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Patterns in microbiome composition differ with ocean acidification in anatomic compartments of the Mediterranean coral *Astroides calycularis* living at CO_2 vents

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ABSTRACT

Coral microbiomes, the complex microbial communities associated with the different anatomic compartments of the coral, provide important functions for the host's survival, such as nutrient cycling at the host's surface, prevention of pathogens colonization, and promotion of nutrient uptake. Microbiomes are generally referred to as plastic entities, able to adapt their composition and functionality in response to environmental change, with a possible impact on coral acclimatization to phenomena related to climate change, such as ocean acidification. Ocean sites characterized by natural gradients of pCO_2 provide models for investigating the ability of marine organisms to acclimatize to decreasing seawater pH. Here we compared the microbiome of the temperate, shallow water, non-symbiotic solitary coral *Astroides calycularis* that naturally lives at a volcanic CO_2 vent in Ischia Island (Naples, Italy), with that of corals living in non-acidified sites at the same island. Bacterial DNA associated with the different anatomic compartments (mucus, tissue and skeleton) of *A. calycularis* was differentially extracted and a total of 68 samples were analyzed by 16S rRNA gene sequencing. In terms of phylogenetic composition, the microbiomes associated with the different coral anatomic compartments were different from each other and from the microbial communities of the surrounding seawater. Of all the anatomic compartments, the mucus-associated microbiome differed the most between the control and acidified sites. The differences detected in the microbial communities associated to the three anatomic compartments included a general increase in subdominant bacterial groups, some of which are known to be involved in different stages of the nitrogen cycle, such as potential nitrogen fixing bacteria and bacteria able to degrade organic nitrogen. Our data therefore suggests a potential increase of nitrogen fixation and recycling in *A. calycularis* living close to the CO_2 vent system.

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

INTRODUCTION

Over the past hundred years, human activities have acted as drivers of global environmental change, altering natural habitats and their biodiversity (Cardinale et al., 2012). Increasing atmospheric carbon dioxide (CO_2) is causing the oceans to warm and acidify (Doney et al., 2009). Ocean acidification (OA) actively contributes to altering the marine environment, with negative consequences for the survival, growth and reproduction of its inhabitants, both at microbial and multicellular levels (Kroeker et al., 2010; Gattuso et al., 2015; Yu & Chen, 2019). Atmospheric CO_2 uptake by the ocean surface lowers the seawater pH and the carbonate ion concentration (Caldeira & Wickett, 2003), with potential detrimental consequences for a variety of calcifying organisms (e.g. mollusks and sea urchins), including corals (Hoegh-Guldberg et al., 2017). Ocean acidity has increased by 25-30% (0.1 pH units) since the industrial revolution and a further increase of 150–200% is projected for the end of the century, which is equivalent to a drop of 0.3 pH units (Stocker et al., 2013). The Mediterranean basin will likely be one of the regions most affected by climate change, making it a natural focus of interest for research (Cramer et al., 2018; Lejeusne et al., 2010).

