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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 $\mu\text{C g}^{-1}$, 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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All data are included in the text



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 Oceanography* 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

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4 **1 Benthic deep-sea fungi in submarine canyons of the Mediterranean Sea**

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9 3 Giulio Barone¹, Eugenio Rastelli², Cinzia Corinaldesi³, Michael Tangherlini², Roberto
10 4 Danovaro^{1,2}, Antonio Dell'Anno^{1*}

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20 **Running title:** Fungal abundance and diversity in Mediterranean canyons

61
62
63 **Abstract**
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66 Fungi are ubiquitous components of microbial assemblages in aquatic ecosystems, but their
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68 quantitative relevance, ecological role and diversity in benthic deep-sea ecosystems are still
69
70 largely unknown. Here, we investigated patterns and drivers of benthic fungal abundance,
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82 Fungal biomass, ranging from 0.17 to 5.78 $\mu\text{gC g}^{-1}$, and abundance increased with increasing
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88 belonging to all fungal phyla known to date, were found and Ascomycota represented the
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90 dominant phylum. However, only 36% of the reads belonged to known genera. In particular,
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92 Tricase and Crotone canyons hosted the highest proportion of unknown fungal taxa,
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94 suggesting that deep-sea sediments can harbour a high number of novel fungal lineages. Our
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96 findings also reveal that fungal assemblage composition in the investigated canyons was
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98 influenced by trophic and thermo-haline conditions, which may promote a high turnover
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100 diversity of benthic deep-sea fungal assemblages. Overall results reported here indicate that
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102 the submarine canyons of the Mediterranean Sea can represent hot-spots of abundant and
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104 highly diversified fungal assemblages and pave the way for a better understanding of the
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106 ecological role of fungi in the largest ecosystem on Earth.
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112 **Key Words:** Benthic deep-sea ecosystems, fungal abundance, fungal diversity, submarine
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114 canyons, Mediterranean Sea
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123 **1. Introduction**
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126 49 Deep-sea ecosystems represent more than 65% of the world's surface and >95% of the
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128 50 global biosphere (Herring, 2002), and host yet undiscovered biodiversity and a significant
129 51 portion of the world's genetic diversity (Danovaro et al., 2017). In benthic deep-sea
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131 52 ecosystems, biomass is dominated by bacteria and archaea, followed by unicellular
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133 53 eukaryotes and small metazoans (<0.5 mm in size, meiofauna). These organisms are
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135 54 essential for carbon cycling and nutrient regeneration, and thus vital for sustaining
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137 55 oceanic production (Dell'Anno and Danovaro, 2005; Sogin et al., 2006; Jørgensen and
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139 56 Boetius, 2007; Danovaro et al., 2015; Danovaro et al., 2017). Recent findings, based on
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141 57 culture-dependent and independent approaches, revealed that fungi are present in deep-
142
143 58 sea environments across a variety of ecosystem types spanning from hypersaline anoxic
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145 59 basins (Bernhard et al., 2014; Edgcomb et al., 2017) to cold seeps (Nagahama et al.,
146
147 60 2011; Thaler et al., 2012), from hydrothermal vents (Burgaud et al., 2009; Burgaud et al.,
148
149 61 2010; Xu et al., 2017) to surface and subsurface sediments (Orsi et al., 2013; Pachiadaki
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151 62 et al., 2016). Fungi have also been reported as the dominant unicellular eukaryotic group
152
153 63 in the marine snow in bathypelagic waters with biomass similar to that of prokaryotes
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155 64 (Bochdansky et al., 2017).

156
157 65 Theoretical estimates suggest that fungi can be the most diversified component of
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159 66 unicellular eukaryotes on Earth, with more than 5 million species of which only 5% have
160
161 67 been described (Hawksworth, 1997; Blackwell, 2011). This gap applies in particular to
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163 68 open ocean ecosystems where a significant fraction of fungal diversity is still unknown
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165 69 (Jeffries et al., 2016). Recent studies suggest that a variety of environmental factors (e.g.
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167 70 temperature, salinity, nutrients) can influence the diversity and assemblage composition
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169 71 of fungi in marine ecosystems (Li et al., 2016; Tisthammer et al., 2016). However, drivers
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183 72 controlling the distribution and diversity of fungi in benthic deep-sea ecosystems remain
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185 73 to date largely unexplored.

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187 74 Fungi in terrestrial and freshwater ecosystems are among the main decomposers of
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189 75 organic matter and play a key role in the processing of the most refractory fraction of
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191 76 organic carbon (Carlile et al. 2001; Clipson et al. 2006; Hwang et al. 2006; Dighton,
192
193 77 2007). Since deep-sea ecosystems can contain relatively high amounts of organic carbon
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195 78 (Pusceddu et al., 2009), fungi might play a key role in C cycling also in these ecosystems
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197 79 (Hyde et al., 1998; Burgaud et al., 2009; Cathrine and Raghukumar, 2009; Jebaraj et al.,
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199 80 2010).

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202 81 In this study, we investigated the abundance, biomass and taxonomic composition of
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204 82 fungal assemblages along the continental margins of the Central Mediterranean Sea.

205
206 83 Continental margins are characterised by open slopes and submarine canyons, which are
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208 84 essential for C cycling and nutrient regeneration processes at a global scale (Bousquet et
209
210 85 al., 2000; Dickens, 2003). In particular, submarine canyons can channel large amounts of
211
212 86 organic matter photosynthetically produced from the continental shelf down to deep-sea
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214 87 ecosystems (Monaco et al., 1999; Sánchez-Vidal et al., 2008; Allen and Durrieu de
215
216 88 Madron, 2009; Puig et al., 2014). For this reason, we selected three submarine canyons
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218 89 characterised by different environmental conditions and investigated fungal abundance,
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220 90 biomass and diversity at depths ranging from 200 to 1000 m. To identify the factors
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222 91 potentially controlling their quantitative importance and diversity in deep-sea sediments,
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224 92 we explored the role of environmental conditions, including the organic matter quality
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226 93 and quantity.

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234 96 **2. Materials and methods**

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97 *2.1. Study area and sampling design*

98 Sediment sampling was carried out in the Ionian Sea (Central Mediterranean Sea) during
99 the oceanographic cruise “SAND 2016” held on board of the research vessel R/V
100 Minerva Uno in May 2016. Sediment samples were collected within the main axis of
101 three canyons located along the SE Italian margin at 200, 500 and 1000 m depths (Figure
102 1). One of the investigated canyon (hereafter defined “Tricase”) located along the
103 Apulian margin, is far from any continental freshwater inputs. The other two investigated
104 canyons are located along the Calabrian margin and were close to river estuaries. The
105 Northern canyon, extending for about 30 km, is located in front of the Crotone
106 municipality (canyon “Crotone”) and its head is close to a river mouth. The head of the
107 canyon “Squillace” is close to the coastline in front of the Squillace municipality and is
108 characterised by the presence of sporadic, but intense river inputs. Sediment samples
109 were collected at each benthic site by independent multiple corer deployments.
110 The top 1 cm of each sediment sample was used for the analysis of the quantity and
111 biochemical composition of organic matter, fungal abundance (based on q-PCR analysis
112 of 18S rRNA genes), biomass and diversity. At each station, temperature and salinity of
113 bottom waters were measured using CTD casts.

