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(Article begins on next page)

1 **Patterns in microbiome composition differ with ocean acidification in anatomic**
2 **compartments of the Mediterranean coral *Astroides calycularis* living at CO₂ vents**

3
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21
22 **Running title:** Coral microbiome at Mediterranean CO₂ vents

26 **ABSTRACT**

27 Coral microbiomes, the complex microbial communities associated with the different anatomic
28 compartments of the coral, provide important functions for the host's survival, such as nutrient
29 cycling at the host's surface, prevention of pathogens colonization, and promotion of nutrient
30 uptake. Microbiomes are generally referred to as plastic entities, able to adapt their composition and
31 functionality in response to environmental change, with a possible impact on coral acclimatization
32 to phenomena related to climate change, such as ocean acidification. Ocean sites characterized by
33 natural gradients of $p\text{CO}_2$ provide models for investigating the ability of marine organisms to
34 acclimatize to decreasing seawater pH. Here we compared the microbiome of the temperate,
35 shallow water, non-symbiotic solitary coral *Astroides calycularis* that naturally lives at a volcanic
36 CO_2 vent in Ischia Island (Naples, Italy), with that of corals living in non-acidified sites at the same
37 island. Bacterial DNA associated with the different anatomic compartments (mucus, tissue and
38 skeleton) of *A. calycularis* was differentially extracted and a total of 68 samples were analyzed by
39 16S rRNA gene sequencing. In terms of phylogenetic composition, the microbiomes associated
40 with the different coral anatomic compartments were different from each other and from the
41 microbial communities of the surrounding seawater. Of all the anatomic compartments, the mucus-
42 associated microbiome differed the most between the control and acidified sites. The differences
43 detected in the microbial communities associated to the three anatomic compartments included a
44 general increase in subdominant bacterial groups, some of which are known to be involved in
45 different stages of the nitrogen cycle, such as potential nitrogen fixing bacteria and bacteria able to
46 degrade organic nitrogen. Our data therefore suggests a potential increase of nitrogen fixation and
47 recycling in *A. calycularis* living close to the CO_2 vent system.

48

49 **KEYWORDS:** microbiota, non-symbiotic coral, mucus, skeleton, tissue, Scleractinia

50

51 **INTRODUCTION**

52 Over the past hundred years, human activities have acted as drivers of global environmental change,
53 altering natural habitats and their biodiversity (Cardinale et al., 2012). Increasing atmospheric
54 carbon dioxide (CO₂) is causing the oceans to warm and acidify (Doney et al., 2009). Ocean
55 acidification (OA) actively contributes to altering the marine environment, with negative
56 consequences for the survival, growth and reproduction of its inhabitants, both at microbial and
57 multicellular levels (Kroeker et al., 2010; Gattuso et al., 2015; Yu & Chen, 2019). Atmospheric
58 CO₂ uptake by the ocean surface lowers the seawater pH and the carbonate ion concentration
59 (Caldeira & Wickett, 2003), with potential detrimental consequences for a variety of calcifying
60 organisms (e.g. mollusks and sea urchins), including corals (Hoegh-Guldberg et al., 2017). Ocean
61 acidity has increased by 25-30% (0.1 pH units) since the industrial revolution and a further increase
62 of 150–200% is projected for the end of the century, which is equivalent to a drop of 0.3 pH units
63 (Stocker et al., 2013). The Mediterranean basin will likely be one of the regions most affected by
64 climate change, making it a natural focus of interest for research (Cramer et al., 2018; Lejeune et
65 al., 2010).

66 In the blooming of the microbiome era, it has been proposed that the microbial communities
67 inhabiting all kinds of animals are involved in survival mechanisms in difficult living conditions,
68 such as extreme environments and habitats variations (Bang et al., 2018). In plants, for instance,
69 bacteria that populate the rhizosphere can increase the plant tolerance to a salt-enriched soil or other
70 abiotic environmental stresses, by influencing developmental and physiological plant processes
71 through production and exchange of bioactive molecules (Waller et al, 2005; Lugtenberg &
72 Kamilova, 2009; Mendes et al., 2011). Another example is provided by invertebrates living in the
73 intertidal zone, with daily fluctuations in light, temperature and oxygen (Mortzfeld et al., 2016).
74 Indeed, it has been observed that bacterial communities associated with corals in such conditions
75 change rapidly with tidal cycles (Sweet et al., 2017). Microbial mechanisms to facilitate holobiont

76 acclimatization to environmental changes include proportional changes in microbiome members
77 and loss or acquisition of microbes, as well as horizontal gene transfer (Bang et al., 2018).

78 Coral microbiomes include thousands of bacterial and archaeal phylotypes in species-specific
79 associations across broad geographical and temporal scales, which populate the different habitats of
80 coral anatomic compartments, such as surface mucus, tissue and skeleton (Apprill et al., 2016).

81 Microbiomes are critical to the health and survival of coral holobionts as they provide their hosts
82 with a variety of functions, such as assistance in recovering nutrients, protection from pathogens,
83 production of chemicals that drive larval settlement (McDevitt-Irwin et al., 2017; McFall-Ngai et
84 al., 2013; Morrow et al., 2012; O'Brien et al., 2019; Ritchie & Smith, 1997; Rohwer et al., 2001;
85 Rohwer et al., 2002; Rosenberg et al., 2007). Microbiomes are generally referred to as capable of
86 shifting their composition and functionality in response to environmental variables, conferring
87 plasticity to the ecological services provided to the host (Torda et al., 2017). The unprecedented rate
88 of environmental change that characterizes the Anthropocene has boosted pioneering research
89 exploring the possible role of microbiomes in the phenotypic plasticity of corals, particularly for
90 what concerns the ability to respond to rapid changes in the environment and, generally, to climate
91 change (McDevitt-Irwin et al., 2017; Ziegler et al., 2017). The plastic response of corals to a
92 warming and acidifying ocean could indeed rely on the microbiome ability to rapidly shift in
93 composition in a changing environment and provide fine-tuned functions for the host fitness (Torda
94 et al., 2017). Studies aimed at investigating the impact of OA can benefit from areas characterized
95 by natural gradients of $p\text{CO}_2$ to provide realistic insights into the ability of the marine biota to react
96 to the decrease in ocean pH (Fabricius et al., 2014; Goffredo et al., 2014; Hall-Spencer et al., 2008).

97 In fact, acting as natural laboratories, CO_2 vents incorporate a range of environmental factors, such
98 as gradients of nutrients, currents and species interactions that cannot be replicated in aquaria or
99 mesocosms (Foo et al., 2018), thus representing exceptional opportunities to study organisms that
100 naturally live in acidic habitats (Caroselli et al., 2019). Recently, various new vent systems have
101 been discovered along the coast of Ischia across depths of 3-48 m and span a variety of habitats,

102 including *Posidonia oceanica* seagrass meadows, gravel and sandy bottoms, caves, and
103 coralligenous outcrops (Gambi et al., 2019). The predominant gas is CO₂ (92-95% CO₂, without
104 hydrogen sulphide) and does not elevate temperature. There is one population of the coral *Astroides*
105 *calycularis* that naturally occurs in the semi-submersed cave affected by CO₂ venting (5 m depth).
106 *Astroides* is abundant in the vent system with 50% cover at 1-2 m depth (Teixido et al., 2016).