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

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

Coral microbiomes include thousands of bacterial and archaeal phylotypes in species-specific associations across broad geographical and temporal scales, which populate the different habitats of coral anatomic compartments, such as surface mucus, tissue and skeleton (Apprill et al., 2016). Microbiomes are critical to the health and survival of coral holobionts as they provide their hosts with a variety of functions, such as assistance in recovering nutrients, protection from pathogens, production of chemicals that drive larval settlement (McDevitt-Irwin et al., 2017; McFall-Ngai et al., 2013; Morrow et al., 2012; O'Brien et al., 2019; Ritchie & Smith, 1997; Rohwer et al., 2001; Rohwer et al., 2002; Rosenberg et al., 2007). Microbiomes are generally referred to as capable of shifting their composition and functionality in response to environmental variables, conferring plasticity to the ecological services provided to the host (Torda et al., 2017). The unprecedented rate of environmental change that characterizes the Anthropocene has boosted pioneering research exploring the possible role of microbiomes in the phenotypic plasticity of corals, particularly for what concerns the ability to respond to rapid changes in the environment and, generally, to climate change (McDevitt-Irwin et al., 2017; Ziegler et al., 2017). The plastic response of corals to a warming and acidifying ocean could indeed rely on the microbiome ability to rapidly shift in composition in a changing environment and provide fine-tuned functions for the host fitness (Torda et al., 2017). Studies aimed at investigating the impact of OA can benefit from areas characterized by natural gradients of pCO_2 to provide realistic insights into the ability of the marine biota to react to the decrease in ocean pH (Fabricius et al., 2014; Goffredo et al., 2014; Hall-Spencer et al., 2008). In fact, acting as natural laboratories, CO_2 vents incorporate a range of environmental factors, such as gradients of nutrients, currents and species interactions that cannot be replicated in aquaria or mesocosms (Foo et al., 2018), thus representing exceptional opportunities to study organisms that naturally live in acidic habitats (Caroselli et al., 2019). Recently, various new vent systems have been discovered along the coast of Ischia across depths of 3-48 m and span a variety of habitats,

including *Posidonia oceanica* seagrass meadows, gravel and sandy bottoms, caves, and coralligenous outcrops (Gambi et al., 2019). The predominant gas is CO_2 (92-95% CO_2, without hydrogen sulphide) and does not elevate temperature. There is one population of the coral *Astroides calycularis* that naturally occurs in the semi-submersed cave affected by CO_2 venting (5 m depth). *Astroides* is abundant in the vent system with 50% cover at 1-2 m depth (Teixido et al., 2016).

A very small number of studies have so far explored the coral microbiome along with lowering pH in naturally acidified sites (Meron et al., 2012; Morrow et al., 2015; O'Brien et al., 2018), showing a different microbiome response depending on the host species. For instance, at natural CO_2 seeps in Papua New Guinea, the endolithic community associated with massive *Porites* spp. does not change significantly between ambient and low pH sites (Marcelino et al., 2017), while large shifts in tissue-associated bacterial communities were found in *Acropora millepora* and *Porites cylindrica* across the same CO_2 seep (Morrow et al, 2015). Concerning the Mediterranean Sea, no changes in coral bacterial communities have been detected following translocation of two symbiotic coral species (i.e. *Balanophyllia europaea* and *Cladocora caespitosa*) along a natural pH gradient in the Gulf of Naples, where they grew for 7 months (Meron et al., 2012). However, in these studies the microbiomes of the different anatomic compartment (i.e. surface mucus, tissue and skeleton) were not separated during the analysis.

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

In order to provide some glimpses on the possible importance of coral microbiomes for acclimatization to acidification, in this first study on the microbiome of a temperate, shallow water, non-symbiotic solitary coral, we explored the differences in microbiome composition in mucus, tissue and skeleton of *A. calycularis*, that naturally lives at a volcanic CO_2 vent along the coast of Ischia Island, in comparison to corals living in non-acidified control sites with ambient pH.

MATERIALS AND METHODS

Selection of target sites and sampling. Coral colonies of *Astroides calycularis* (**Figure 1A**), mucus cotton swabs from each colony, and water samples were collected at Ischia Island (Gulf of Naples, Italy) (**Figure 1B**; **Table 1**) in June 2017 and April 2018. Colonies were sampled inside the naturally acidified semi-submerged cave, Grotta del Mago (**Figure 1C**), and in two control sites with ambient pH and no vent activity. Grotta del Mago consists of a main chamber of 10 m wide x 30 m long. The control sites were chosen based on the criterion that they hosted similar habitats and depths as the CO_2 vent site and there was no venting activity: Punta Vico, PV2, another semi-submerged cave (with a main chamber 10 m wide x 30 m long, 5 m maximum depth), and Sant'Angelo, SA2, an overhang located on a natural arch (with an opening of 10 m wide x 10 m height, 10 m maximum depth). Since the CO_2 emissions originate from the bottom of the cave (5 m depth), sampling at Grotta del Mago was performed at two depths to analyze corals living under a moderate (2 m depth, GM2) or intense (3 m depth, GM3) acidification condition. Coral sampling in the reference areas was performed at 2 m depth. Mean pH values in the three study sites are: pH_T = 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 in PV2 (ambient pH site) and pH_T = 8.02 in SA2 (ambient pH site) The CO_2 vents in Grotta del Mago do not elevate temperature. Hourly measurements taken by *in situ* hobo sensors from 2016 to 2019 at 2 m depth at the three study sites revealed seasonal fluctuations from 14.8 ± 0.2°C (mean ± SD) in winter to 26.1 ± 0.3 °C in summer. Temperature differences among sites were ~ 0.1°C. Nutrients (nitrite, nitrate, ammonium, phosphate, and silicate) and salinity did not change among

