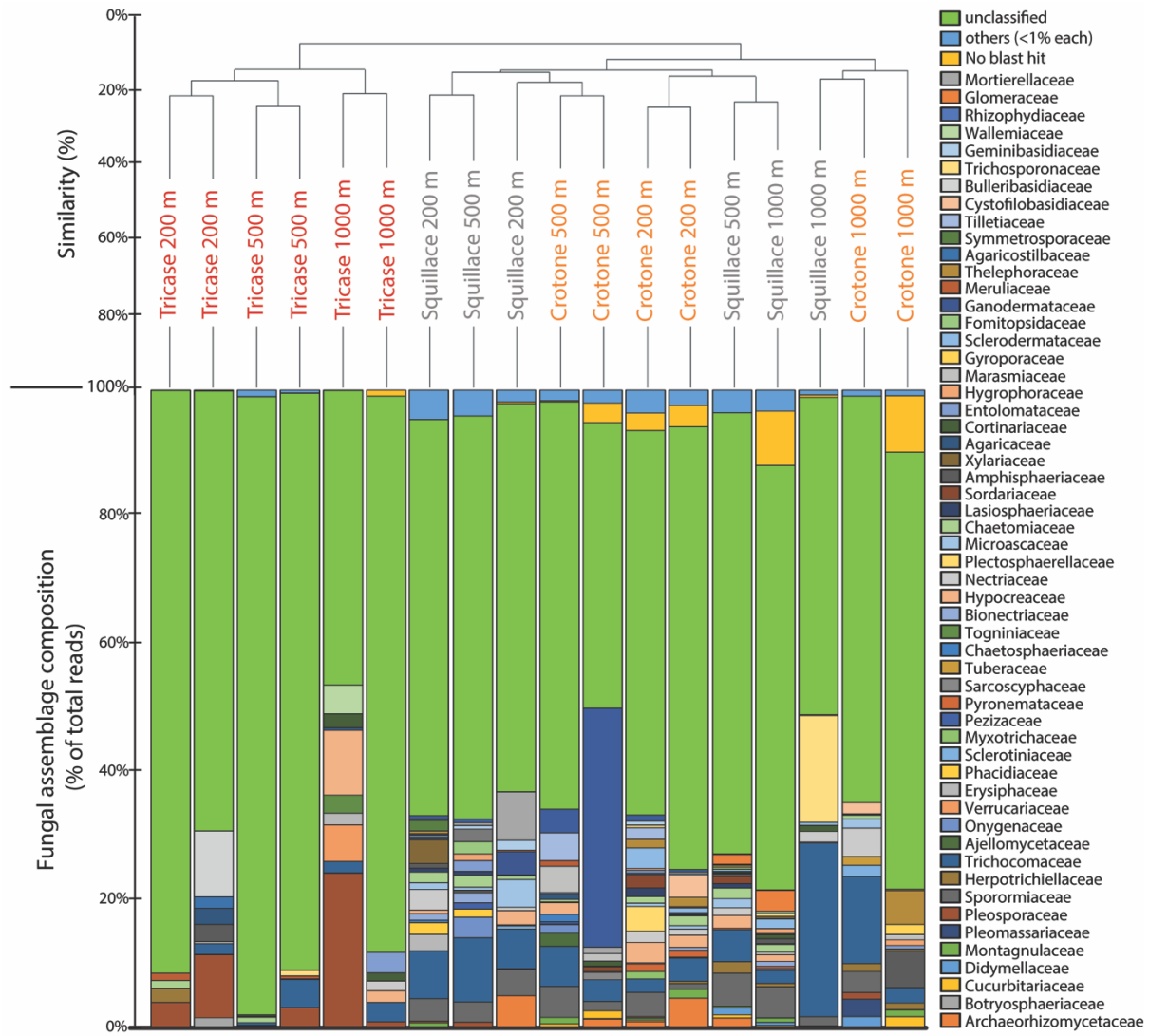

OTU number
400
300
200
100
0
200 m
500 m
1000 m
200 m
500 m
1000 m
200 m
500 m
1000 m
Tricase
Crotone
Squillace