114
115 *2.2. Quantity and biochemical composition of organic matter*

116 The three major biochemical classes of organic compounds (proteins, carbohydrates and
117 lipids) in deep-sea sediments were determined according to previously described
118 procedures (Danovaro, 2010). Protein, carbohydrate and lipid concentrations were
119 determined spectrophotometrically and expressed as albumin, glucose and tripalmitin
120 equivalents, respectively. All analyses were carried out in 3 replicates. Protein,
121 carbohydrate and lipid concentrations were then converted to carbon equivalents

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303 122 (conversion factors: 0.49, 0.40 and 0.75 gC g⁻¹, respectively) to determine biopolymeric C
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305 123 content (Dell'Anno et al., 2002).
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309 125 *2.3. Fungal biomass*

310 126 To detect and quantify fungi in the sediment samples, fluorescence in-situ hybridisation
311
312 127 (FISH) coupled with Calcofluor white staining (which targets chitin, cellulose and
313
314 128 carboxylated polysaccharides) have been used following procedures previously described
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316 129 (Bochdansky et al., 2016). The FISH reaction was performed using the Pan-Fungal probe
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318 130 PF2 (5'-CTC TGG CTT CAC CCT ATT C-3') Cy-3 labelled (Kempf et al., 2000).
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320 131 Briefly, about 1 g of sediment was first treated using 4 ml of a mix containing EDTA,
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322 132 Tween 80, sodium-pyrophosphate and methanol and ultrasounds treatment to separate
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324 133 fungi from the sediment matrix. After centrifugation, sediment samples were washed
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326 134 twice with PBS buffer and then treated with increasing concentrations of ethanol (50, 80
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328 135 and 96%, for 3 min each). The sediment was then suspended in 500 µl hybridisation
329
330 136 buffer containing 0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris-HCl pH 7.2, 30 %v/v
331
332 137 formamide and 1 µM PF2 (Kempf et al., 2000), then incubated for 3 h at 46°C in the
333
334 138 dark. Samples were then transferred in sterile tubes containing pre-warmed washing
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336 139 buffer (20 mM Tris-HCl pH 8.0, 0.01% w/v SDS, 5 mM EDTA, 0.112M NaCl) and
337
338 140 incubated for 30 minutes at 48°C. After centrifugation and resuspension of the sediment
339
340 141 samples with 0.2 µm pre-filtered water, aliquots of the slurry (n=3) were filtered on 0.2
341
342 142 µm polycarbonate filters (Millipore) conditions. Filters were then stained with 0.5 mM
343
344 143 Calcofluor white and incubated in the dark for 5 min. Subsequently, slides were washed
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346 144 with 0.02 µm pre-filtered water and analysed under epifluorescence microscopy. The
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348 145 whole filter was examined, and length and width measures were taken for each fungal-
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350 146 like structure. Then, the average width and cumulative length were converted to a
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147 cylinder with half-spheres at ends, and the biovolume was converted into fungal biomass,
148 assuming 1 μm^3 of fungal biovolume equivalent to 1 pg C (Damare and Raghukumar
149 2008).

151 *2.4 DNA extraction and purification for molecular analysis*

152 The DNA was extracted and purified from the sediment samples using the PowerSoil
153 DNA isolation kit (QIAGEN)) following the manufacturer's instruction with slight
154 modifications to remove extracellular DNA (based on three subsequent washing steps)
155 before DNA extraction (Danovaro, 2009; Danovaro et al., 2016).

157 *2.5 Quantitative real-time PCR of fungal 18S rRNA gene sequences*

158 DNA extracted from two sediment samples collected at each study site by independent
159 multiple corer deployments was used for quantitative real-time PCR (qPCR) analysis
160 which was performed as described in Taylor et al. (2016) with slight modifications.
161 Briefly, fungi-specific primers FR1 5'-AIC CAT TCA ATC GGT AIT-3' and FF390 5'-
162 CGA TAA CGA ACG AGA CCT-3' (Prevost-Boure et al., 2011) were used with the
163 Sensi-FAST SYBR Q-PCR kit (Bioline, London, UK). The 15 μl reactions contained 8 μl
164 Sensi-FAST master mix, 1 μl of each primer (final concentration 1 μM), 1 μl of DNA
165 template and 5 μl nuclease-free molecular-grade water (Taylor and Cunliffe, 2016). A
166 Bio-Rad iQ5 was used to perform qPCR. The following qPCR thermal cycles were used:
167 94°C for 3min, then 40 cycles of 94 °C for 10 s, annealing at 50 °C for 15 s, elongation at
168 72°C for 20 s and acquisition of fluorescence data at 82°C. Standard curves were
169 generated using known concentration of *Aspergillus niger* 18S rDNA.

171 *2.6 Fungal diversity*

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423 172 DNA extracted from two sediment samples collected at each study site by independent
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425 173 multiple corer deployments was amplified using the primer set ITS1F (5'-
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427 174 GGAAGTAAAAGTCGTAACAAGG-3') and ITS2 (5'-
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429 175 GCTGCGTTCTTCATCGATGC-3') which amplify the internal transcribed spacer-1
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431 176 (ITS1) region of the fungal rRNA gene (Walters et al., 2015). Amplicons were
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433 177 sequenced on an Illumina MiSeq platform by LGC group (Berlin, Germany) following
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435 178 Earth Microbiome Project protocols ([http://www.earthmicrobiome.org/emp-standard-](http://www.earthmicrobiome.org/emp-standard-protocols/)
436
437 179 [protocols/](http://www.earthmicrobiome.org/emp-standard-protocols/)). Barcodes and ITS1 primer pairs were removed before demultiplexing. Paired-
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439 180 end sequences were then merged with FLASH (Magoč and Salzberg, 2011). Merged
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441 181 sequences were quality filtered using the SEARCH tool (Edgar, 2010) to remove
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443 182 sequences with expected error >1.0 and analysed with the QIIME software package
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445 183 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were assigned with a
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447 184 threshold of 98.5% pairwise identity as indicated by the UNITE fungal ITS database
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449 185 (<http://unite.ut.ee/>). Then, OTUs were classified taxonomically against the UNITE
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451 186 database (<http://unite.ut.ee/>, Version 7.1, November 20, 2016). To allow a proper
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453 187 comparison among samples, we followed the approach by Gihring et al. (2012) with
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455 188 sample normalisation to 2500 randomly-selected sequences (corresponding to the lowest
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457 189 read count obtained in our samples). Rarefaction curves highlighted that 2500 sequences
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459 190 used for the comparison among all samples were generally sufficient to describe the
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461 191 fungal diversity in the different benthic deep-sea ecosystems investigated (Figure S1).
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468 193 *2.7 Statistical analyses*

