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

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5 Giulio Barone[1], Eugenio Rastelli[2], Cinzia Corinaldesi[3], Michael Tangherlini[2], Roberto
6 Danovaro[1,2], Antonio Dell'Anno[1*]
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9 [1]*Department of Life and Environmental Sciences, Polytechnic University of Marche, 60131*
10 *Ancona Italy*
11 [2]*Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy*
12 [3]*Department of Sciences and Engineering of Materials, Environment and Urbanistics,*
13 *Polytechnic University of Marche, 60131 Ancona, Italy*
14
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18 *Address for correspondence: a.dellanno@univpm.it
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22 **Running title**: Fungal abundance and diversity in Mediterranean canyons
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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 represent one of the most 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

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 (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

74 controlling the distribution and diversity of fungi in benthic deep-sea ecosystems remain
75 to date largely unexplored.
76 In terrestrial and freshwater ecosystems, fungi are among the main decomposers of
77 organic matter, and they play an important role in the processing of the most refractory
78 fraction of organic carbon (Carlile et al., 2001; Clipson et al., 2006; Hwang et al., 2006;
79 Dighton, 2007). In marine sediments, and especially in benthic deep-sea ecosystems, the
80 sedimentary organic matter is typically composed of a large fraction of recalcitrant
81 compounds, resistant to biological degradation (Pusceddu et al., 2009). Fungi are
82 expected to be specialized in the decomposition of refractory organic compounds, yet
83 their role in C cycling in benthic deep-sea ecosystems remains poorly understood (Hyde
84 et al., 1998; Burgaud et al., 2009; Cathrine and Raghukumar, 2009; Jebaraj et al., 2010).
85 In this study, we investigated the abundance, biomass and taxonomic composition of
86 fungal assemblages along the continental margins of the Central Mediterranean Sea.
87 Continental margins are characterised by open slopes and submarine canyons, which are
88 essential for C cycling and nutrient regeneration processes at a global scale (Bousquet et
89 al., 2000; Dickens, 2003). In particular, submarine canyons can channel large amounts of
90 organic matter photosynthetically produced from the continental shelf down to deep-sea
91 ecosystems (Monaco et al., 1999; Sànchez-Vidal et al., 2008; Allen and Durrieu de
92 Madron, 2009; Puig et al., 2014). For this reason, we selected three submarine canyons
93 characterised by different environmental conditions and investigated fungal abundance,
94 biomass and diversity at depths ranging from 200 to 1000 m. To identify the factors
95 potentially controlling their quantitative importance and diversity in deep-sea sediments,
96 we explored the role of environmental conditions, including the organic matter quality
97 and quantity.

98 **2. Materials and methods**

99 *2.1. Study area and sampling design*

100 Sediment sampling was carried out in the Ionian Sea (Central Mediterranean Sea) during
101 the oceanographic cruise "SAND 2016" held on board of the research vessel R/V
102 Minerva Uno in May 2016. Sediment samples were collected within the main axis of
103 three canyons located along the SE Italian margin at 200, 500 and 1000 m depths (Figure
104 1). One of the investigated canyon (hereafter defined "Tricase") located along the
105 Apulian margin, is far from any continental freshwater inputs. The other two investigated
106 canyons are located along the Calabrian margin and were close to river estuaries. The
107 Northern canyon, extending for about 30 km, is located in front of the Crotone
108 municipality (canyon "Crotone") and its head is close to Neto river mouth. The head of
109 the canyon "Squillace" is close to the coastline in front of the Squillace municipality and
110 is characterised by the presence of sporadic, but intense river inputs from Ghetterllo
111 stream. Sediment samples were collected at each benthic site by independent multiple
112 corer deployments.
113 The top 1 cm of each sediment sample was used for the analysis of the quantity and
114 biochemical composition of organic matter, fungal abundance (based on q-PCR analysis
115 of 18S rRNA genes), biomass and diversity. At each station, temperature and salinity of
116 bottom waters were measured using CTD casts.
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123 *2.2. Quantity and biochemical composition of organic matter*

124 The three major biochemical classes of organic compounds (proteins, carbohydrates and
125 lipids) in deep-sea sediments were determined according to previously described

126 procedures (Danovaro, 2010). Briefly, about 0.5 g of wet sediment was used for each
127 analysis. Protein concentration was obtained via colorimetric method, based on the
128 reaction of proteins with rameic tartrate and the Folin–Ciocalteau reagent in basic
129 environment (pH 10). The reaction provides a stable blue colour whose intensity is
130 proportional to the protein concentration. Carbohydrate concentration was obtained via
131 colorimetric assay based on the reaction between sugars and phenol in the presence of
132 concentrated sulfuric acid. Lipids were extracted by direct elution with chloroform and
133 methanol followed by reaction with sulfuric acid. Protein, carbohydrate and lipid
134 concentrations were determined spectrophotometrically and expressed as albumin,
135 glucose and tripalmitin equivalents, respectively. All analyses were carried out in 3
136 replicates. Protein, carbohydrate and lipid concentrations were then converted to carbon
137 equivalents (conversion factors: 0.49, 0.40 and 0.75 gC g^{-1}, respectively) to determine
138 biopolymeric C content in the sediment (Dell'Anno et al., 2002).
139
140 *2.3. Fungal biomass*
141 To detect and quantify fungi in the sediment samples, fluorescence in-situ hybridisation
142 (FISH) coupled with Calcofluor white staining (which targets chitin, cellulose and
143 carboxylated polysaccharides) have been used following procedures previously described
144 (Bochdansky et al., 2017). The FISH reaction was performed using the Pan-Fungal probe
145 PF2 (5'-CTCTGGCTTCACCCTATTC-3') Cy-3 labelled (Kempf et al., 2000). Briefly,
146 about 1 g of sediment was fixed for 1 h in pre-filtered (with 0.2 μm pore size filters)
147 buffered formaldehyde solution (2% vol/vol; Pernthaler et al., 2002). After fixation,
148 samples were centrifuged twice to remove formaldehyde residues and resuspended in
149 PBS. Then, samples were treated using 4 ml of a mix containing EDTA, Tween 80,
150 sodium-pyrophosphate, methanol and ultrasounds treatment to separate fungi from the