Figure 4


Similarity (%)
0%
20%
40%
60%
80%
100%
Tricase 200 m
Tricase 200 m
Tricase 500 m
Tricase 500 m
Tricase 1000 m
Tricase 1000 m
Squillace 200 m
Squillace 500 m
Squillace 200 m
Crotone 500 m
Crotone 500 m
Crotone 200 m
Crotone 200 m
Squillace 500 m
Squillace 1000 m
Squillace 1000 m
Crotone 1000 m
Crotone 1000 m
Fungal assemblage composition
(% of total reads)
unclassified
others (<1% each)
No blast hit
Mortierellaceae
Glomeraceae
Rhizophydiaceae
Wallemiaceae
Geminibasidiaceae
Trichosporonaceae
Bulleribasidiaceae
Cystofilobasidiaceae
Tilletiaceae
Symmetrosporaceae
Agaricostilbaceae
Thelephoraceae
Meruliaceae
Ganodermataceae
Fomitopsidaceae
Sclerodermataceae
Gyroporaceae
Marasmiaceae
Hygrophoraceae
Entolomataceae
Cortinariaceae
Agaricaceae
Xylariaceae
Amphisphaeriaceae
Sordariaceae
Lasiosphaeriaceae
Chaetomiaceae
Microascaceae
Plectosphaerellaceae
Nectriaceae
Hypocreaceae
Bionectriaceae
Togniniaceae
Chaetosphaeriaceae
Tuberaceae
Sarcoscyphaceae
Pyronemataceae
Pezizaceae
Myxotrichaceae
Sclerotiniaceae
Phacidiaceae
Erysiphaceae
Verrucariaceae
Onygenaceae
Ajellomycetaceae
Trichocomaceae
Herpotrichiellaceae
Sporormiaceae
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Montagnulaceae
Didymellaceae
Cucurbitariaceae
Botryosphaeriaceae
Archaeorhizomycetaceae

Figure 5

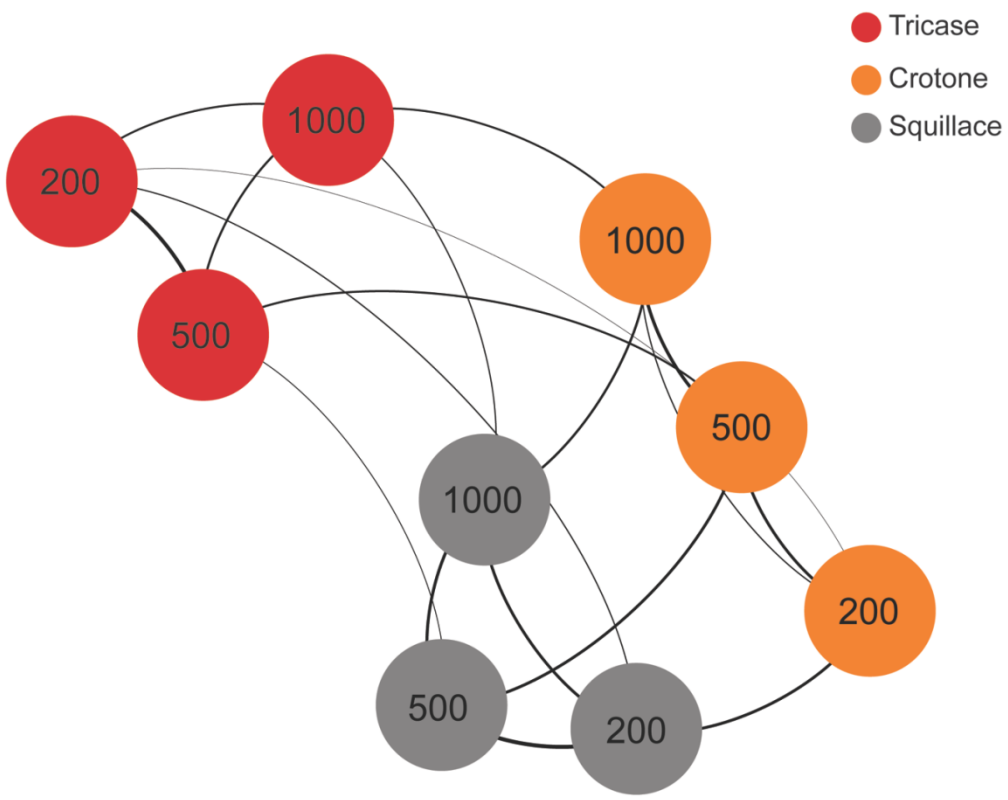
Tricase
Crotone
Squillace
1000
200
500
1000
500
1000
200
500
200