470 194 Two-way analysis of variance (ANOVA) was performed to test for differences in organic
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472 195 matter content, fungal abundance, biomass and OTU richness among canyons and depths.
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474 196 When significant differences were encountered, post-hoc tests were also carried out.
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483 197 ANOSIM analysis was performed to test for the presence of statistical differences in the
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485 198 trophic conditions at the seafloor between canyons. Permutational multivariate analysis of
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487 199 variance (PERMANOVA) was used based on Bray-Curtis similarity matrix and
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489 200 visualised using cluster analysis to test for differences in fungal community composition
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491 201 among canyons and depths. Distance-based multivariate analysis for a linear model
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493 202 (DistLM) forward (Anderson, 2008) was performed to identify potential factors
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495 203 influencing fungal abundance, biomass, OTU richness and assemblage composition. P
496
497 204 values were obtained with 9,999 permutations of residuals under the reduced model
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499 205 (Anderson, 2008). Temperature, salinity and trophic resources (as protein, carbohydrate
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501 206 and lipid concentrations) were used as predictor variables. Distance-based redundancy
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503 207 analysis (dbRDA) was finally used to visualise the relationships between fungal
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505 208 assemblage composition of the different canyon systems and thermo-haline and trophic
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507 209 variables. All statistical analyses were performed using Primer 6+ software.
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516 213 **3. Results and discussion**

517
518 214 The thermo-haline conditions of bottom waters of the benthic systems investigated in the
519
520 215 present study changed across depths and canyons, with temperature values ranging from
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522 216 13.77 to 15.20 °C, and salinity values ranging from 38.75 to 38.93 (Table 1). Lowest
523
524 217 temperature and salinity values were generally observed at the greatest depth (i.e. 1000
525
526 218 m). Also, the analysis of organic matter quantity in the sediments revealed differences
527
528 219 among the investigated canyons (Tables 1, TableS1), with concentrations of proteins and
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530 220 carbohydrates significantly higher in Crotone and Squillace canyons than in Tricase
531
532 221 canyon ($p < 0.05$ and $p < 0.01$, for proteins and carbohydrates, respectively). The highest
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534 222 organic matter content in the sediments of Crotone and Squillace canyons is likely due to
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543 223 their proximity to the coast and the presence of nearby river inputs which amplify the
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545 224 magnitude of organic matter exported from the water column and settling on the seafloor
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547
548 225 (Lopez-Fernandez et al., 2013).

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550 226 The amount of organic matter in deep-sea sediments represents a significant factor
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552 227 influencing the abundance and distribution of benthic assemblages (Danovaro et al.,
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554 228 2014). Fungal abundance, expressed as number of fungal 18S rDNA copies ranged from
555
556 229 1.4×10^6 to 5.1×10^7 copies g⁻¹ and was significantly lower in Tricase ($0.38 \pm 0.04 \times 10^7$
557
558 230 copies g⁻¹) than in Crotona and Squillace canyons (2.7 ± 0.5 and $1.3 \pm 0.4 \times 10^7$ copies
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560 231 g⁻¹, respectively; $p < 0.01$; Figure 2a). Our results fall within previously reported ranges for
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562 232 deep-sea sediments of the Pacific Ocean (3.5×10^6 - 5.2×10^7 28S rDNA copies g⁻¹; Xu
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564 233 et al., 2014), providing the first evidence of the quantitative importance of fungi also in
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566 234 benthic deep-sea ecosystems of the Mediterranean Sea. In all canyons, the 18S rDNA
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568 235 copy number changed significantly with water depth, with highest values at the
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570 236 shallowest depth in Crotona and Squillace canyons and at 500 m depth in Tricase
571
572 237 canyons.

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574
575 238 Fungal biomass ranged from 0.17 to 5.78 $\mu\text{gC g}^{-1}$, with values significantly lower in the
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577 239 sediments of Tricase ($0.63 \pm 0.14 \mu\text{gC g}^{-1}$) than in Crotona and Squillace canyons ($2.40 \pm$
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579 240 0.43 and $2.73 \pm 0.49 \mu\text{gC g}^{-1}$, respectively; $p < 0.01$) (Figure 2b). The distribution of
580
581 241 fungal biomass along the bathymetric gradients within each canyon was similar to that of
582
583 242 18S rDNA copy number. Data on fungal biomass are practically no existent for deep-sea
584
585 243 surface sediments (Damare and Raghukumar, 2008). However, the fungal biomass values
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587 244 reported here are similar to those of other benthic components reported at equal depths in
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589 245 the whole Mediterranean Sea (Gambi et al., 2017) suggesting that fungi can represent a
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591 246 significant component of benthic biomass in deep-sea sediments.

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593
594 247 We found a significant relationship between fungal abundance and biomass (Figure S2).
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603 248 From the slope of this relationship, we estimated that 1 µg of fungal biomass could be
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605 249 equivalent to 7.8×10^6 fungal 18S rDNA copies. Although such relationship should be
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608 250 view with caution and needs to be better refined with a broader spatial scale investigation,
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610 251 it can provide useful information on the quantitative relevance of deep-sea fungi based on
611
612 252 copy number determinations (Taylor and Cunliffe, 2016).

613
614 253 Significant positive relationships between carbohydrate concentrations and fungal
615
616 254 abundance and biomass were found ($r=0.715$ and $r=0.893$, both $p<0.01$, for the
617
618 255 abundance and biomass, respectively; Figure 3). Also, multivariate multiple regression
619
620 256 analysis provided evidence that carbohydrate concentration in the sediment was the
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622 257 primary factor explaining the distribution of the abundance and biomass of fungi in the
623
624 258 benthic deep-sea ecosystems investigated (Table S2). Since fungi are osmotrophic (i.e.
625
626 259 feed by secreting enzymes into the environment to degrade organic matter externally
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628 260 before taking the resulting metabolites into the cell; Richards and Talbot, 2013; Richards
629
630 261 et al., 2015), our results suggest that they could be highly specialized in the utilisation of
631
632 262 carbohydrates which are typically characterised by a highly recalcitrant fraction,
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634 263 especially in benthic deep-sea ecosystems (Dell'Anno et al., 2000; Dell'Anno et al.,
635
636 264 2013).

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639 265 Our results also show that the clustering of the 1203476 fungal ITS sequences (obtained
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641 266 after quality check) allowed us to identify a total of 1742 fungal OTUs, belonging to all
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643 267 fungal phyla known to date. Ascomycota represented the dominant phylum (accounting
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645 268 for 68% of the total reads), followed by Basidiomycota (10%) and Chytridiomycota (4%).
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647 269 The dominance of such phyla has been consistently reported in other benthic deep-sea
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649 270 ecosystems (Zhang et al., 2016).