151 sediment matrix. After centrifugation, sediment samples were washed twice with PBS
152 buffer and then treated with increasing concentrations of ethanol (50, 80 and 96%, for 3
153 min each). The sediment was then suspended in 500 µl hybridisation buffer containing
154 0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris–HCl pH 7.2, 30 %v/v formamide and 1 µM
155 PF2 (Kempf et al., 2000), then incubated for 3 h at 46°C in the dark. Samples were then
156 transferred in sterile tubes containing pre-warmed washing buffer (20 mM Tris–HCl pH
157 8.0, 0.01% w/v SDS, 5 mM EDTA, 0.112M NaCl) and incubated for 30 minutes at 48°C.
158 After centrifugation and resuspension of the sediment samples with 0.2 µm pre-filtered
159 water, aliquots of the slurry (n=3) were filtered on 0.2 µm polycarbonate filters
160 (Millipore). Filters were then stained with 0.5 mM Calcofluor white and incubated in the
161 dark for 5 min. Subsequently, slides were washed with 0.02 µm pre-filtered water and
162 analysed under epifluorescence microscopy. The whole filter was examined, and length
163 and width measures were taken for each fungal-like structure. A positive FISH signal was
164 detected in almost all (i.e., more than 90%) of the calcofluor-stained structures identified.
165 However, consistently with previous findings (Gonçalves et al., 2006; Bochdansky et al.,
166 2017), FISH signal was typically weaker and less homogeneously distributed over the
167 fungal structures compared with the calcofluor signal. This could be due to the low and/or
168 uneven distribution of nucleic acids in fungal hyphae (Teertstra et al., 2004), as well as to
169 the low permeability of fungal cells to FISH probes (Bochdansky et al., 2017). Thus, for a
170 more reliable assessment of fungal biomass the calcofluor signals were used (according to
171 Damare and Raghukumar 2008), while the FISH approach was used to exclude unspecific
172 calcofluor signals (i.e., chitin-containing structures but not identified as fungi by FISH).
173 The average width and cumulative length of each identified fungal structure were
174 converted to a cylinder with half-spheres at ends, and the biovolume was converted into

175 fungal biomass, assuming 1 μm^3 of fungal biovolume equivalent to 1 pg C (Damare and
176 Raghukumar 2008).
177
178 *2.4 DNA extraction and purification for molecular analysis*
179 The DNA was extracted and purified from the sediment samples using the PowerSoil
180 DNA isolation kit (QIAGEN)) following the manufacturer's instruction with slight
181 modifications to remove extracellular DNA (based on three subsequent washing steps)
182 before DNA extraction (Danovaro, 2009; Danovaro et al., 2016).
183
184 *2.5 Quantitative real-time PCR of fungal 18S rRNA gene sequences*
185 The DNA extracted from two sediment samples collected at each study site by
186 independent multiple corer deployments was used for quantitative real-time PCR (qPCR)
187 analysis which was performed as described in Taylor et al. (2016) with slight
188 modifications. Briefly, fungi-specific primers FR1 5′-AIC CAT TCA ATC GGT AIT-3′
189 and FF390 5′-CGA TAA CGA ACG AGA CCT-3′ (Prevost-Boure et al., 2011) were used
190 with the Sensi-FAST SYBR Q-PCR kit (Bioline, London, UK). The 15 µl reactions
191 contained 8 µl Sensi-FAST master mix, 1 µl of each primer (final concentration 1 µM),
192 1µl of DNA template and 5 µl nuclease-free molecular-grade water (Taylor and Cunliffe,
193 2016). A Bio-Rad iQ5 was used to perform qPCR. The following qPCR thermal cycles
194 were used: 94°C for 3min, then 40 cycles of 94 °C for 10 s, annealing at 50 °C for 15 s,
195 elongation at 72°C for 20 s and acquisition of fluorescence data at 82°C. Standard curves
196 were generated using known concentration of *Aspergillus niger* 18S rDNA.
197
198 *2.6 Fungal diversity*

199 DNA extracted from two sediment samples collected at each study site by independent
200 multiple corer deployments was amplified using the primer set ITS1F (5′-
201 GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′-
202 GCTGCGTTCTTCATCGATGC-3′) which amplify the internal transcribed spacer-1
203 (ITS1) region of the fungal rRNA gene (Walters et al., 2015). Amplicons were
204 sequenced on an Illumina MiSeq platform by LGC group (Berlin, Germany) following
205 Earth Microbiome Project protocols (http://www.earthmicrobiome.org/emp-standard-
206 protocols/). Raw sequences were demultiplexed and barcodes and ITS1 primer pairs were
207 removed afterwards. Paired-end sequences were then merged with FLASH (Magoč and
208 Salzberg, 2011). Merged sequences were quality filtered using the USEARCH tool
209 (Edgar, 2010) to remove sequences with expected error >1.0 and analysed with the
210 QIIME software package (Caporaso et al., 2010). Operational taxonomic units (OTUs)
211 were assigned with a threshold of 98.5% pairwise identity as indicated by the UNITE
212 fungal ITS database (http://unite.ut.ee/). Prior to taxonomic identification, OTUs were
213 checked by means of the ChimeraSlayer tool within the QIIME software package against
214 the UNITE ITS database to identify and remove potential chimeras. Then, non-chimeric
215 OTUs were classified taxonomically against the UNITE database (http://unite.ut.ee/,
216 Version 7.1, November 20, 2016). To allow a proper comparison among samples, we
217 followed the approach by Gihring et al. (2012) with sample normalisation to 2500
218 randomly-selected sequences (corresponding to the lowest read count obtained in our
219 samples). Rarefaction curves highlighted that 2500 sequences used for the comparison
220 among all samples were generally sufficient to describe the fungal diversity in the
221 different benthic deep-sea ecosystems investigated (Figure S1).
222
223 *2.7 Statistical analyses*

224 Two-way analysis of variance (ANOVA) was performed to test for differences in organic
225 matter content, fungal abundance, biomass and OTU richness among canyons and depths.
226 When significant differences were encountered, post-hoc tests were also carried out.
227 ANOSIM analysis was performed to test for the presence of statistical differences in the
228 trophic conditions at the seafloor between canyons. Permutational multivariate analysis of
229 variance (PERMANOVA) was used based on Bray-Curtis similarity matrix and
230 visualised using cluster analysis to test for differences in fungal community composition
231 among canyons and depths. Distance-based multivariate analysis for a linear model
232 (DistLM) forward (Anderson, 2008) was performed to identify potential factors
233 influencing fungal abundance, biomass, OTU richness and assemblage composition. P
234 values were obtained with 9,999 permutations of residuals under the reduced model
235 (Anderson, 2008). Temperature, salinity and trophic resources (as protein, carbohydrate
236 and lipid concentrations) were used as predictor variables. Distance-based redundancy
237 analysis (dbRDA) was finally used to visualise the relationships between fungal
238 assemblage composition of the different canyon systems and thermohaline and trophic
239 variables. All statistical analyses were performed using Primer 6+ software.
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243 **3. Results and discussion**
244 The thermohaline conditions of bottom waters of the benthic systems investigated in the
245 present study changed across depths and canyons, with temperature values ranging from
246 13.77 to 15.20 °C, and salinity values ranging from 38.75 to 38.93 (Table 1). Lowest
247 temperature and salinity values were generally observed at the greatest depth (i.e. 1000
248 m). Also, the analysis of organic matter quantity in the sediments revealed differences
249 among the investigated canyons (Tables 1, TableS1), with concentrations of proteins and