Figure 6

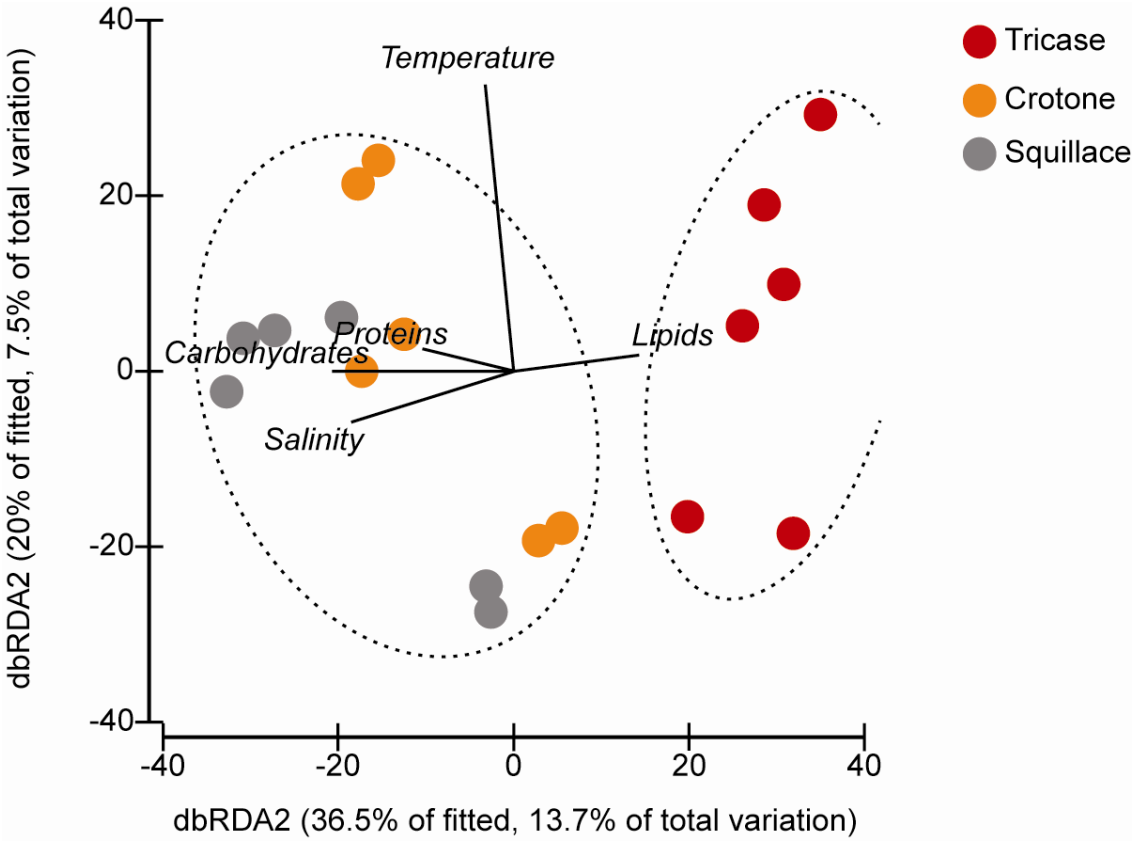
Tricase
Crotone
Squillace
Temperature
Proteins
Carbohydrates
Lipids
Salinity
40
20
0
-20
-40
-40
-20
0
20
40
dbRDA2 (20% of fitted, 7.5% of total variation)
dbRDA2 (36.5% of fitted, 13.7% of total variation)