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652 271 The number of fungal OTUs we found in the sediments of the different canyons was
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654 272 similar compared with that reported in other deep-sea ecosystems (Zhang et al., 2016).
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273 The Tricase canyon displayed a significantly lower OTU number (range: 64-71 OTUs)
274 compared to Crotone and Squillace canyons (range: 113-325 and 173-221 OTUs,
275 respectively; $p < 0.01$; Figure 4).

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

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

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

292 The majority of fungal OTUs were unclassified below the order level and overall
293 represented up to 69% of the total sequences. The quantitative relevance of unclassified
294 sequences in our study was much higher than that reported for coastal sediments (Picard,
295 2017), indicating that deep-sea ecosystems might harbour a higher richness of novel
296 fungal lineages compared with shallow benthic ecosystems.

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

302 The analysis of the turnover (β -)diversity highlighted that the similarity of the fungal
303 assemblage composition among different sites was very low (Table S3 and Figure 6).
304 Indeed, the within-canyon similarity (i.e., the similarity of fungal assemblage
305 composition among samples collected at a different depth within the same canyon) was
306 on average 11%, while the inter-canyon comparisons resulted in an average similarity of
307 7% (Table S3). Moreover, the Tricase canyon showed the highest percentage of unique
308 OTUs (i.e., OTUs found in Tricase but not in Squillace nor Crotone canyons; Table S4).
309 Overall, the three canyons shared only 46 out of 1742 OTUs, that cumulatively accounted
310 for only 22% of the total sequences. Twenty-seven of these 46 shared OTUs (overall
311 accounting for 14% of the total sequences) were not classified, while the others shared
312 OTUs (each of them contributing for $\leq 0.45\%$ of the total sequences) included taxa
313 belonging to *Epicoccum nigrum*, *Illyonectria robusta*, *Trichoderma bissettii*,
314 *Cryptococcus victoriae*, *Aspergillus sydowii*, *Fusarium sp.*, *Penicillium halotolearns* and
315 *Thermomyces lanuginosus*.

316 Distance-based redundancy analysis highlighted that the fungal assemblage composition
317 in the sediments of the different canyons was related to an array of factors including
318 organic matter content (as carbohydrates and lipid concentrations, $r = -0.624$ and $r = 0.434$,
319 respectively) and temperature ($r = 0.980$) and salinity ($r = -0.560$; Figure 7). These results
320 confirm that also in the deep-sea sediments investigated trophic availability and thermo-
321 haline conditions are important drivers of fungal assemblage composition (Hanson et al.,

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322 2008; McGuire et al., 2010; Li et al., 2016; Taylor and Cunliffe, 2016; Tisthammer et al.,
323 2016). Our findings also suggest that changes in the thermo-haline and trophic conditions
324 among submarine canyons may promote a high turnover diversity of benthic deep-sea
325 fungal assemblages.

326 Overall results of the present study indicate that the submarine canyons of the
327 Mediterranean Sea host abundant and highly diversified fungal assemblages most of
328 which still unidentified and pave the way for a better understanding of the ecological role
329 of fungi in the largest ecosystem on Earth.

330
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336
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339 G.B., E.R., M.T. and A.D. contributed to data elaboration and interpretation. G.B., E.R., and
340 A.D. wrote the first draft of the manuscript. All authors contributed to results discussion and
341 finalization of the manuscript.

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343 **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), Crotono (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, Crotono 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, Crotono 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.

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

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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

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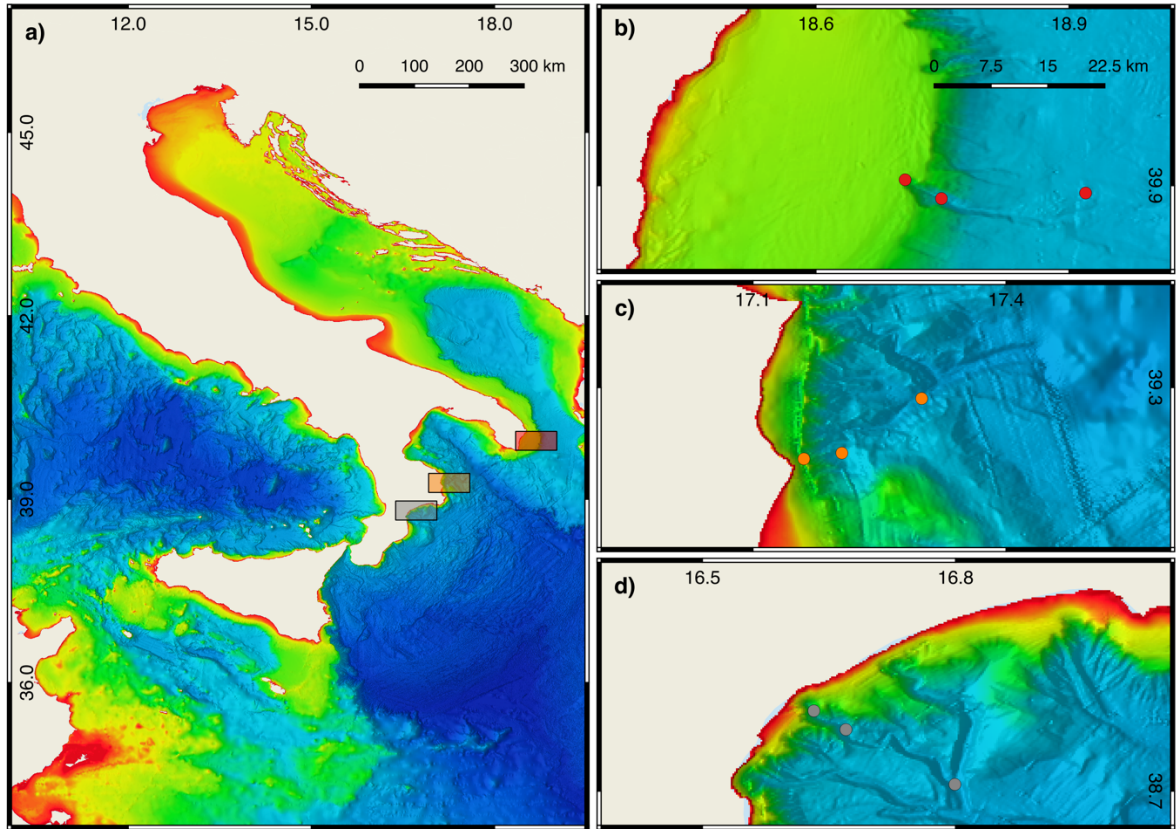