250 carbohydrates significantly higher in Crotone and Squillace canyons than in Tricase
251 canyon ($p<0.05$ and $p<0.01$, for proteins and carbohydrates, respectively). The highest
252 organic matter content in the sediments of Crotone and Squillace canyons is likely due to
253 their proximity to the coast and the presence of nearby river inputs which amplify the
254 magnitude of organic matter exported from the water column and settling on the seafloor
255 (Lopez-Fernandez et al., 2013).
256 The amount of organic matter in deep-sea sediments represents a significant factor
257 influencing the abundance and distribution of benthic assemblages (Danovaro et al.,
258 2014). Fungal abundance, expressed as number of fungal 18S rDNA copies ranged from
259 1.4×10^6 to 5.1×10^7 copies g^{-1} and was significantly lower in Tricase ($0.38 \pm 0.04 \times 10^7$
260 copies g^{-1}) than in Crotone and Squillace canyons (2.7 ± 0.5 and $1.3 \pm 0.4 \times 10^7$ copies
261 g^{-1}, respectively; $p<0.01$; Figure 2a). Our results fall within previously reported ranges for
262 deep-sea sediments of the Pacific Ocean (3.5×10^6 - 5.2×10^7 28S rDNA copies g^{-1}; Xu
263 et al., 2014), providing the first evidence of the quantitative importance of fungi also in
264 benthic deep-sea ecosystems of the Mediterranean Sea. In all canyons, the 18S rDNA
265 copy number changed significantly with water depth, with highest values at the
266 shallowest depth in Crotone and Squillace canyons and at 500 m depth in Tricase
267 canyons.
268 Fungal biomass ranged from 0.17 to 5.78 μgC g^{-1}, with values significantly lower in the
269 sediments of Tricase ($0.63 \pm 0.\ 14$ μgC g^{-1}) than in Crotone and Squillace canyons ($2.40 \pm$
270 0.43 and 2.73 ± 0.49 μgC g^{-1}, respectively; $p<0.01$) (Figure 2b). The distribution of
271 fungal biomass along the bathymetric gradients within each canyon was similar to that of
272 18S rDNA copy number. Data on fungal biomass are practically non-existent for deep-sea
273 surface sediments (Damare and Raghukumar, 2008). However, the fungal biomass values
274 reported here are similar to those of other benthic components reported at equal depths in

275 the whole Mediterranean Sea (Gambi et al., 2017) suggesting that fungi can represent a
276 significant component of benthic biomass in deep-sea sediments.
277 We found a significant relationship between fungal biomass estimated by using
278 microscopy and fungal abundance assessed on the basis of qPCR analysis (Figure S2).
279 From the slope of this relationship, we estimated that 1 μg of fungal biomass could be
280 equivalent to 7.8×10^6 fungal 18S rDNA copies. Although such relationship should be
281 viewed with caution and needs to be better refined with a broader spatial scale
282 investigation, it can provide useful information on the quantitative relevance of deep-sea
283 fungi based on copy number determinations (Taylor and Cunliffe, 2016).
284 Significant positive relationships between carbohydrate concentrations and fungal
285 abundance and biomass were found ($r=0.715$ and $r=0.893$, both $p<0.01$, for abundance
286 and biomass, respectively; Figure 3). Also, multivariate multiple regression analysis
287 provided evidence that carbohydrate concentration in the sediment was the primary factor
288 explaining the distribution of the abundance and biomass of fungi in the benthic deep-sea
289 ecosystems investigated (Table S2).
290 Fungi are heterotrophic organisms known to utilise carbohydrates (Richards and Talbot,
291 2013; Richards et al., 2015, Couturier et al., 2016), which in deep-sea sediments represent
292 one of the most refractory compounds of organic matter (Dell'Anno et al., 2000;
293 Dell'Anno et al., 2013). Therefore, our findings suggest that benthic deep-sea fungi,
294 besides prokaryotes, can be actively involved in the decomposition and utilisation of
295 highly refractory compounds, thus contributing to their cycling.
296 Our results also show that the clustering of the 1203476 fungal ITS sequences (obtained
297 after quality check) allowed us to identify a total of 1742 fungal OTUs, belonging to all
298 fungal phyla known to date. Ascomycota represented the dominant phylum (accounting
299 for 68% of the total reads), followed by Basidiomycota (10%) and Chytridiomycota (4%).

300 The dominance of such phyla has been consistently reported in other benthic deep-sea
301 ecosystems (Zhang et al., 2016).
302 The number of fungal OTUs we found in the sediments of the different canyons was
303 similar compared with that reported in other deep-sea ecosystems (Zhang et al., 2016).
304 The Tricase canyon displayed a significantly lower OTU number (range: 64-71 OTUs)
305 compared to Crotone and Squillace canyons (range: 113-325 and 173-221 OTUs,
306 respectively; $p<0.01$; Figure 4).
307 In our dataset, the OTUs affiliating to currently known fungal families were represented
308 by only 19-38% of the total reads (Figure 5). The classified fungal OTUs affiliated to 206
309 genera belonging to 132 families, 66 orders and 27 classes.
310 At all benthic sites, Pleosporales was the most represented fungal order (accounting for
311 ca. 20% of the total reads in each sample). This group is commonly present in marine
312 environment and can account for a relevant fraction of the fungal diversity (up to 18% of
313 all OTUs and sequences) in benthic deep-sea ecosystems (Li et al., 2016). Moreover,
314 members belonging to the Pleosporales order are known to be adapted to high hydrostatic
315 pressure (Nagano and Nagahama, 2012), possibly contributing to the ecological success
316 of such taxon in deep-sea ecosystems.
317 Most of the fungi that we successfully classified were affiliated to genera such as
318 *Aspergillus*, *Penicillium*, *Epicoccum*, *Cryptococcus* and *Candida* previously encountered
319 in other deep-sea environments (Nagahama et al., 2003; Edgcomb et al., 2011; Rédou et
320 al., 2014). However, these genera represented overall only ca. 36% of the total reads,
321 indicating that the majority of fungal taxa belonged to genera not represented in UNITE
322 database (Kõljalg et al., 2013).
323 The majority of fungal OTUs were unclassified below the order level and overall
324 represented up to 69% of the total sequences. The quantitative relevance of unclassified