107 A very small number of studies have so far explored the coral microbiome along with lowering pH
108 in naturally acidified sites (Meron et al., 2012; Morrow et al., 2015; O'Brien et al., 2018), showing
109 a different microbiome response depending on the host species. For instance, at natural CO₂ seeps
110 in Papua New Guinea, the endolithic community associated with massive *Porites* spp. does not
111 change significantly between ambient and low pH sites (Marcelino et al., 2017), while large shifts
112 in tissue-associated bacterial communities were found in *Acropora millepora* and *Porites cylindrica*
113 across the same CO₂ seep (Morrow et al, 2015). Concerning the Mediterranean Sea, no changes in
114 coral bacterial communities have been detected following translocation of two symbiotic coral
115 species (i.e. *Balanophyllia europaea* and *Cladocora caespitosa*) along a natural pH gradient in the
116 Gulf of Naples, where they grew for 7 months (Meron et al., 2012). However, in these studies the
117 microbiomes of the different anatomic compartment (i.e. surface mucus, tissue and skeleton) were
118 not separated during the analysis.

119 *A. calycularis* (Pallas, 1766) is a non-symbiotic scleractinian coral commonly found in the
120 southwestern Mediterranean Sea (Casado-Amezua et al., 2013; Goffredo et al, 2011), with some
121 sparse colonies also observed on the Atlantic Coast of the Iberian Peninsula (Ocaña et al, 2015), and
122 some spreading colonies in the northeastern part of the Adriatic Sea (Casellato et al., 2007; Kruzic
123 et al., 2002). This coral covers relatively large surfaces of vertical walls, cave entrances, overhangs
124 and slopes (Zibrowius, 1995). It is characterized by a bright orange coenosarc and polyps
125 (Zibrowius, 1995) and is found in abundance from the intertidal fringe to 40 m depth (Kruzic et al.,
126 2002).

127 In order to provide some glimpses on the possible importance of coral microbiomes for
128 acclimatization to acidification, in this first study on the microbiome of a temperate, shallow water,
129 non-symbiotic solitary coral, we explored the differences in microbiome composition in mucus,
130 tissue and skeleton of *A. calycularis*, that naturally lives at a volcanic CO₂ vent along the coast of
131 Ischia Island, in comparison to corals living in non-acidified control sites with ambient pH.

132

133 MATERIALS AND METHODS

134 **Selection of target sites and sampling.** Coral colonies of *Astroides calycularis* (**Figure 1A**),
135 mucus cotton swabs from each colony, and water samples were collected at Ischia Island (Gulf of
136 Naples, Italy) (**Figure 1B; Table 1**) in June 2017 and April 2018. Colonies were sampled inside the
137 naturally acidified semi-submerged cave, Grotta del Mago (**Figure 1C**), and in two control sites
138 with ambient pH and no vent activity. Grotta del Mago consists of a main chamber of 10 m wide x
139 30 m long. The control sites were chosen based on the criterion that they hosted similar habitats and
140 depths as the CO₂ vent site and there was no venting activity: Punta Vico, PV2, another semi-
141 submerged cave (with a main chamber 10 m wide x 30 m long, 5 m maximum depth), and
142 Sant'Angelo, SA2, an overhang located on a natural arch (with an opening of 10 m wide x 10 m
143 height, 10 m maximum depth). Since the CO₂ emissions originate from the bottom of the cave (5 m
144 depth), sampling at Grotta del Mago was performed at two depths to analyze corals living under a
145 moderate (2 m depth, GM2) or intense (3 m depth, GM3) acidification condition. Coral sampling in
146 the reference areas was performed at 2 m depth. Mean pH values in the three study sites are: pH_T =
147 7.62-7.74 at 3 m (GM3) and pH_T = 7.65-7.88 at 2 m (GM2) in the vent system, and pH_T = 8.04-8.05
148 in PV2 (ambient pH site) and pH_T = 8.02 in SA2 (ambient pH site) The CO₂ vents in Grotta del
149 Mago do not elevate temperature. Hourly measurements taken by *in situ* hobo sensors from 2016 to
150 2019 at 2 m depth at the three study sites revealed seasonal fluctuations from 14.8 ± 0.2°C (mean ±
151 SD) in winter to 26.1 ± 0.3 °C in summer. Temperature differences among sites were ~ 0.1°C.
152 Nutrients (nitrite, nitrate, ammonium, phosphate, and silicate) and salinity did not change among

153 the study sites with differences $< 0.1 \mu\text{mol L}^{-1}$ for nutrients and ~ 0.1 for salinity. $\text{NO}_2(\mu\text{mol L}^{-1})$,
154 $\text{NO}_3(\mu\text{mol L}^{-1})$, $\text{NO}_x(\text{NO}_2+\text{NO}_3) (\mu\text{mol L}^{-1})$, $\text{NH}_4(\mu\text{mol L}^{-1})$, $\text{PO}_4(\mu\text{mol L}^{-1})$, $\text{SiO}_2(\mu\text{mol L}^{-1})$ were reported
155 as (mean \pm SD): 0.016 ± 0.002 , 0.124 ± 0.023 , 0.140 ± 0.024 , 0.089 ± 0.013 , 0.032 ± 0.002 , and $0.949 \pm$
156 0.068 at GM2; 0.018 ± 0.003 , 0.118 ± 0.003 , 0.137 ± 0.001 , 0.147 ± 0.021 , 0.032 ± 0.005 , and $1.175 \pm$
157 0.067 at PV1; and 0.005 ± 0.001 , 0.059 ± 0.001 , 0.064 ± 0.001 , 0.069 ± 0.013 , 0.049 ± 0.007 and $0.698 \pm$
158 0.018 , respectively. Salinity ranged from 37.3 (GM2, GM3, PV1) to 37.4 (SA) (Teixido et al.,
159 unpublished). Corals were collected by SCUBA divers using a hammer and chisel, and placed in
160 plastic bags, while mucus cotton swabs were collected on the boat immediately after coral
161 collection (Glasl et al., 2016; Sweet et al., 2011). At each site, close to the collected corals, 2 liters
162 of seawater were collected with a Niskin bottle. All samples were transported in ice to the
163 laboratory where they were frozen at -80°C .

164 **Samples processing and DNA extraction from coral mucus, tissue and skeleton.** The collected
165 samples were processed to physically separate the main components associated with the coral:
166 mucus, tissue and skeleton (Apprill et al., 2016; Rubio-Portillo et al., 2016). The cotton tip of each
167 mucus swab was transferred into a 2-ml Eppendorf tube to which 500 μl of sterile artificial seawater
168 were added. To detach mucus specimens from swabs, each sample was vortexed for 1 min and
169 sonicated for 2 min, repeating these steps twice. Cotton swabs were then discarded and the
170 suspension centrifuged at 9,000 g for 5 min at 4°C . Pellets were then stored frozen at -80°C until
171 further processing.