Figure 1

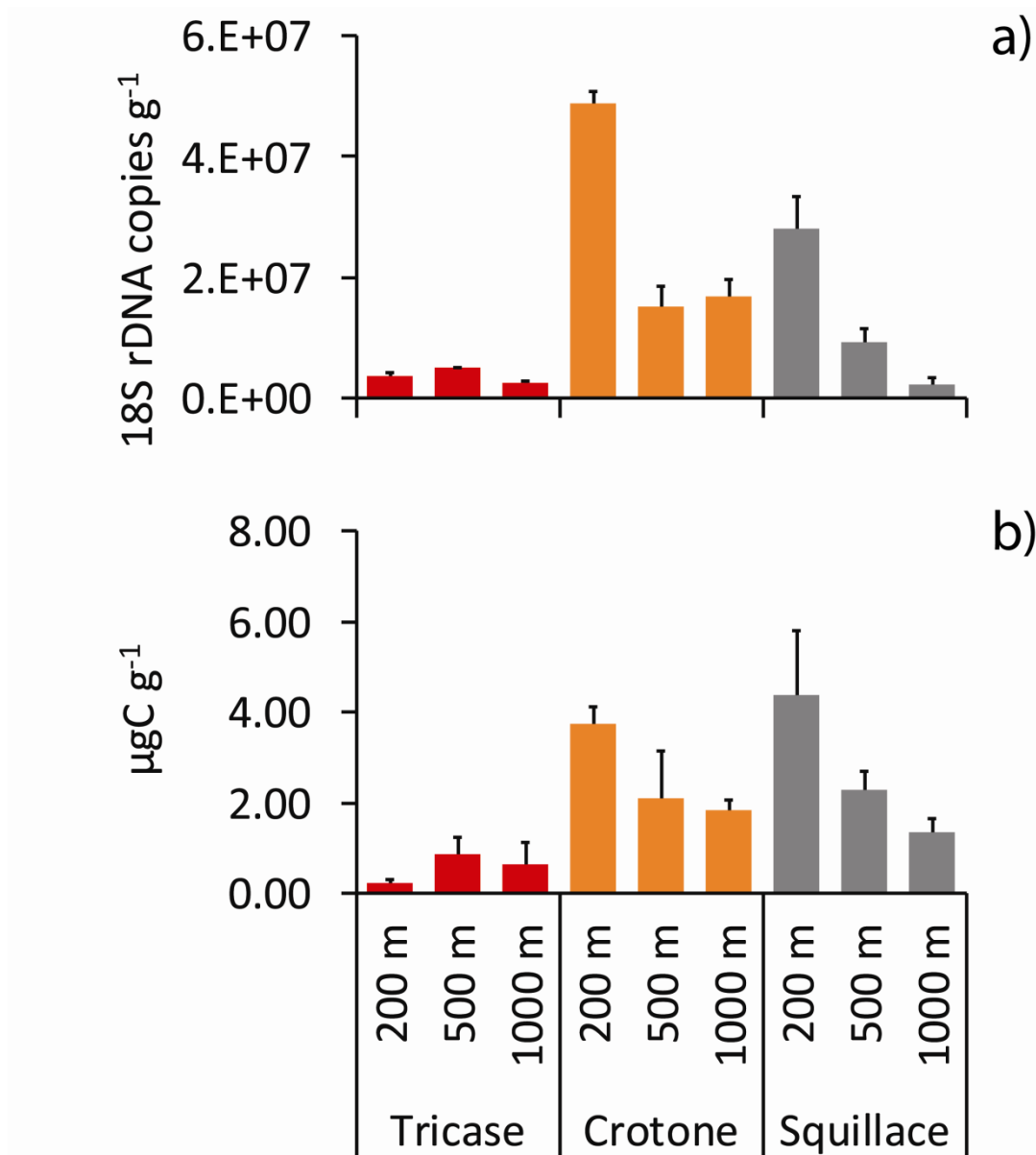
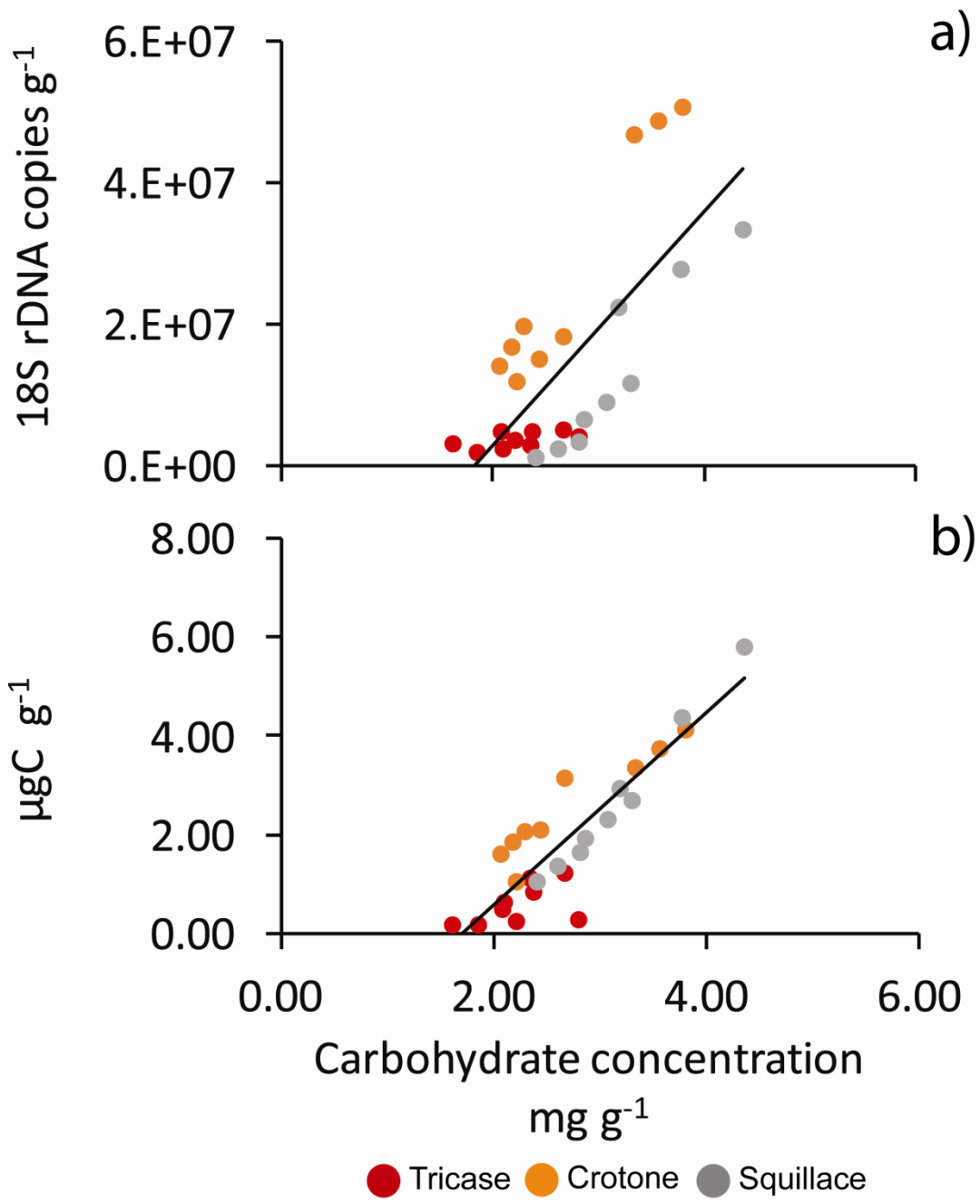