325 sequences in our study was much higher than that reported for coastal sediments (Picard,
326 2017), indicating that deep-sea ecosystems might harbour a higher richness of novel
327 fungal lineages compared with shallow benthic ecosystems.
328 The composition of fungal assemblage in the sediments of the Tricase canyon was
329 significantly different ($p<0.01$) from that of the other canyons, which otherwise showed
330 no significant differences (Figure 5). These results suggest that submarine canyons far
331 from the coastline and lacking river inputs can host distinct fungal assemblages from
332 those close to river estuaries.
333 The analysis of the turnover (β-)diversity highlighted that the similarity of the fungal
334 assemblage composition among different sites was very low (Table S3 and Figure 6).
335 Indeed, the within-canyon similarity (i.e., the similarity of fungal assemblage
336 composition among samples collected at a different depth within the same canyon) was
337 on average 11%, while the inter-canyon comparisons resulted in an average similarity of
338 7% (Table S3). Moreover, the Tricase canyon showed the highest percentage of unique
339 OTUs (i.e., OTUs found in Tricase but not in Squillace nor Crotone canyons; Table S4).
340 Overall, the three canyons shared only 46 out of 1742 OTUs, that cumulatively accounted
341 for only 22% of the total sequences. Twenty-seven of these 46 shared OTUs (overall
342 accounting for 14% of the total sequences) were not classified, while the others shared
343 OTUs (each of them contributing for $\leq$0.45% of the total sequences) included taxa
344 belonging to *Epicoccum nigrum*, *Illyonectria robusta*, *Trichoderma bissettii*,
345 *Cryptococcus victoriae*, *Aspergillus sydowii*, *Fusarium sp*, *Penicillum halotolearns* and
346 *Thermomyces lanuginosus*.
347 Distance-based redundancy analysis highlighted that the fungal assemblage composition
348 in the sediments of the different canyons was related to an array of factors including
349 organic matter content (as carbohydrates and lipid concentrations, r= -0.624 and r= 0.434,

350 respectively), temperature (r= 0.980) and salinity (r= -0.560; Figure 7). These results
351 confirm that also in the deep-sea sediments investigated trophic availability and
352 thermohaline conditions are important drivers of fungal assemblage composition (Hanson
353 et al., 2008; McGuire et al., 2010; Li et al., 2016; Taylor and Cunliffe, 2016; Tisthammer
354 et al., 2016). Our findings also suggest that changes in the thermohaline and trophic
355 conditions among submarine canyons may promote a high turnover diversity of benthic
356 deep-sea fungal assemblages.
357 Overall results of the present study indicate that the submarine canyons of the
358 Mediterranean Sea host abundant and highly diversified fungal assemblages most of
359 which still unidentified and pave the way for a better understanding of the ecological role
360 of fungi in the largest ecosystem on Earth.
361
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367
368 **Author Contributions:** R.D., C.C., and A.D. conceived the study. G.B. participated in the
369 oceanographic cruise for collecting sediment samples and performed laboratory analyses.
370 G.B., E.R., M.T. and A.D. contributed to data elaboration and interpretation. G.B., E.R., and
371 A.D. wrote the first draft of the manuscript. All authors contributed to results discussion and
372 finalization of the manuscript.
373
374 **Conflict of interest:** All the other authors declare no competing financial interests.

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610 **Captions of figures**

611

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

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

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

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

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

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

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

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

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Canyon	Water depth	Temperature	Salinity	PRT	CHO	LIP	Biopolymeric C
	m	°C		mg g^{-1}	mg g^{-1}	mg g^{-1}	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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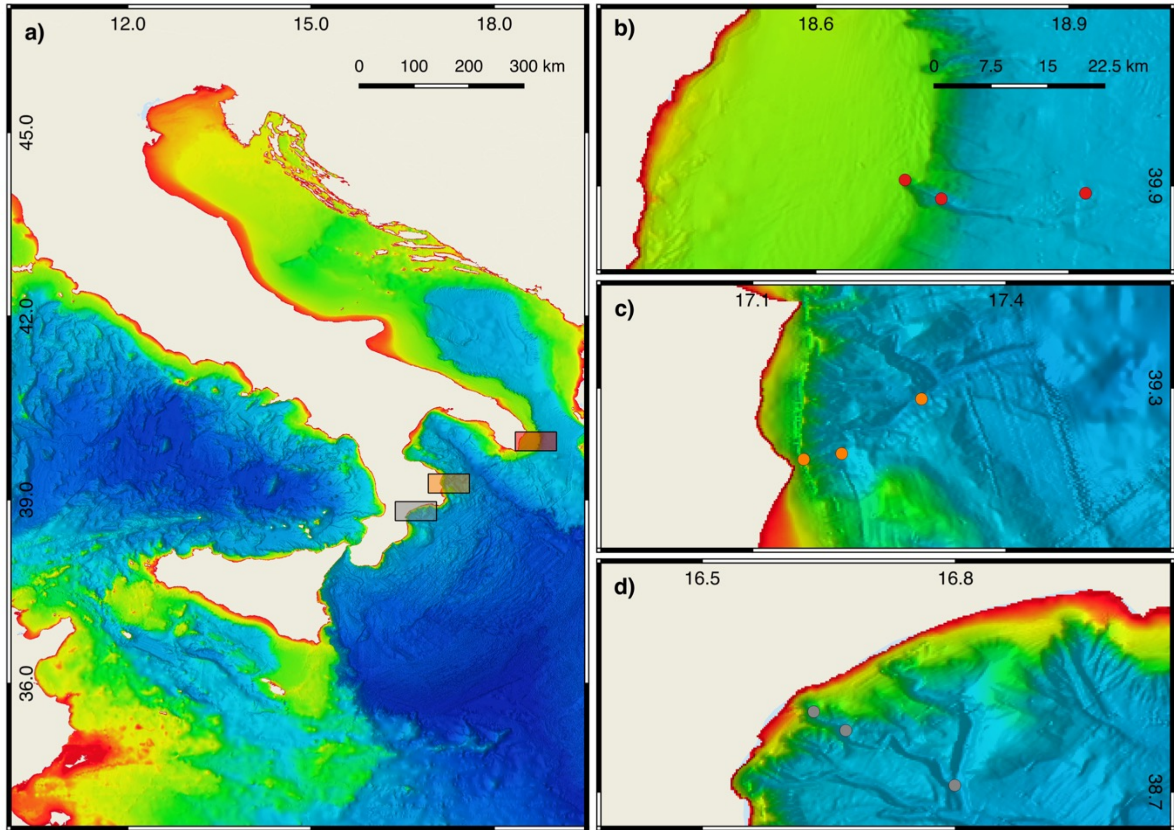