172 The coral tissue was separated from the carbonate skeletal matrix by mechanical fragmentation
173 (Rubio-Portillio et al., 2016). Coral specimens were transferred into an agate mortar using sterile
174 forceps and fragmented with the pestle in 10 ml of sterile artificial seawater (NaCl 450 mM, KCl 10
175 mM, CaCl_2 9 mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 30 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 16 mM, pH 7.8). Additional 20 ml of
176 artificial seawater were used to wash mortar and pestle from coral residues. The holobiont
177 homogenate was then transferred into a 250-ml Becher and incubated at room temperature for 15
178 min to allow the skeletal fragments to settle. The seawater suspension was aliquoted into two 50-ml

179 Corex tubes and centrifuged at 9,300 g for 15 min at 4°C to pellet the coral tissue fraction.
180 Supernatants were then discarded and pellets re-suspended in 1.5 ml of artificial seawater, vortexed
181 briefly and transferred into a 2-ml Eppendorf tube. Following a further centrifugation step at 9,300
182 g for 15 min at 4°C, the supernatant was discarded and the pellet stored frozen at -80°C until
183 processing. Coral skeleton fragments were washed three times using 10 ml of sterile artificial
184 seawater, with the last washing volume being discarded after 10 min of fragment settling. Skeletal
185 fragments were then transferred into a 2-ml Eppendorf tube and stored frozen at -80°C until
186 processing.

187 Bacterial DNA was extracted from each sample (mucus, skeleton, and tissue) using the DNeasy
188 PowerBiofilm kit (QIAGEN, Hilden, Germany) as previously described (Weber et al., 2017).
189 Mucus and tissue pellets were resuspended in 350 µl of MBL solution and transferred into
190 PowerBiofilm Bead Tubes, while 100 mg of skeleton sample were directly transferred into the Bead
191 Tubes. DNA extraction was performed according to the manufacturer's protocol, using 200 µl of
192 IRS solution for mucus and tissue samples. The FastPrep instrument (MP Biomedicals, Santa Ana,
193 CA) was used for the bead-beating step, by homogenizing samples with three treatments at 5.5
194 movements s⁻¹ for 1 min, and incubating samples on ice between treatments. The elution step was
195 repeated twice and the final DNA concentration determined by using NanoDrop ND-1000 (Thermo
196 Fisher Scientific, Wilmington, DE) and stored at -20°C until library preparation.

197 **DNA extraction from seawater samples.** Each seawater sample (2 L) was aseptically filtered
198 using 50-mm diameter, 0.45-µm pore-size sterile Durapore membrane filters (Millipore, Boston,
199 MA) via vacuum filtration. Each membrane filter was folded and transferred directly into a
200 PowerBiofilm Bead Tube (QIAGEN) using sterile forceps (Campbell et al., 2015; Staley et al.,
201 2017). Total bacterial DNA was then extracted using the DNeasy PowerBiofilm kit (QIAGEN)
202 according to the manufacturer's instructions. The FastPrep instrument (MP Biomedicals) was used
203 for the bead-beating step, by homogenizing samples with one treatment at 5.5 movements s⁻¹ for 1
204 min. All samples were stored at -20°C until further processing.

205 **16S rRNA gene PCR amplification and sequencing.** Following isolation of microbial DNA from
206 coral holobionts and seawater samples, the V3-V4 hypervariable region of the 16S rRNA gene was
207 PCR-amplified using the 341F and 785R primers with Illumina overhang adapter sequences (Biagi
208 et al., 2018). Amplicon purification was performed by using AMPure XP magnetic beads (Beckman
209 Coulter, Brea, CA). For indexed library preparation, the Nextera XT DNA Library Prep Kit
210 (Illumina, San Diego, CA) was used. A further magnetic bead-based purification step was
211 performed and libraries were quantified using the Qubit 3.0 fluorimeter (Invitrogen), then pooled at
212 4 nM. The library pool was denatured with NaOH 0.2 N and diluted to 6 pM. Sequencing was
213 performed on Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the
214 manufacturer's instructions (Illumina).

215 **Bioinformatics and statistics.** Raw sequences were processed using a pipeline combining
216 PANDAseq and QIIME 2 (Bolyen et al., 2019; <https://qiime2.org>). Sequencing reads were
217 deposited in SRA-NCBI (project number PRJNA601621, coral samples from SRR10902270 to
218 SRR10902270, water samples from SRR10902443 to SRR10902458). After chimera sequences
219 removal, high-quality reads were filtered and binned into high-resolution operational taxonomic
220 units (OTUs) according to the taxonomic threshold of 99% through an open-reference strategy
221 performed with dada2 (Callahan et al., 2016). Taxonomy was assigned using the vsearch classifier
222 (Rognes et al., 2016) and SILVA database as a reference (Quast et al., 2013). Evenness of the
223 microbial community was measured using the Shannon diversity index, whereas phylogenetic
224 diversity (measured as Faith PD index) and the number of observed OTUs were used to estimate
225 community richness. Statistics was performed using R Studio version 1.0.136 running on R 3.1.3
226 (<https://www.r-project.org/>), implemented with the libraries vegan, made4, PMCMR, vcd, ggtern,
227 ggplot2, and IndicSpecies. Beta diversity (i.e. how samples vary against each other in terms of
228 bacterial species composition) was estimated by computing weighted UniFrac distances and
229 visualized by Principal Coordinates Analysis (PCoA). The significance of separation among groups
230 of samples was tested by permutational multivariate analysis of variance using the function

231 “Adonis” of the vegan package. Bacterial phylogenetic groups (genus, family, class, phylum)
232 showing a minimum relative abundance of 0.5% in at least two of the considered samples were kept
233 for further analysis. P values were corrected for multiple comparisons using the Benjamini-
234 Hochberg method. A false discovery rate of 5% was used. Bacterial families enriched in mucus,
235 skeleton, or coral tissue were explored based on genus-level relative abundance values, and ternary
236 plots were chosen as graphical representation, as inspired by D’Amico et al. (2018). The statistical
237 package IndicSpecies was used to identify bacterial genera whose abundance was significantly
238 associated with acidification conditions (Ziegler et al., 2017).

239

240 **RESULTS**

241 The 16S rRNA amplicons obtained from a total of 68 DNA samples (20 samples from coral tissue,
242 20 from coral skeleton, 12 from coral mucus, and 16 from seawater) (**Table 1**) were sequenced,
243 resulting in 4,718,656 high-quality sequences, ranging between a minimum of 13,154 and a
244 maximum of 710,890 sequences per sample, with an average value of 69,392 sequences per sample.
245 Reads were clustered into 14,453 operational taxonomic units (OTUs) based on 99% similarity.

246 The composition of the microbiota isolated from seawater was distinct from those found in the coral
247 anatomic compartments (Adonis test, $P = 0.001$), with the coral mucus samples being the most
248 similar to the water ones, with respect to coral tissue and skeleton samples in the Principal
249 Coordinates Analysis (PCoA) based on weighted UniFrac distances (**Figure 2a**). Interestingly,
250 tissue and skeleton microbiomes showed the highest inter-individual diversity, indicating greater
251 individual specificity in microbiota composition. The microbial communities found in the coral
252 surface mucus were separated according to the sampling site (**Figure 2c**) ($P = 0.01$).