the study sites with differences < 0.1 µmol L^{-1} for nutrients and ~ 0.1 for salinity. NO_2(µmol L^{-1}), NO_3(µmol L^{-1}), NO_x(NO_2+NO_3) (µmol L^{-1}), NH_4(µmol L^{-1}), PO_4(µmol L^{-1}), SiO_2(µmol L^{-1}) were reported as (mean ± SD): 0.016 ± 0.002, 0.124 ± 0.023, 0.140 ± 0.024, 0.089 ± 0.013, 0.032 ± 0.002, and 0.949 ± 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 ± 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 ± 0.018, respectively. Salinity ranged from 37.3 (GM2, GM3, PV1) to 37.4 (SA) (Teixido et al., unpublished). Corals were collected by SCUBA divers using a hammer and chisel, and placed in plastic bags, while mucus cotton swabs were collected on the boat immediately after coral collection (Glasl et al., 2016; Sweet et al., 2011). At each site, close to the collected corals, 2 liters of seawater were collected with a Niskin bottle. All samples were transported in ice to the laboratory where they were frozen at -80°C.

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

The coral tissue was separated from the carbonate skeletal matrix by mechanical fragmentation (Rubio-Portillio et al., 2016). Coral specimens were transferred into an agate mortar using sterile forceps and fragmented with the pestle in 10 ml of sterile artificial seawater (NaCl 450 mM, KCl 10 mM, $CaCl_2$ 9 mM, $MgCl_2 \cdot 6H_2O$ 30 mM, $MgSO_4 \cdot 7H_2O$ 16 mM, pH 7.8). Additional 20 ml of artificial seawater were used to wash mortar and pestle from coral residues. The holobiont homogenate was then transferred into a 250-ml Becher and incubated at room temperature for 15 min to allow the skeletal fragments to settle. The seawater suspension was aliquoted into two 50-ml

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

DISCUSSION

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

The mucus bacterial ecosystem of *A. calycularis* was the most clearly affected by coral growth in acidified sites, with respect to both tissue and skeletal microbiomes. This might be related to the fact that the surface mucus is a more exposed niche, at the connection with the surrounding environment, and that the resident microbiome, with its plasticity, can dynamically respond to environmental changes, possibly playing a key role in coral survival and health upon environmental 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

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

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

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

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

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

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

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

In our study, ambient and low pH sites were carefully chosen to minimize the effect of site differences. The locations shared similar habitats and depths (semi-submersed cave for Grotta del Mago and the control site Punta Vico, and an overhang located on a natural arch for the second control site, Sant'Angelo). Furthermore, the measured environmental conditions of the study sites (i.e. temperature, salinity, and nutrients) were consistent among sites, only pH and the associated carbonate system parameters being affected by the presence of the CO_2 vents (Teixido et al., unpublished), thereby limiting the possible presence of other confounding factors.