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645 Figure 1

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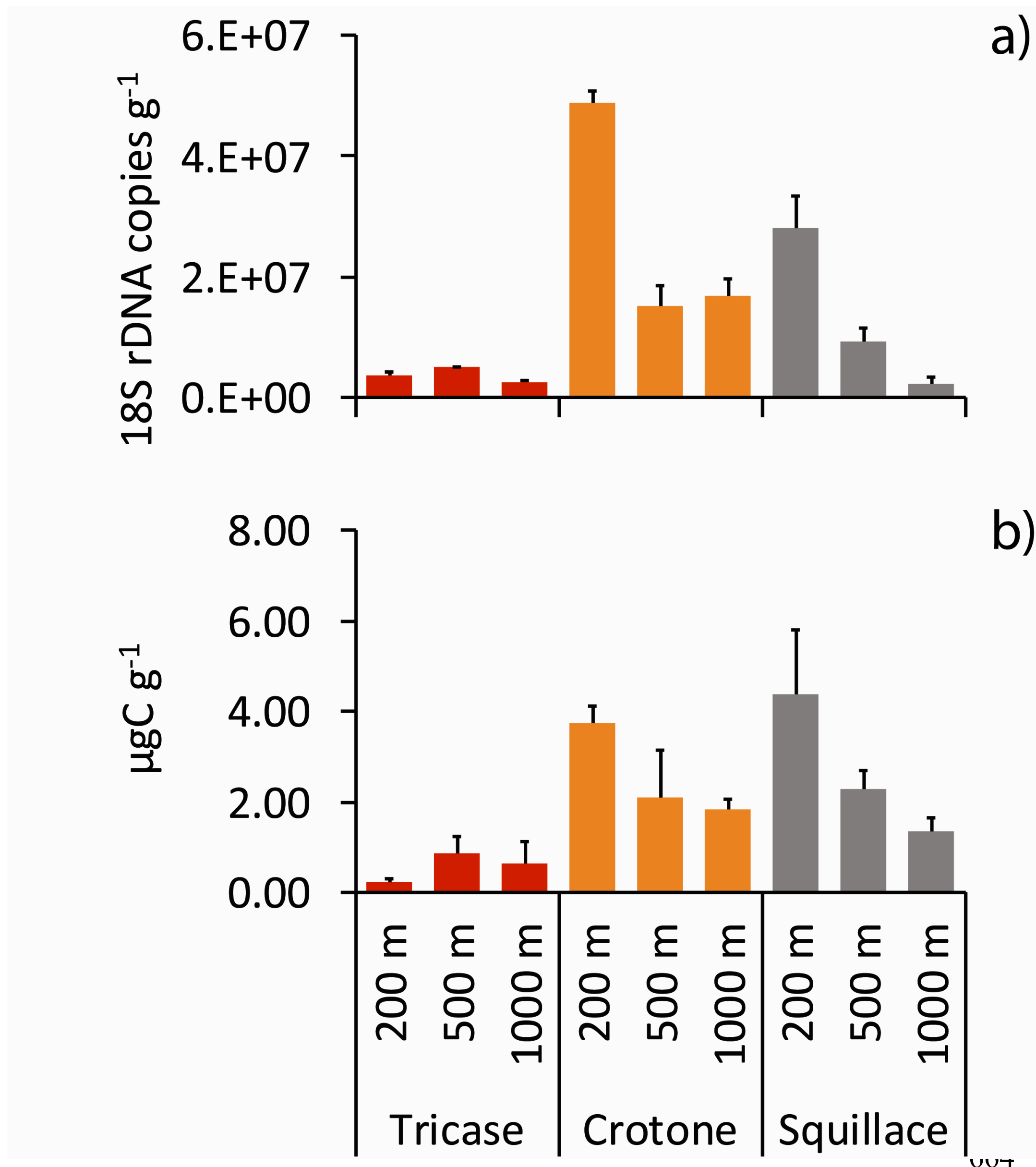

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666 Figure 2

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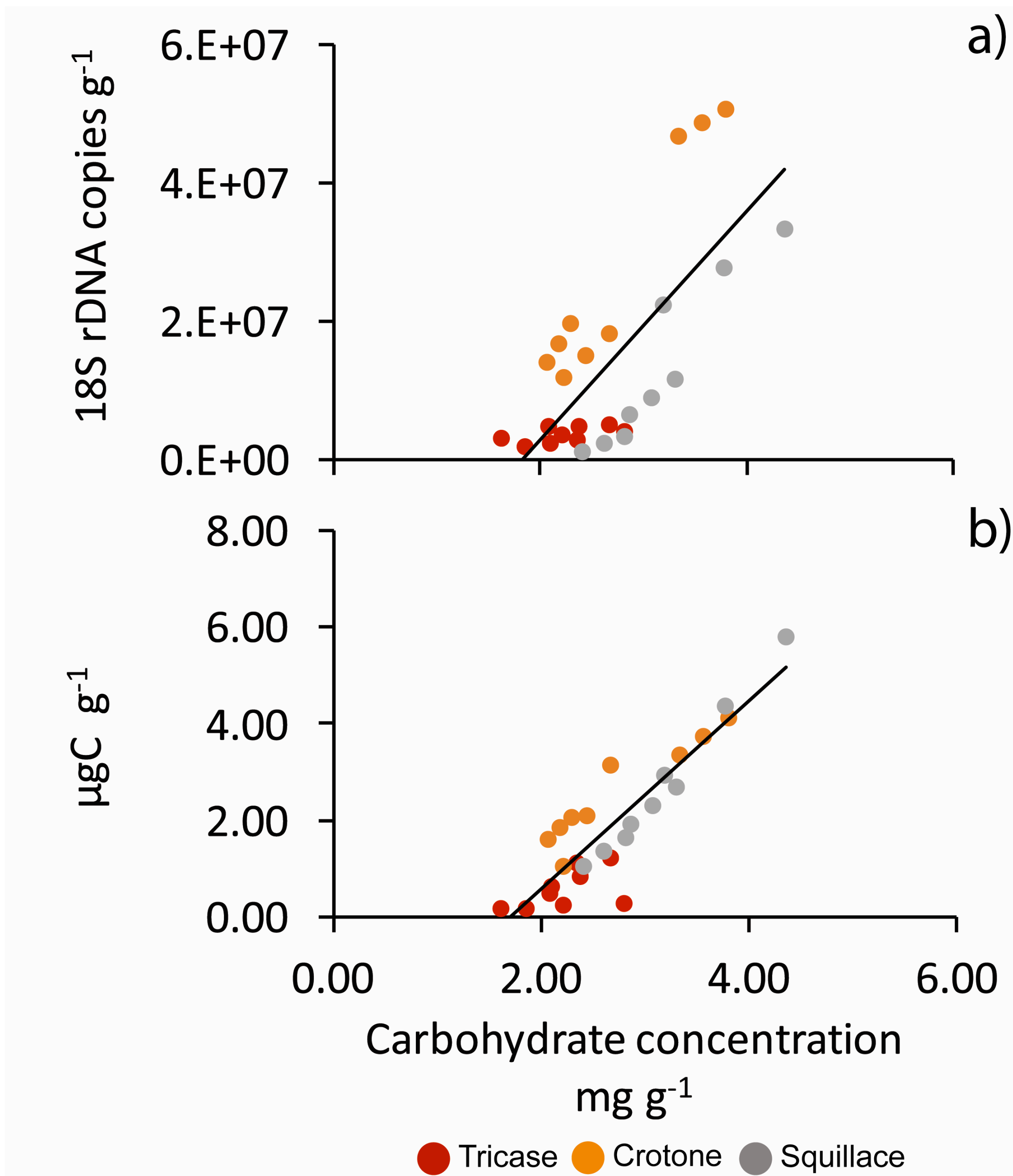