253 The most represented bacterial groups in the three coral compartments and water samples, at class
254 level, were Alphaproteobacteria and Gammaproteobacteria, representing on average 39% and 23%
255 in water, 34% and 19% in mucus, 20% and 23% in tissue, and 18% and 21% in skeleton,
256 respectively (**Supplementary Figure S1**). Water and mucus samples also showed a considerable

257 average relative abundance of Flavobacteria (13% and 9%, respectively), whereas skeleton and
258 tissue samples were averagely more enriched in Acidimicrobiia (7% and 6%, respectively) and
259 members of the Chloroflexi phylum, such as Caldilineae and SAR202 clade (**Supplementary**
260 **Figure S1**). The different coral anatomic compartments did not show significantly different
261 community alpha-diversity, as calculated by the Shannon diversity index, Faith PD index, and
262 number of observed OTUs (**Supplementary Figure S2**). However, the microbial communities
263 found in the skeleton samples (mean±SD: Shannon index, 7.1±0.7; Faith PD index, 33.7±13.2;
264 observed OTUs, 308±161) tended to show higher average biodiversity than both mucus (Shannon
265 index, 6.5±1.4; Faith PD index, 29.8±9.3; observed OTUs, 267±100) and tissue samples (Shannon
266 index, 6.3±0.9; Faith PD index, 28.6±13.7; observed OTUs, 227±141). Unlike the mucus and tissue
267 compartments, the skeleton microbiome also showed significantly higher alpha-diversity than the
268 microbiome found in the surrounding seawater (Shannon index, 6.0±0.4; Faith PD index, 17.8±6.8;
269 observed OTUs, 145±49; P = 0.001, P = 0.0004, P = 0.001, respectively) (**Supplementary Figure**
270 **S2**). Within each anatomic compartment, no differences in bacterial alpha-diversity were detected
271 among sampling sites.

272 In an attempt to focus on specific acidification-related microbiome differences, we considered the
273 samples taken at the two ambient pH sites (Punta Vico, PV2, and Sant'Angelo, SA2) as a single
274 control group, representative of non-acidified conditions, for subsequent analyses. The visualization
275 of the coral microbiome structure by means of a ternary plot (**Figure 3**) allowed highlighting the
276 peculiarities of each anatomic compartment. Interestingly, each of the coral microbiomes shows
277 peculiarities related to the living sites (comparison between **Figures 3a, 3b and 3c**). In particular,
278 the ternary plots seem to highlight for all three compartments a loss in terms of dominant (i.e. most
279 abundant) bacterial families, in favor of subdominant groups. This was particularly evident in the
280 case of the mucus ecosystem, where subdominant taxa belonging to the Bacteroidetes,
281 Verrucomicrobia, and Cyanobacteria phyla are plotted closer to the mucus (MS) vertex and increase
282 in average abundance (i.e. width of circles) in the samples taken at the highly acidified site (**Figure**

283 **3c)**. Similarly, subdominant Planctomycetaceae (phylum Planctomycetes, corresponding to light
284 green circles), which were more associated with skeleton and tissue compartments, tended to be
285 elevated in acidified sites (average relative abundance in skeleton and tissue, 5% and 1.1% in
286 control samples, 6.6% and 4.8% in samples from GM2, 8.5% and 3.7% in samples from GM3)
287 (**Supplementary Table S1**). In addition, Flavobacteriaceae (phylum Bacteroidetes), which was one
288 of the most abundant families associated with the mucus compartment, showed higher relative
289 abundance at the acidified sites (average relative abundance, 5.1% in controls, 8.3% and 8.8% in
290 samples from GM2 and GM3, respectively), and the corresponding blue circles are plotted closer to
291 the MS vertex with decreasing pH (**Figure 3, Supplementary Table S1**).

292 Our IndicSpecies analysis revealed genus-level groups of bacteria that were strongly associated
293 with samples taken in acidified conditions. In the case of the mucus compartment, few genera were
294 strongly associated with acidified sites, meaning both moderate and intense acidification (GM2 and
295 GM3 together): *Luteolibacter* (family Verrucomicrobiaceae) ($P = 0.002$), uncultured members of
296 the Sva0996 marine group ($P = 0.01$), and members of the OM27 clade of the family
297 Bdellovibrionaceae ($P = 0.02$) (**Figure 4a-4c**). Interestingly, a bacterial OTU assigned to the
298 species *Luteolibacter algae* (strain A5J-41-2, isolated by Yoon et al., 2008, according to the
299 BLAST analysis), was the only OTU-level group found to be strongly associated with acidified
300 sites ($P = 0.03$) when the package IndicSpecies was used on OTU-level abundance. Additionally,
301 intense acidification (site GM3) was associated with higher abundances of members of the genus
302 *Legionella* ($P = 0.007$) (**Figure 4d**).

303 In the coral tissue microbiome, genera of the family Planctomycetaceae were strongly associated
304 with acidified sites (both GM2 and GM3); in particular, the genera *Planctomyces* ($P = 0.01$),
305 *Rhodopirellula* ($P = 0.009$), and members of the Pir4 lineage ($P = 0.03$) were detected as indicators
306 of corals grown in acidified sites (**Figure 4e-g**). Furthermore, the samples taken at the heavily
307 acidified site were strongly associated with higher abundances of NS5 marine group (family
308 Flavobacteriaceae) ($P = 0.01$) (**Figure 4h**).

309 In the coral skeleton compartment, an increased abundance of members of the genus *Nitrospina*,
310 family Nitrospinaceae, was associated with acidified sites (both GM2 and GM3) ($P = 0.002$)
311 (**Figure 4i**), whereas significantly higher abundances of the genus *Synechococcus* were associated
312 with the heavily acidified site (GM3) ($P = 0.002$) (**Figure 4k**).

313 In order to test the involvement of the seawater microbial component in the detected associations
314 between acidification and coral microbiomes, the same IndicSpecies analysis was applied to the
315 collected seawater samples, and no association was found.

316

317 **DISCUSSION**

318 Corals have shown to be able to acclimatize, at least to some extent, to the warming and acidifying
319 oceans, thanks to transgenerational plasticity processes that may be facilitated by the complex and
320 species-specific microbiomes associated with them (Torda et al., 2017). The study of how
321 microbiomes can contribute to the acclimatization to climate changes fits in between marine
322 biology and molecular microbiology, and it is a research field still in its infancy (van Oppen &
323 Blackall, 2019); nonetheless, indications that distinct coral species show differences in microbiome
324 composition under acidified conditions have already been provided by the available literature
325 (Meron et al., 2012; Morrow et al., 2015; McDevitt-Irwin et al., 2017; Ziegler et al., 2017; Torda et
326 al., 2017; O'Brien et al., 2018). In our study, we exploited the vent system present at the Grotta del
327 Mago cave, along the coast of Ischia island, Italy, to attempt to dissect the effect of low pH
328 conditions on the microbiomes colonizing the non-symbiotic coral *A. calycularis*.