Figure 2



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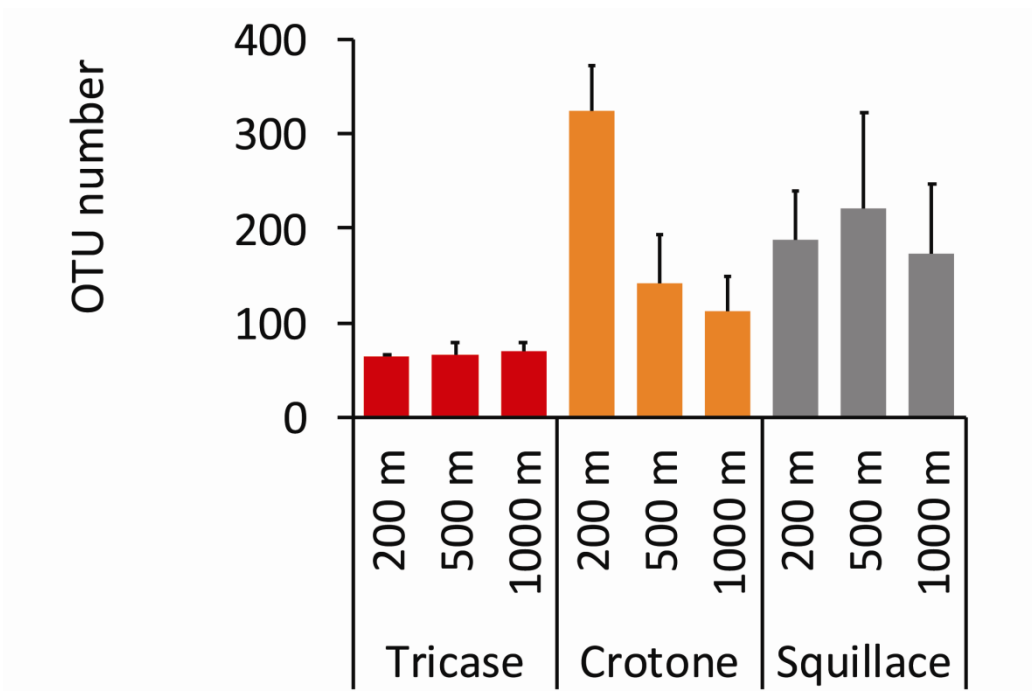


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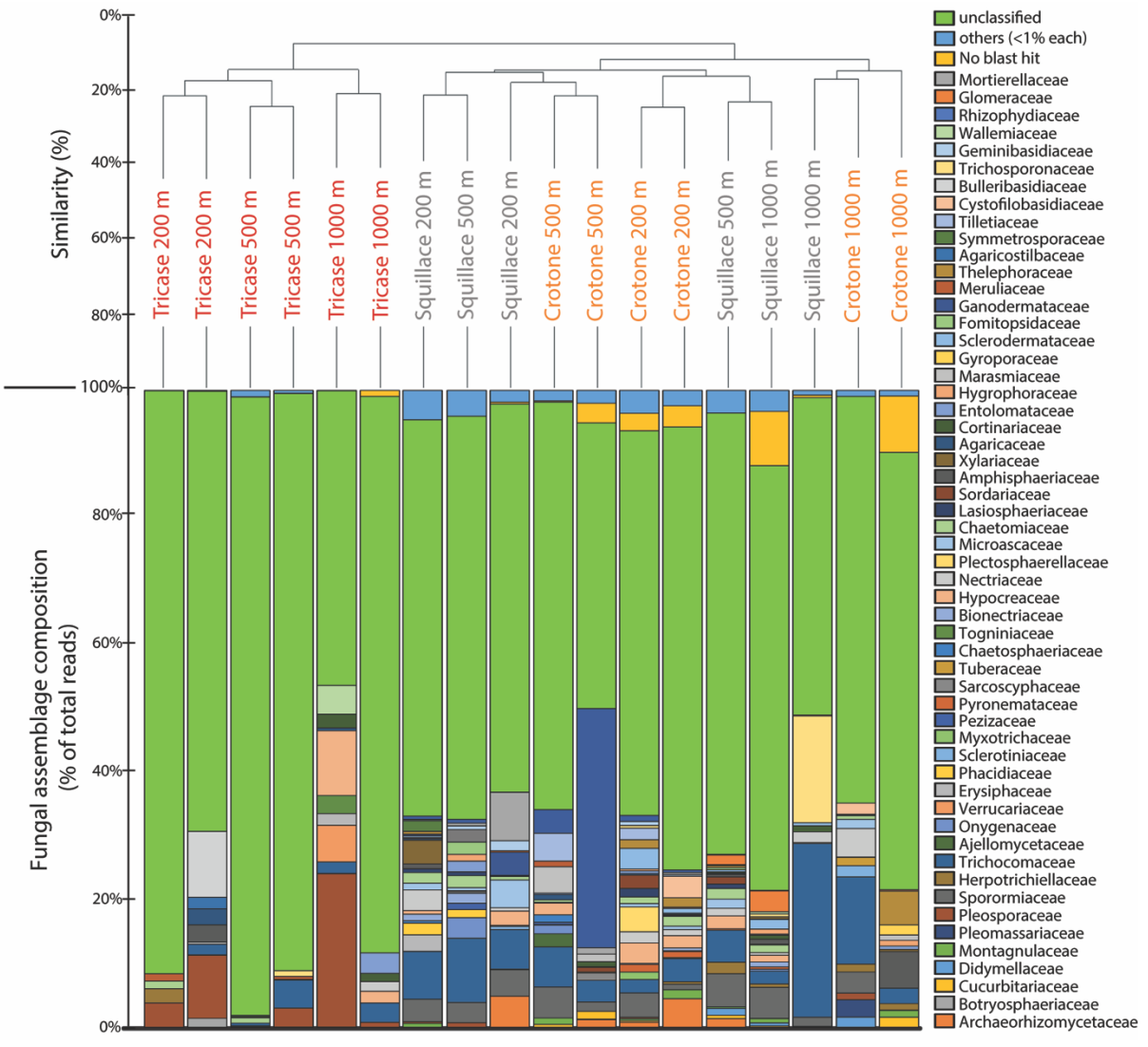
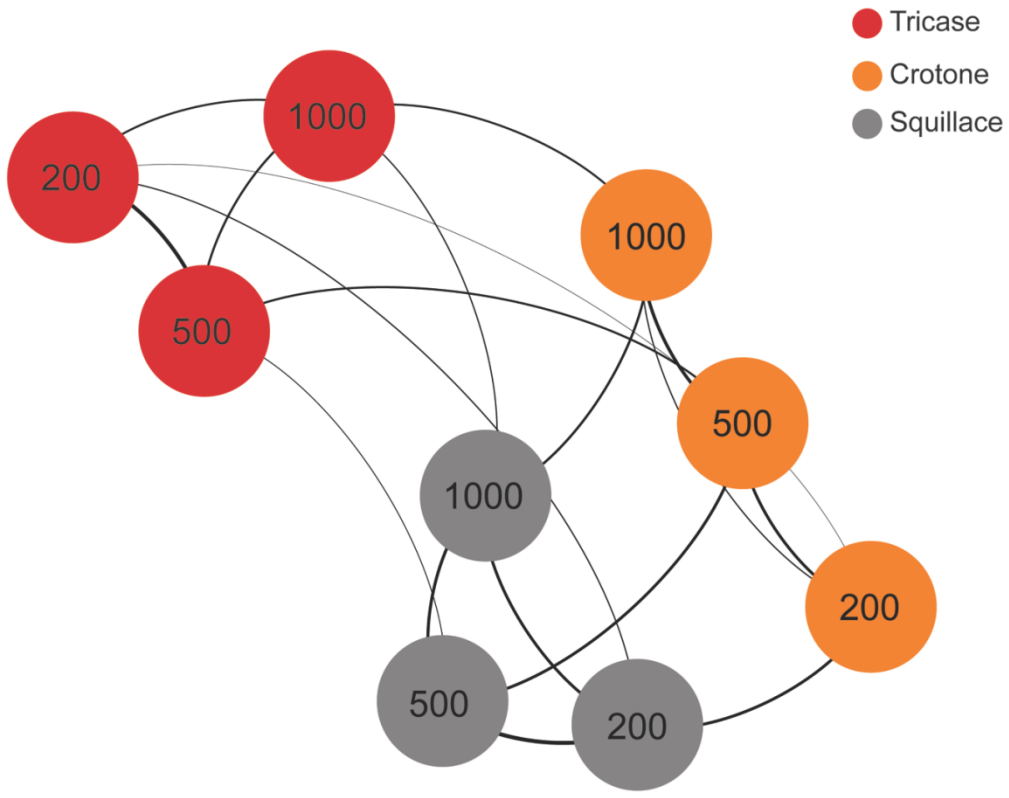