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669 Figure 3

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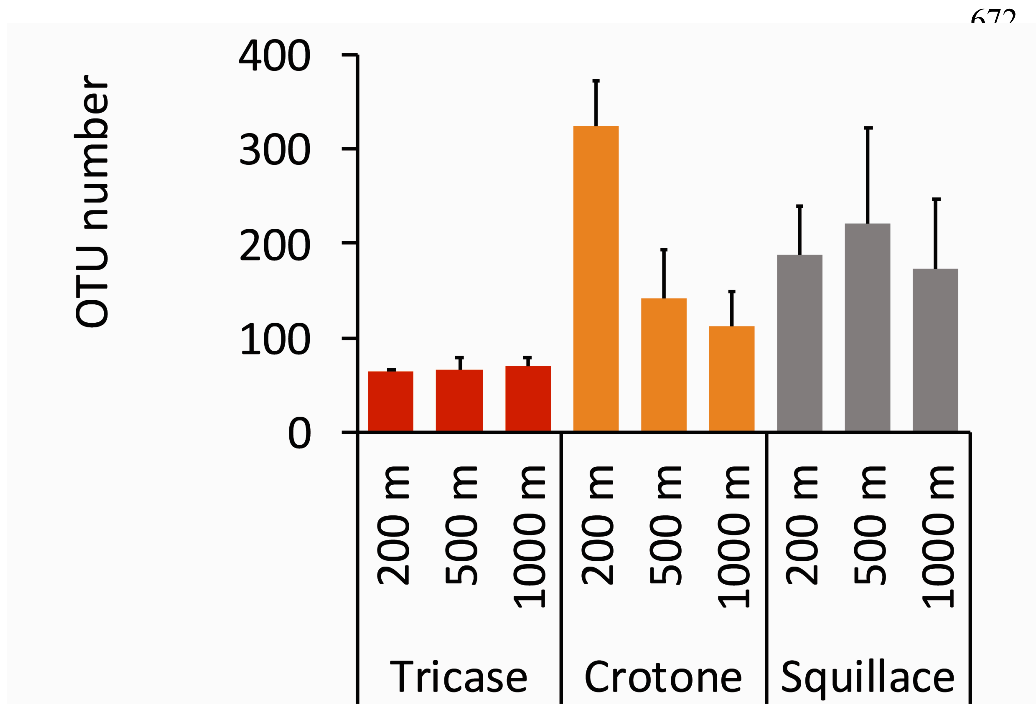

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690 Figure 4

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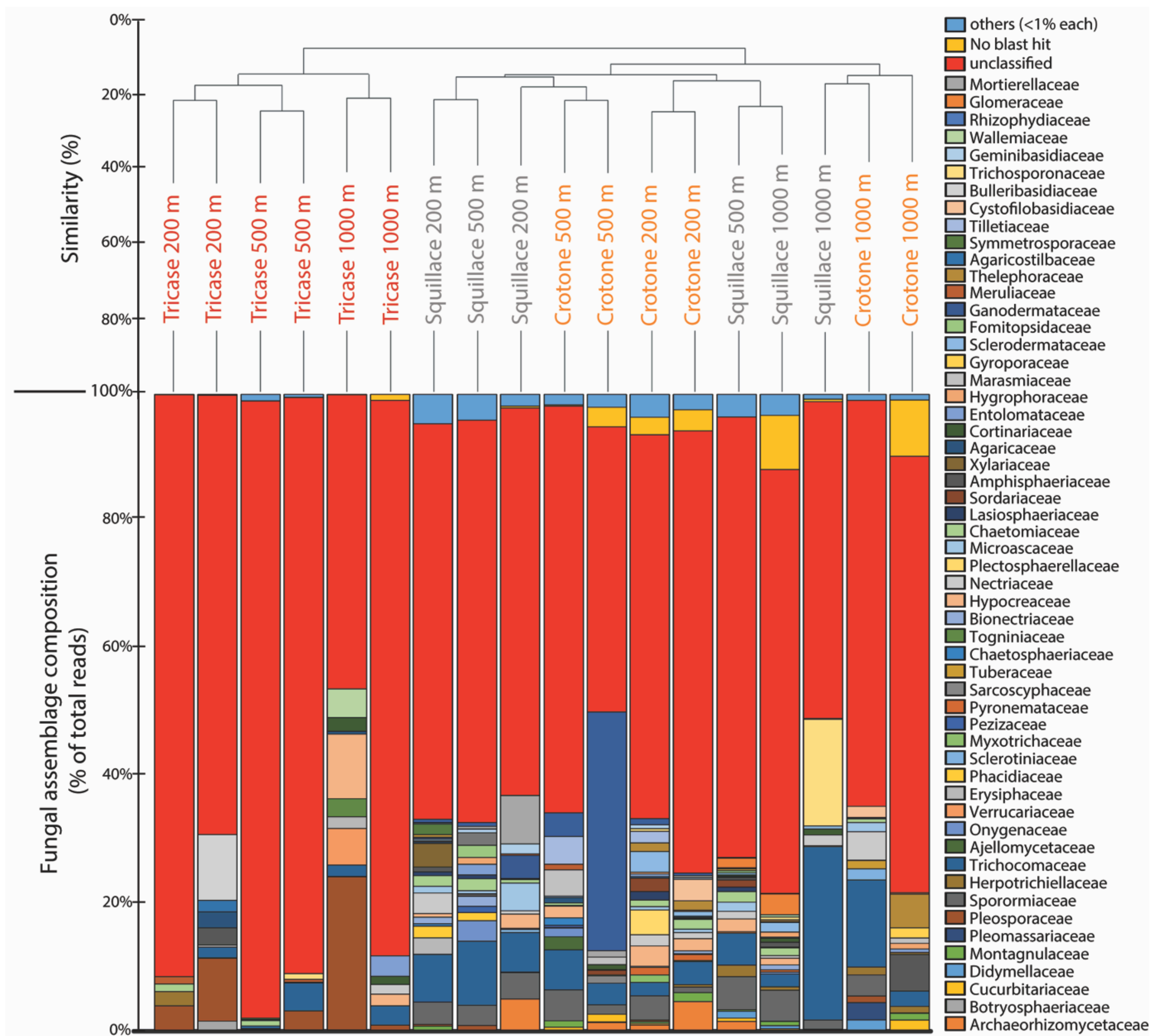