329 The mucus bacterial ecosystem of *A. calycularis* was the most clearly affected by coral growth in
330 acidified sites, with respect to both tissue and skeletal microbiomes. This might be related to the
331 fact that the surface mucus is a more exposed niche, at the connection with the surrounding
332 environment, and that the resident microbiome, with its plasticity, can dynamically respond to
333 environmental changes, possibly playing a key role in coral survival and health upon environmental
334 disturbances (Glasl et al., 2016). Indeed, some of the literature that does not report changes in the

335 microbiomes of different coral species in response to decreasing pH is based on analyses performed
336 without distinguishing between mucus and tissue environment (Meron et al., 2012; O'Brien et al.,
337 2018). Few subdominant bacterial groups present in coral mucus were strongly associated with
338 corals living in the acidified sites, some of which might be involved in nitrogen cycling at the
339 interface between the coral and the water column. In particular, members of the OM27 clade, of the
340 family Bdellovibrionaceae, are an uncultivated group of microorganisms with the highest
341 percentage of OTUs related to protein assimilation, and known to be part of the particle-associated
342 community actively cycling dissolved organic nitrogen (Orsi et al., 2016). Members of the Sva0996
343 clade, which are a still enigmatic and uncultured clade of marine Actinobacteria, can utilize
344 dissolved protein (Orsi et al., 2016), but their functional and ecological aspects remain poorly
345 understood. A possible, even if only speculative, explanation of the increase in these bacterial
346 groups capable of utilizing organic nitrogen might reside in differences in the composition of the
347 mucus itself. In some reef-building corals, stressed individuals produce mucus with higher protein
348 content (Wright et al., 2019), which might support the proliferation of bacteria with higher ability to
349 metabolize these nutrients.

350 Mucus microbiota modifications in corals growing at acidified sites included augmented relative
351 abundance of the Verrucomicrobiaceae genus *Luteolibacter*, and in particular, of an OTU assigned
352 to the species *Luteolibacter algae*, studied because of its ability to degrade fucoidan produced by
353 brown seaweed (Ohshiro et al., 2012; Nagao et al., 2018). Bacteria of the family
354 Verrucomicrobiaceae are known members of the coral surface mucus, and have been shown to
355 increase in suboptimal conditions such as warming (Lee et al., 2015) or aged surface mucus (Glasl
356 et al., 2016). Finally, bacteria belonging to the genus *Legionella*, detected in higher abundance in
357 the mucus environment of *A. calycularis* living in the intensely acidified site (GM3) as compared to
358 non-acidified sites (PV2, SA2), have been found in the skeletons of *C. caespitosa* and *B. europaea*
359 (Meron et al., 2012) but also detected in diseased colonies of the gorgonian coral *Eunicella*
360 *verrucosa* (Ransome et al., 2014). Members of the order Legionellales comprise intracellular

361 parasites mostly of protists, thus their direct association with the coral host has yet to be confirmed
362 (Kellogg et al., 2016).

363 Even if the endolithic community was less affected by acidification, bacterial groups potentially
364 involved in nitrogen cycling were also detected in the skeleton as associated with acidification,
365 including the genera *Nitrospina*, which encompasses chemolithoautotrophic nitrite-oxidizing
366 bacteria (Ngugi et al., 2016), and *Synechococcus*, a diazotrophic group of Cyanobacteria, known to
367 grow faster under acidified conditions when in association with sponges (Bragg et al., 2010;
368 Morrow et al., 2015). *Synechococcus* has also been proposed to be involved in a symbiotic
369 relationship for nitrogen fixation in another non-symbiotic coral, *Lophelia pertusa* (Neulinger et al.,
370 2008).

371 Lastly, the microbial communities associated with coral tissue highlighted differences associated to
372 corals living at the acidified sites that involves genera belonging to Planctomycetes, one of the
373 dominant phyla of the tissue ecosystem, which is known to thrive in acidified conditions when
374 associated with algae or sediments (Huggett et al., 2018; Roth-Schulze et al., 2018; Tait et al.,
375 2013). Also, Planctomycetes increased with acidification in the gut ecosystem of the seaweed-
376 grazer crustacean, *Synisoma nadejda* (Aires et al., 2018), where they may increase the degradation
377 capacity of algal polymers, because of their ability to decompose algal cell wall sugars, namely L-
378 fucose and L-rhamnose (Lage & Bondoso, 2014). Since corals feed on zooplankton, which includes
379 crustaceans like *S. nadejda*, this observation offers a curious and interesting perspective on the
380 microbiome circulation along the trophic chain, even if confirmation of this coincidence still has to
381 be provided. More generally, Planctomycetes are important inhabitants of marine organisms and
382 macro-aggregates, intervening into the global nitrogen cycle by providing diazotrophic nitrogen
383 fixation (DeLong et al., 1993; Fuerst & Sagulenko, 2011; Delmont et al., 2018). The nitrogen cycle
384 has been thoroughly studied in zooxanthellate corals, always in relation to the importance of
385 nitrogen availability in the acquisition and retention of symbiotic algae, as well as to support
386 photosynthesis. Nitrogen-cycling bacteria appear to be essential for maintaining this homeostasis

387 (Radecker et al., 2015) and they might be important for zooxanthellate coral resilience to OA, to
388 sustain the higher photosynthetic rate expected in hypercapnic conditions (Marcelino et al., 2017;
389 Radecker et al., 2015; Santos et al., 2014). Conversely, the role of nitrogen-cycling bacteria in non-
390 symbiotic corals is much less explored: the increase in the relative abundance of bacteria with
391 nitrogen-fixing capability is reported in our observational study for the first time in a non-symbiotic
392 scleractinian coral living in acidified conditions.

393 Taken together, our data on the *A. calycularis* microbiome highlights changes in the tissue and
394 skeleton of corals growing in low pH sites. The observed variations mainly involved an increase in
395 bacterial species that may be active in the nitrogen cycle and, in particular, in nitrogen fixation (i.e.
396 *Synechococcus* and genera of the phylum Planctomycetes) and nitrification, such as the nitrite-
397 oxidizing *Nitrospina*. Diazotrophic nitrogen fixation is known to considerably increase with
398 acidification in open sea water (Wannicke et al., 2018), as well as in shallow coral reefs, where
399 ocean acidification is associated with a general increase in the amount of nitrogen fixed (Cardini et
400 al., 2014). Indeed, it has been proposed that nitrogen fixation may represent the primary source of
401 new nitrogen for the benthic environment in oligotrophic acidified seawater (Wannicke et al.,
402 2018). Thus, the changes we observed could be part of the acclimatization of the coral holobiont to
403 acidified conditions, as they could supply the coral with an augmented source of ammonia and
404 nitrates to be used for nutrition. On the other hand, the microbiome in the surface mucus of corals
405 from acidified sites seems to respond by increasing the abundance of bacteria with a propensity to
406 recycle organic nitrogen (i.e. Bdellovibrionaceae and OM27 clade). Even if the data made available
407 by our study do not allow any speculation about the possible role of these bacteria in the
408 acclimatization of non-symbiotic corals, the increase in mucus microbes capable of metabolizing
409 organic nitrogen compounds could be linked to the augmented nitrogen fixation induced in tissue
410 and skeleton bacteria by coral growth in acidified sites, as part of a mechanism of general increase
411 in nitrogen circulation that can characterize the acidified ocean. Aquarium experiments, involving
412 metabolomics and metatranscriptomic approaches, are needed to better understand if the enrichment

413 in bacteria involved in nitrogen fixation and recycling could favour host acclimatization to acidified
414 conditions.