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

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

CONCLUSIONS

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

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AUTHOR CONTRIBUTION

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

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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

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

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

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

Year	Site	Depth (m)	pH	*A. calycularis* 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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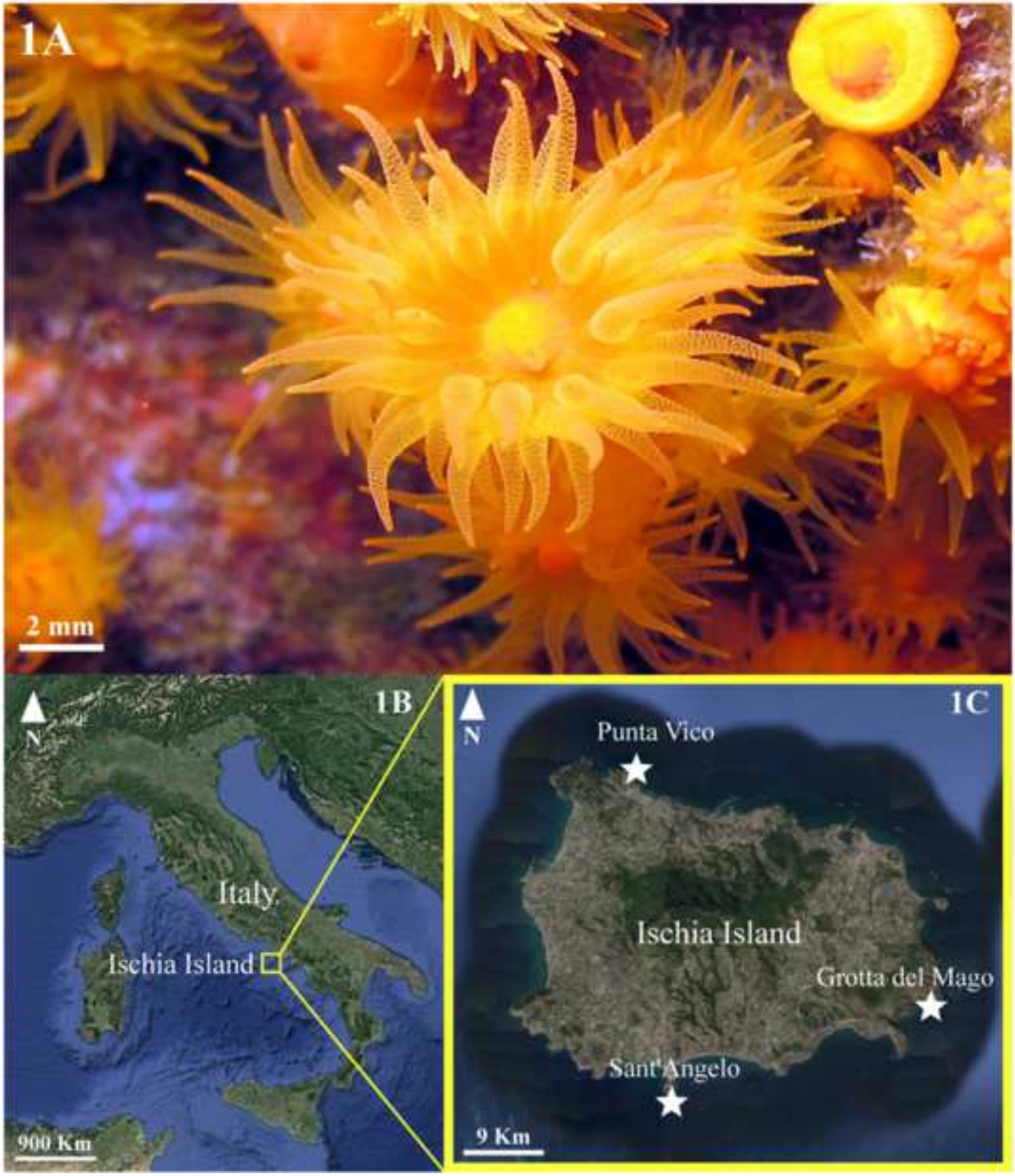