Figure 5

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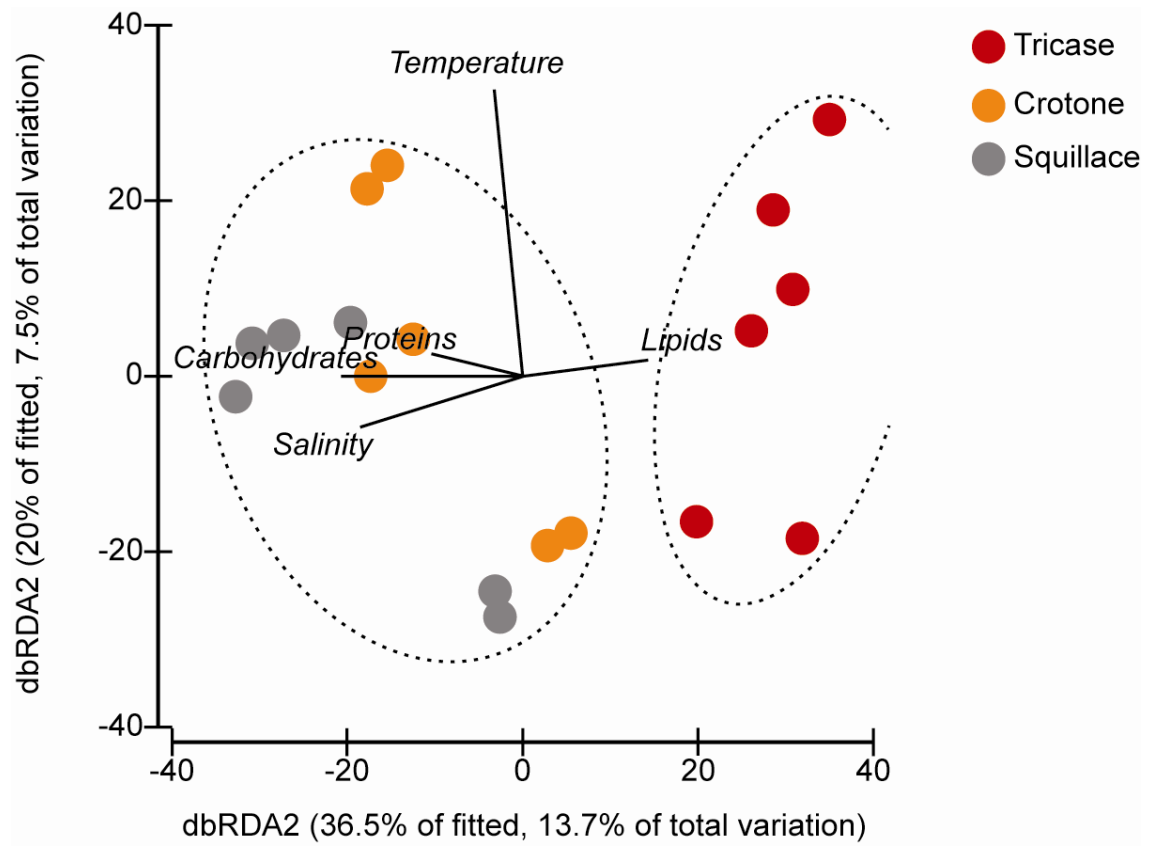


Figure 7

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Supplementary materials

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

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Giulio Barone, Eugenio Rastelli, Cinzia Corinaldesi, Michael Tangherlini, Roberto

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Danovaro, Antonio Dell'Anno

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Supplementary table S1-S4

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Supplementary figures S1 and S2

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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		Dissimilarity	SIMPER		
	R	P		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

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691 **Table S2.** Output of the multivariate multiple regression analysis carried out for testing the
692 effects of organic matter content (proteins, carbohydrates and lipids), temperature and salinity
693 on fungal abundance (as 18S rDNA copies) and biomass. Reported are Pseudo-F and P
694 values (*<0.05; **<0.01; ***<0.001; ns>0.05) and the cumulative variance explained by the
695 significant variables.