415 More generally, our observations regarding microbiome changes in corals living in acidified sites
416 mirror the microbiome plasticity observed for other Cnidarian species and repeatedly proposed as
417 involved in coral acclimatization and stress tolerance (McDevitt-Irwin et al., 2017; Torda et al.,
418 2017; Ziegler et al., 2017; Bang et al., 2018). Such an observation cannot be generalized to different
419 species, since it has been demonstrated that the microbiomes of some corals, such as *Pocillopora*
420 *vericosa*, are unable to rapidly restructure their composition (Pogoreutz et al., 2018). Even if
421 causation has yet to be demonstrated, the degree of coral microbiome compositional plasticity under
422 stress conditions might be a component of the coral resilience or susceptibility to environmental
423 stress, confirming the relevance of this microbiome feature for the survival of coral species affected
424 by environmental changes (Bang et al., 2018; Grottoli et al., 2018).

425 In our study, ambient and low pH sites were carefully chosen to minimize the effect of site
426 differences. The locations shared similar habitats and depths (semi-submersed cave for Grotta del
427 Mago and the control site Punta Vico, and an overhang located on a natural arch for the second
428 control site, Sant'Angelo). Furthermore, the measured environmental conditions of the study sites
429 (i.e. temperature, salinity, and nutrients) were consistent among sites, only pH and the associated
430 carbonate system parameters being affected by the presence of the CO₂ vents (Teixido et al.,
431 unpublished), thereby limiting the possible presence of other confounding factors.

432 Besides observing microbiome variations associated with coral colonies located in acidified sites,
433 we also demonstrated that coral microbial communities associated with the different anatomic
434 compartments are distinct in *A. calycularis* and very different from those found in the surrounding
435 seawater. Indeed, we provide the first study on a non-zooxanthellate coral in which the
436 microbiomes associated with the three anatomic compartments (i.e. surface mucus, tissue and
437 skeleton) are separately characterized. The mucus and water microbiome shared a few phylogenetic
438 features, with respect to skeleton and tissue samples (such as the absence or very low abundance of

439 Acidobacteria, Spirochaetes, and SBR1093), hinting at a certain degree of exchange between the
440 two ecosystems. Yet, the mucus community remained distinct and, most importantly, more
441 biodiverse than the community found in the water column. Indeed, the seawater microbiome
442 showed the lowest alpha-diversity, with respect to all three coral compartments, especially the
443 endolithic community (i.e. skeleton samples), confirming the available literature (Carlos et al.,
444 2013; Hernandez-Zulueta et al., 2016; Kemp et al., 2015; Sunagawa et al., 2010; Trousselier et al.,
445 2017). Taken together, our observations confirm that *A. calycularis* microbiomes are not the result
446 of neutral colonization by bacteria from seawater, but are compartment-specific and selected by the
447 coral itself (Aprill et al., 2016; Huggett & Aprill, 2019; Kemp et al., 2015; Leite et al., 2018;
448 Sharp & Ritchie, 2012).

449

450 **CONCLUSIONS**

451 The study presented here addresses a very up-to-date theme in the field of biology of environmental
452 changes, focusing on the effect that water acidification can have on a species of shallow-water,
453 temperate, non-symbiotic coral. Corals have shown some resilience to environmental variations
454 related to climate change, and it has been proposed that coral microbiomes might be involved in
455 such resilience (McDevitt-Irwin et al., 2017; Ziegler et al., 2017; Torda et al., 2017; van Oppen &
456 Blackall, 2019). The compositional differences of microbiomes in corals from acidified sites
457 concerned bacterial groups involved in different stages of the nitrogen cycle in the benthic
458 environment. The tissue and skeleton of corals from acidified sites were enriched in potential
459 nitrogen-fixing bacteria, whereas in the mucus more bacteria with higher capability to degrade
460 organic nitrogen were reported. Our data seems to hint at a general increase of nitrogen fixation and
461 cycling at the acidified sites, which, if confirmed by aquarium or coral transplantation experiments
462 and metabolomics observations, would be consistent with what is expected based on previous
463 nitrogen cycle observations in seawater and shallow coral reefs.

464

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473

474 **AUTHOR CONTRIBUTION**

475 Conceptualization: EB, EC, SG, MC; Data curation: EB, MS, SR; Formal analysis: EB, SR;
476 Funding acquisition: EC, SG, EB; Investigation: EB, EC, NT, MCG, MB, MP; Methodology: EB,
477 EC, MB; Resources: MC, PB, EC, SG; Writing - original draft: EB, MB, EC, MP; Writing - review
478 & editing: MC, NT, ST, SG.

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873

874

875 **FIGURE LEGENDS**

876

877 **Figure 1. Model organism and sampling location.** (a) Polyps of *Astroides calycularis*. (b) Map
878 of Italy; the yellow square indicates Ischia Island. (c) Map of Ischia Island; the sampling sites
879 (Punta Vico, Sant'Angelo and Grotta del Mago) are indicated by white stars.

880

881 **Figure 2. Diversity of the microbiome of the coral *A. calycularis* in the different anatomic**
882 **compartments and sampling sites.** (a) Principal Coordinates Analysis (PCoA) based on weighted
883 UniFrac distances between the OTU profiles of water (blue diamonds), coral mucus (yellow
884 squares), tissue (light red circles), and skeleton (grey triangles) samples. (b-e) PCoA based on
885 weighted UniFrac distances between the OTU profiles of water (b), coral mucus (c), tissue (d), and
886 skeleton (e) samples. Samples are colored by sampling site: Punta Vico (light green), Sant'Angelo
887 (orange), Grotta del Mago at 2 m (light blue) and at 3 m (dark blue). First and second coordination
888 axes are reported in each plot; the percentages of variation in the datasets explained by each axis are
889 reported.

890

891 **Figure 3. Enrichment of bacterial families in the different anatomic compartments of the**
892 **coral *A. calycularis* and sampling sites.** Ternary plots of bacterial families detected in the dataset
893 with relative abundance >0.5% in at least two samples, in samples taken at non-acidified control
894 sites (Punta Vico and Sant'Angelo, at 2 m depth) (a), a moderately acidified site (Grotta del Mago
895 at 2 m depth) (b), and an intensely acidified site (Grotta del Mago at 3 m depth) (c). The enrichment
896 in the three anatomic compartments is plotted with the mucus (MS), tissue (T) and skeleton (S)
897 niches at the vertexes of the triangles. Each circle represents one bacterial family, and the size is
898 proportional to the weighted relative abundance. Bacterial families are colored according to the
899 phylum (or class, in the case of Proteobacteria) to which they belong (see the color legend at the
900 bottom). The list of bacterial families used for the plots with the average relative abundances in

901 each condition is reported in **Supplementary Table S1**. Within Alphaproteobacteria (red), the
902 largest circle represents the family Rhodobacteraceae. In Gammaproteobacteria (dark red), the two
903 largest circles are classified as Gammaproteobacteria_Other and E01-9C-26 marine group. Within
904 Bacteroidetes (blue), Planctomycetes (light green), Chloroflexi (dark turquoise) and Actinobacteria
905 (yellow), the largest circles identify the families Flavobacteriaceae, Phycisphaeraceae,
906 Caldilineaceae, and Sva0996 marine group, respectively.