Figure 7

Supplementary materials

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

Giulio Barone, Eugenio Rastelli, Cinzia Corinaldesi, Michael Tangherlini, Roberto Danovaro, Antonio Dell'Anno

Supplementary table S1-S4

Supplementary figures S1 and S2

Table S1. Reported are the outputs of the ANOSIM and SIMPER analyses carried out to test for the differences and dissimilarity in sediment organic matter contents between the different canyons investigated and the variables responsible for the estimated differences. Reported are R which represents the sample statistic (global R) and P which is the probability level. **=P <0.01; ns = not significant

	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. Output of the multivariate multiple regression analysis carried out for testing the effects of organic matter content (proteins, carbohydrates and lipids), temperature and salinity on fungal abundance (as 18S rDNA copies) and biomass. Reported are Pseudo-F and P values (*<0.05; **<0.01; ***<0.001; ns>0.05) and 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 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.

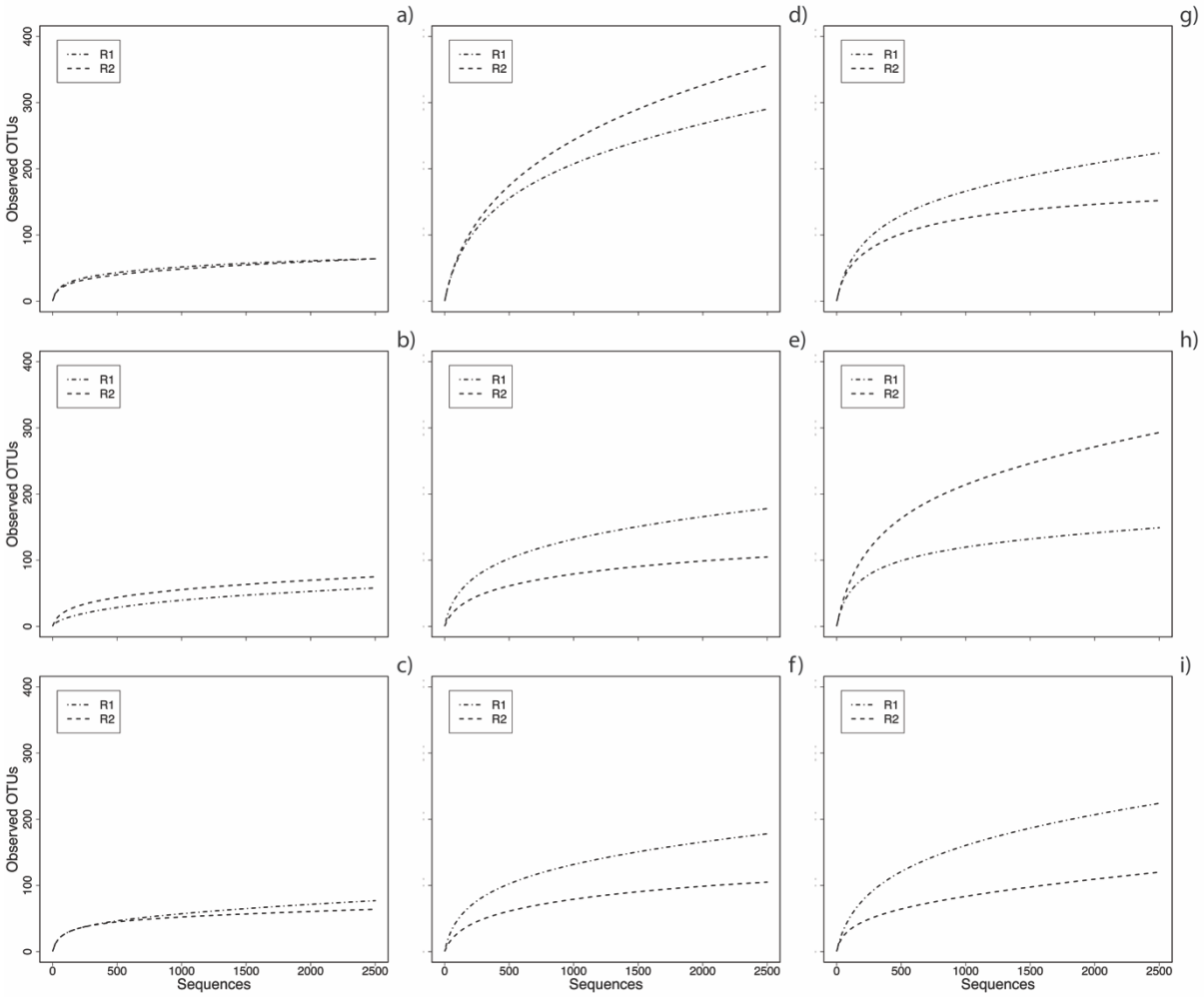
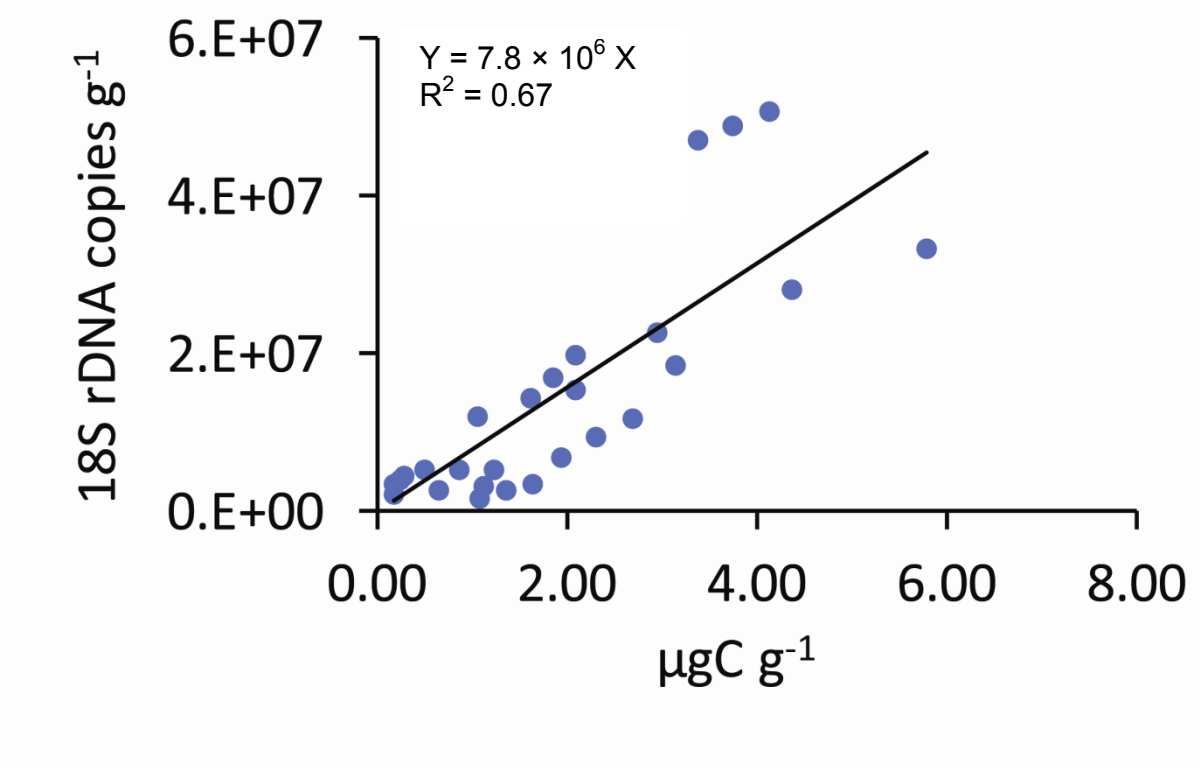
Figure S2. Relationship between benthic fungal abundance (as 18S rDNA copies) and biomass in the sediments of the three canyons