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697 Figure 5

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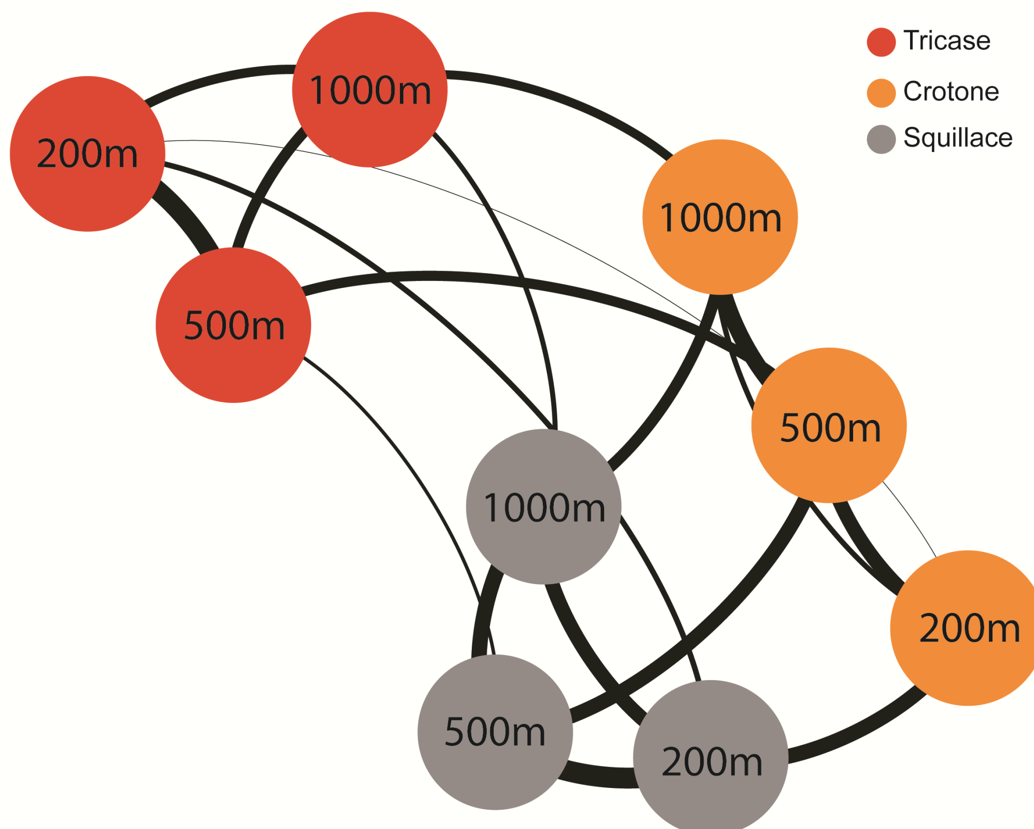

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700 Figure 6

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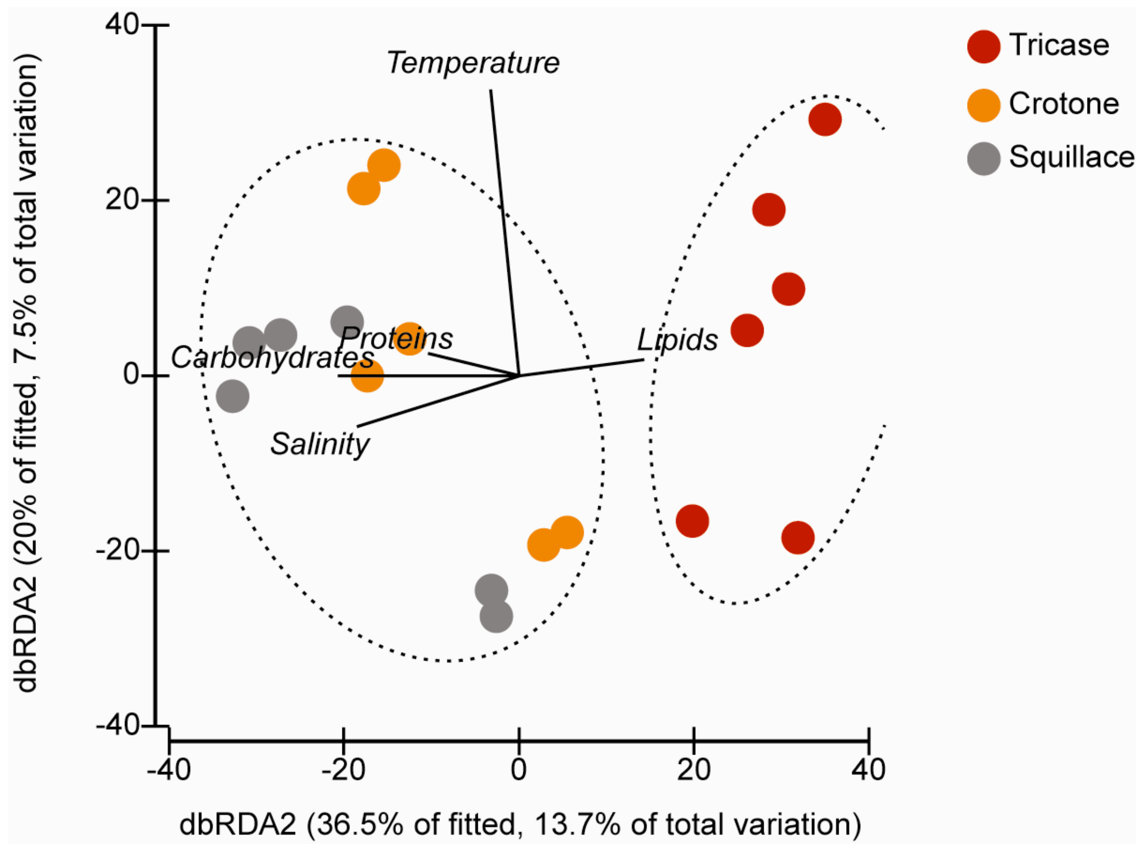

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715 Figure 7

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Supplementary materials

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

721

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

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729 Supplementary table S1-S4
730
731 Supplementary figures S1 and S2
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734 **Table S1.** ANOSIM and SIMPER outputs testing for differences and dissimilarity in
735 sediment organic matter contents between the different canyons investigated and the
736 variables responsible for the estimated differences. R is the sample statistic (global R) and P
737 the probability level (**=P<0.01; ns=P>0.05).