907

908 **Figure 4. Relative abundances of bacterial genera in coral mucus (a-d), tissue (e-h), and**
909 **skeleton (i-k) showing strong association with acidified conditions, as detected by IndicSpecies**
910 **analysis.** Box and whisker distributions of genus-level relative abundances in the samples taken at
911 the two acidified sites (Grotta del Mago at 2 m and 3 m depth, GM2 and GM3) and in the control
912 samples collected at both non-acidified sites (Punta Vico and Sant'Angelo, at 2 m depth). Shades
913 of yellow, light red, and grey are used to distinguish between coral mucus, tissue, and skeleton,
914 respectively.

Table 1. Summary of sample collection activities and features of the sampling sites.

Year	Site	Depth (m)	pH	<i>A. calycularis</i> colonies	Mucus swabs	Seawater samples
2017	Punta Vico	2	Ambient	2	\	2
	Sant'Angelo	2	Ambient	2	\	2
	Grotta del Mago	2	Moderate acidification	2	\	2
	Grotta del Mago	3	Intense acidification	2	\	2
2018	Punta Vico	2	Ambient	3	3	2
	Sant'Angelo	2	Ambient	3	3	2
	Grotta Mago	2	Moderate acidification	3	3	2
	Grotta Mago	3	Intense acidification	3	3	2
Total				20	12	16

Figure 1
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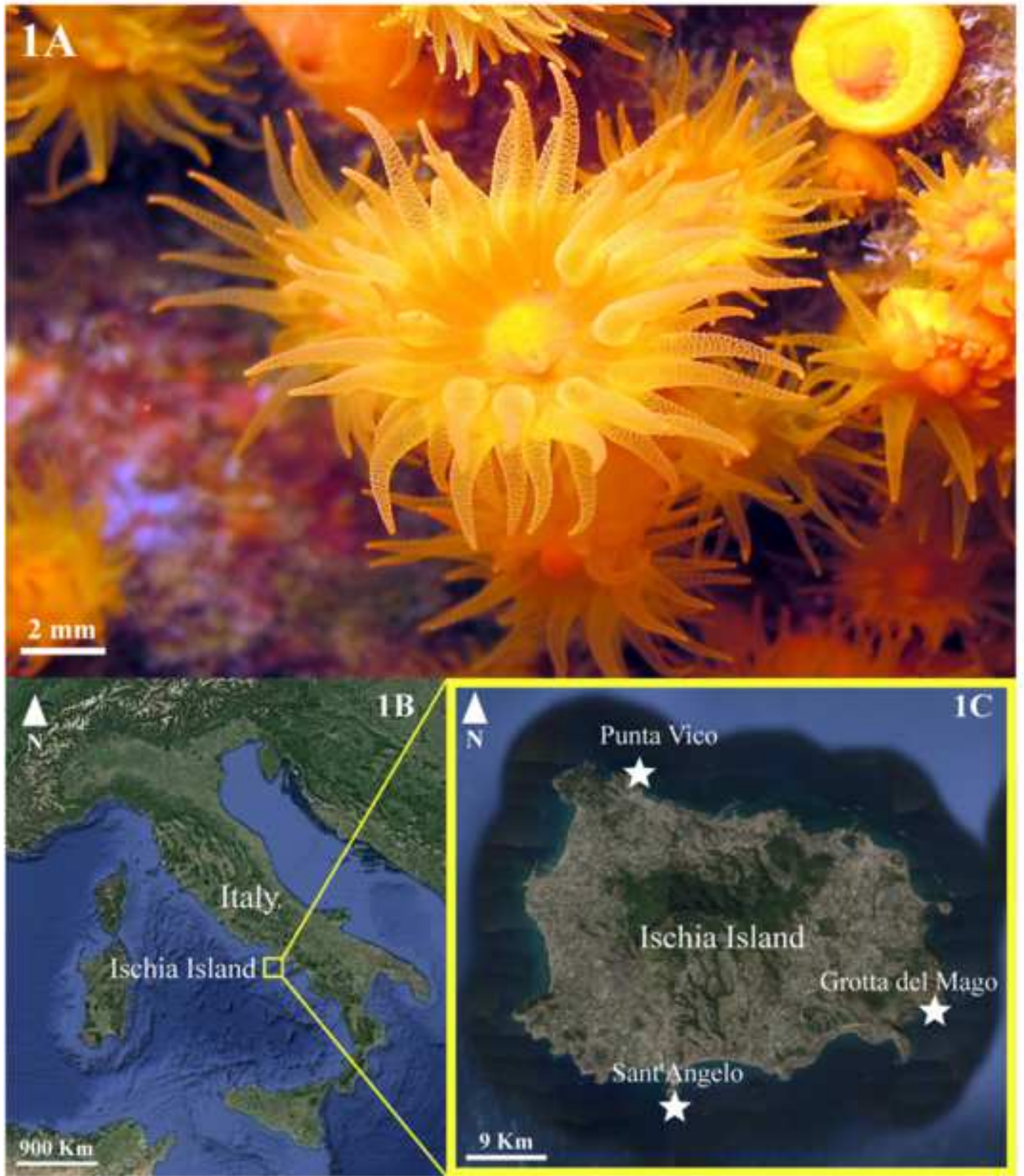


Figure 2
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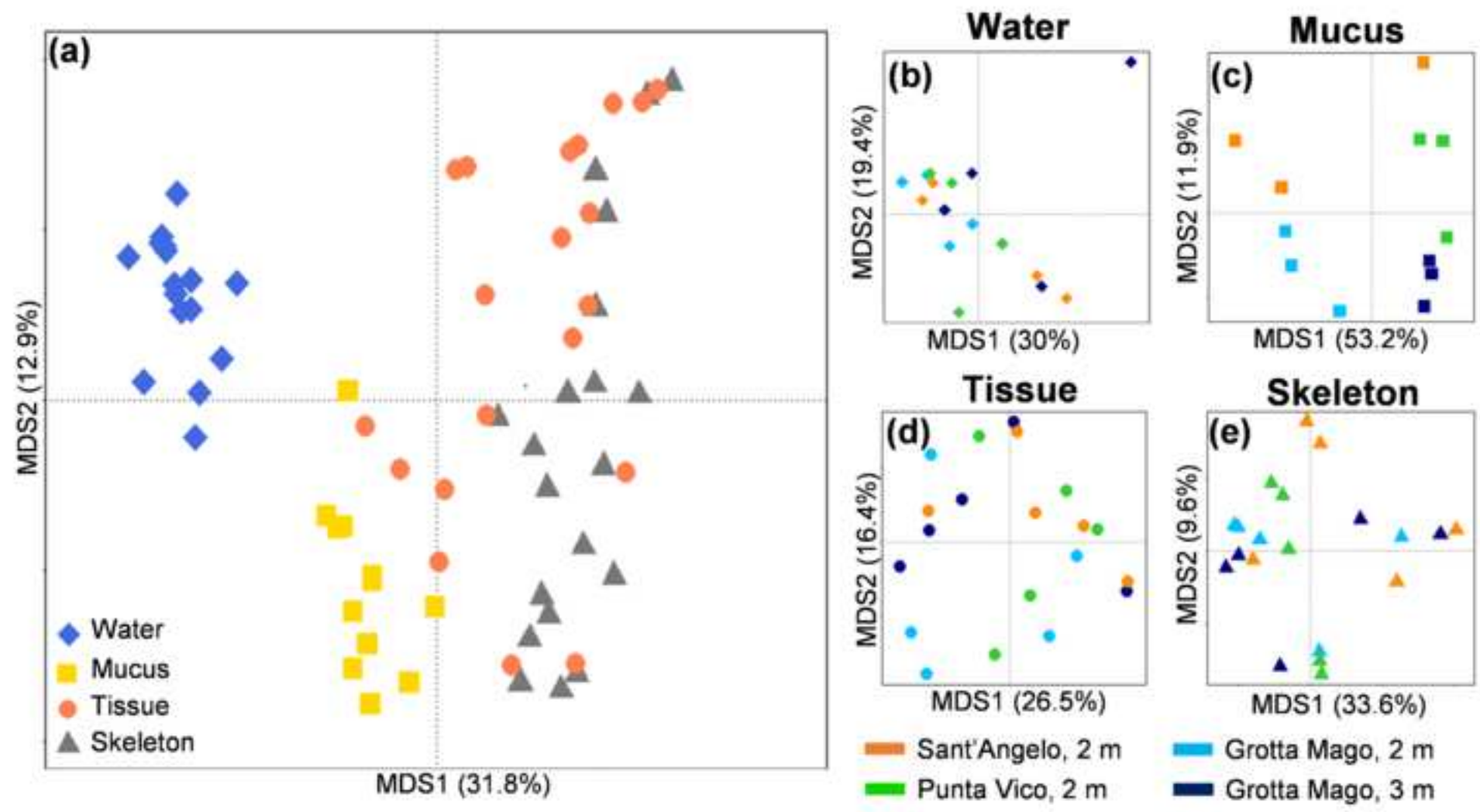