Fungal abundance (18S rDNA copies)			
Variable	Pseudo-F	P	Cumulative variance %
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	-

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

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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

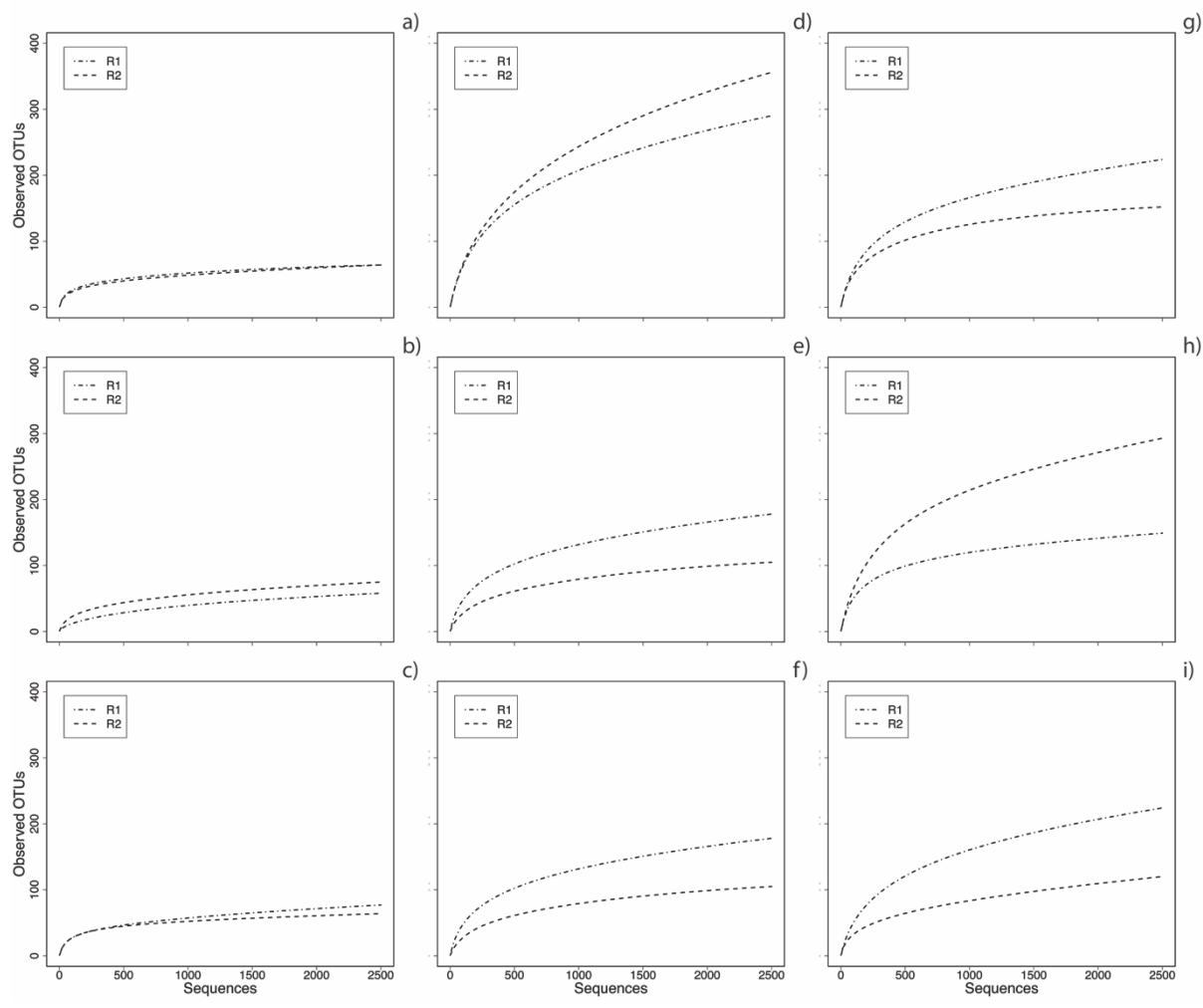
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702 **Table S4.** Percentage of unique and shared OTUs between replicates of the same site, within
703 the canyon and between the canyons
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Type of comparison			Shared %	Unique %
between replicates 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
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
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

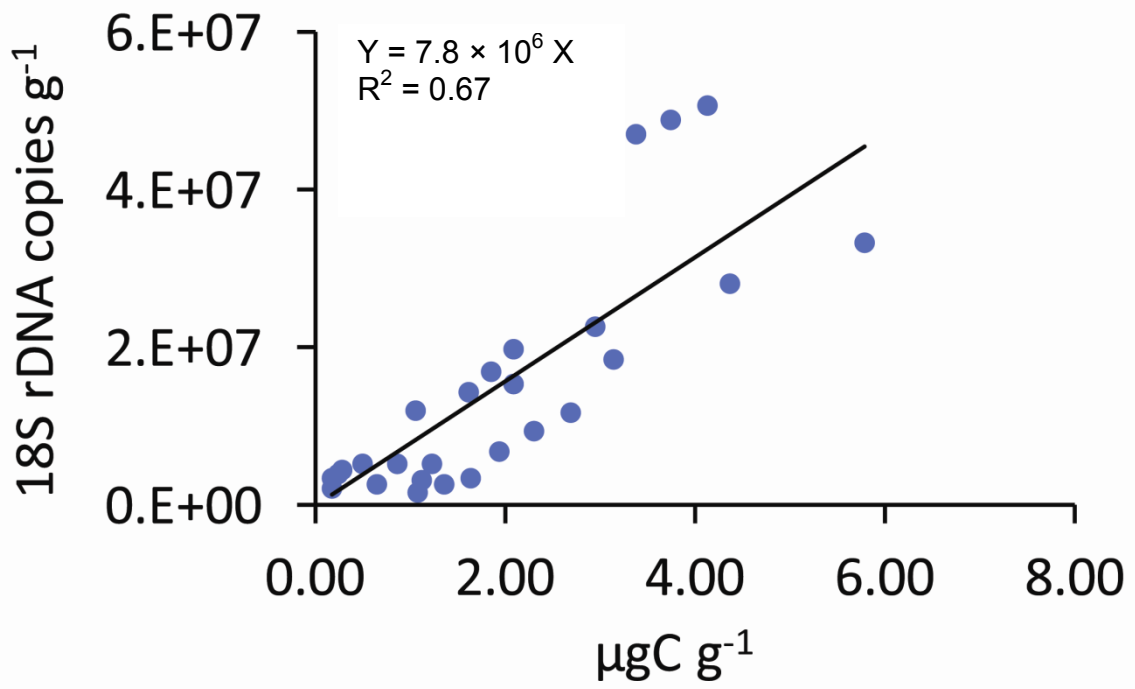
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706 **Figure S1.** Rarefaction curves calculated for each of the two independent replicates (dashed
707 lines, 2500 sequences each) analysed in all benthic deep-sea sites of the canyons investigated.
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721 **Figure S2.** Relationship between benthic fungal abundance (as 18S rDNA copies) and
722 biomass in the sediments of the three canyons



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