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	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	ns	16.82	Proteins	ns	ns
				Lipids	ns	ns
				Carbohydrates	ns	ns

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742 **Table S2.** Multivariate multiple regression analysis outputs testing the effects of organic
743 matter content (proteins, carbohydrates and lipids), temperature and salinity on fungal
744 abundance (as 18S rDNA copies) and biomass. Pseudo-F and P values (*=P<0.05; **=P
745 <0.01; ***=P <0.001; ns=P >0.05) are reported as well as the cumulative variance explained
746 by the 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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749 **Table S3.** Output of SIMPER analysis showing the dissimilarity (turnover diversity) of
750 fungal 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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753 **Table S4.** Percentage of unique and shared OTUs between replicates of the same site, within
754 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**	**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**

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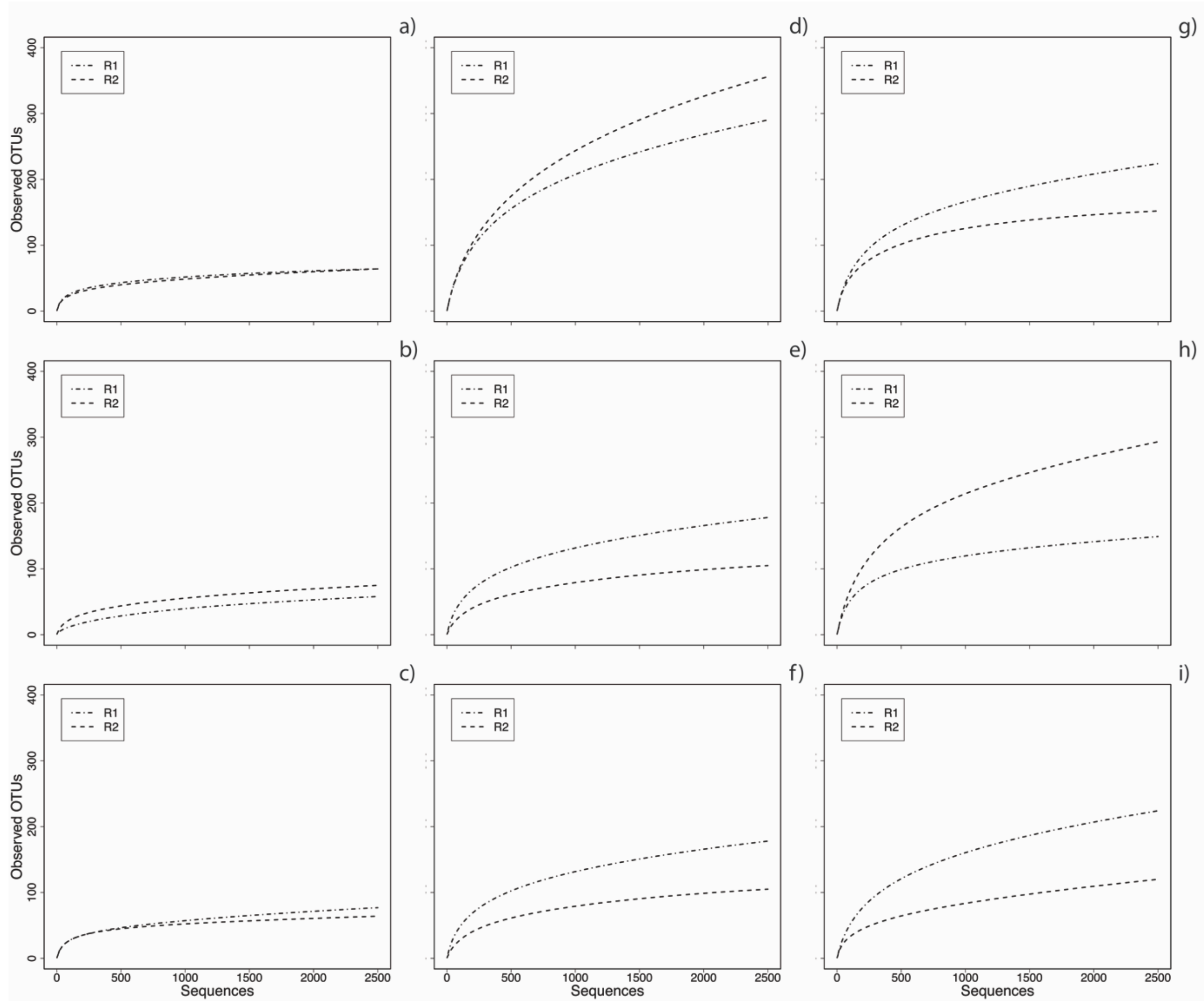
Figure S1. Rarefaction curves calculated for each of the two independent replicates (dashed lines, 2500 sequences each) analysed in all benthic deep-sea sites of the canyons investigated.



772 **Figure S2.** Relationship between benthic fungal abundance (as 18S rDNA copies) and

773 biomass in the sediments of the three canyons

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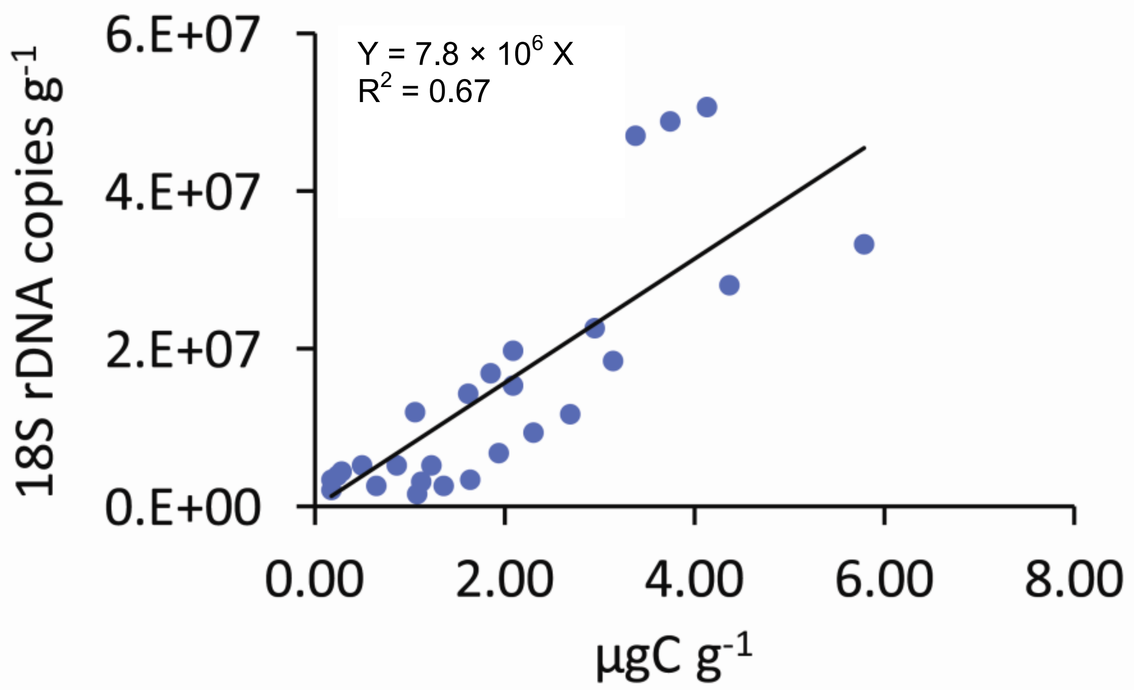

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