Figure 3
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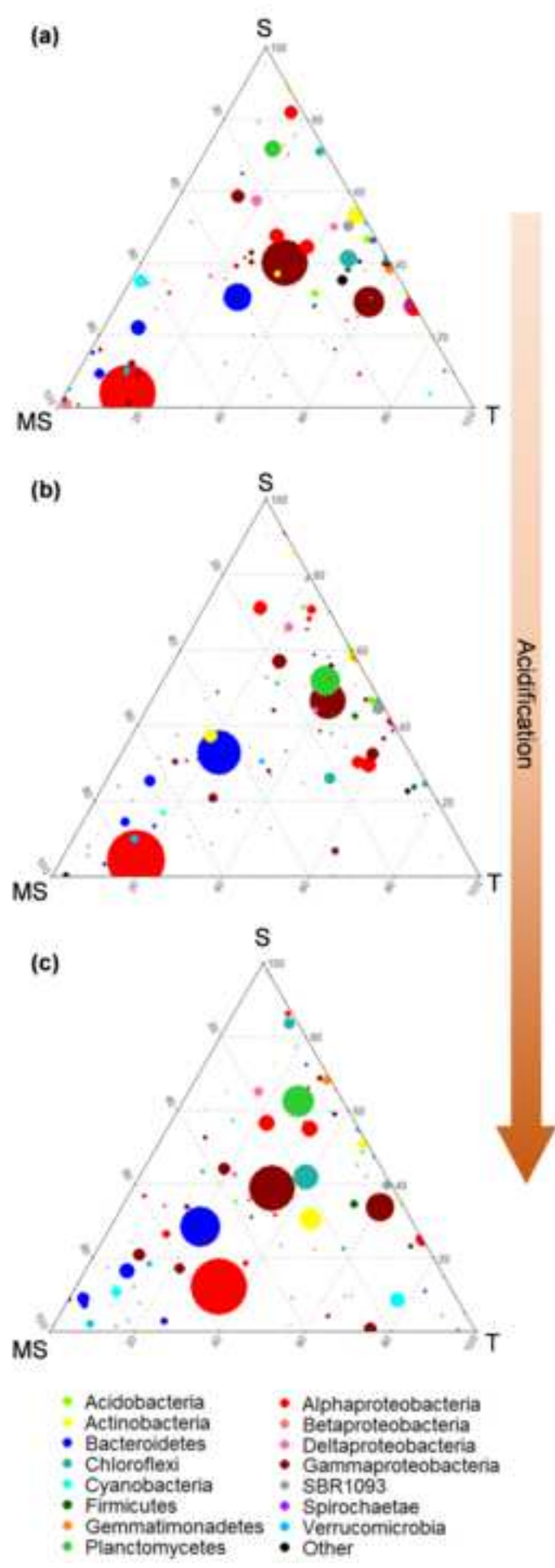
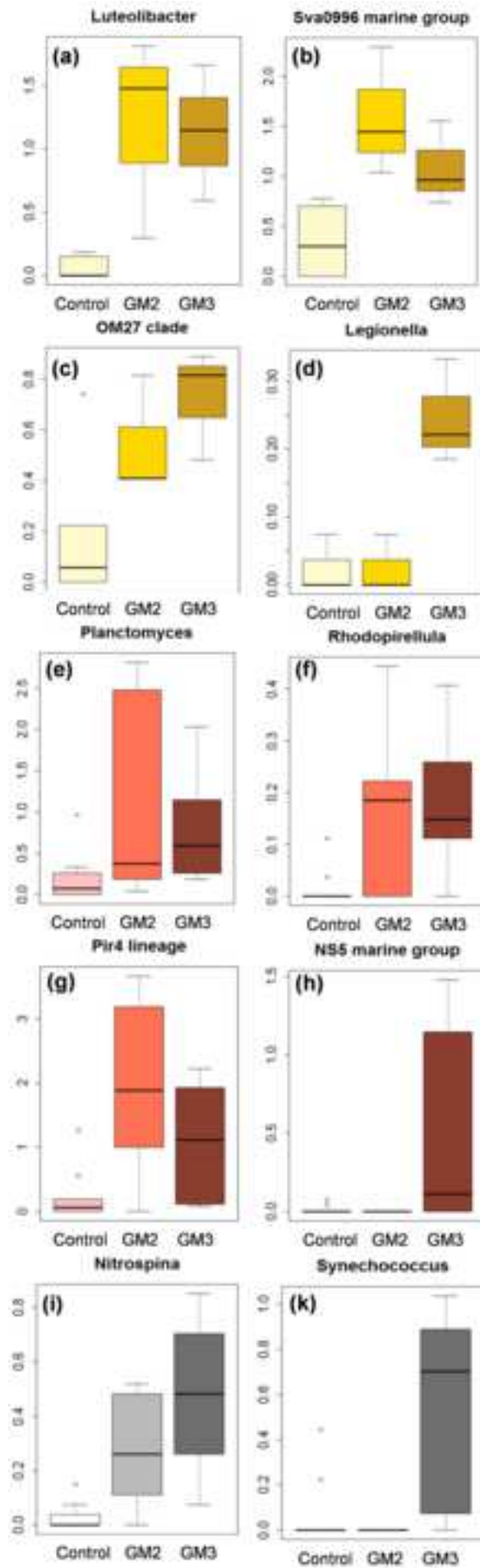


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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CREDIT AUTHOR STATEMENT

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