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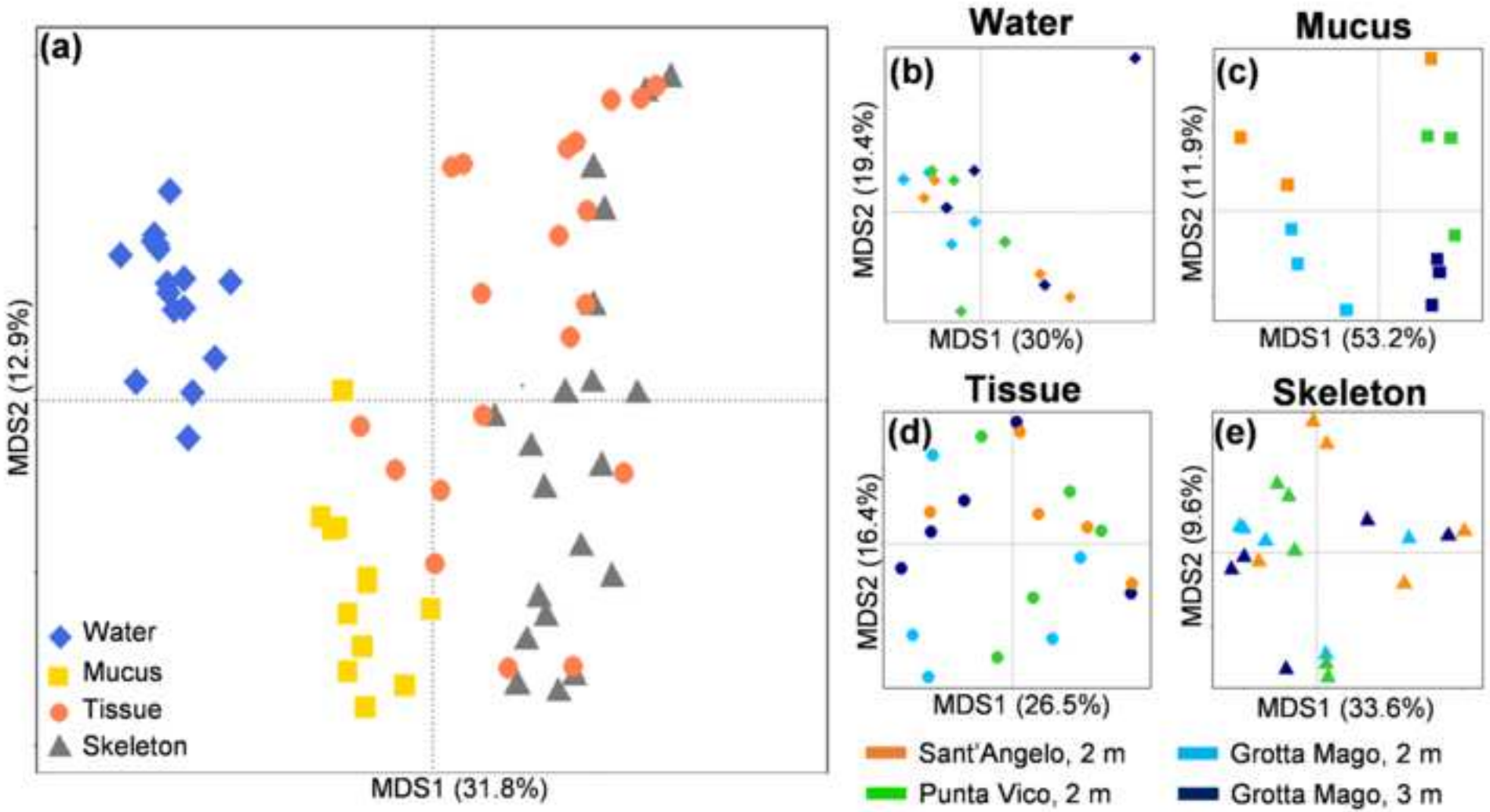

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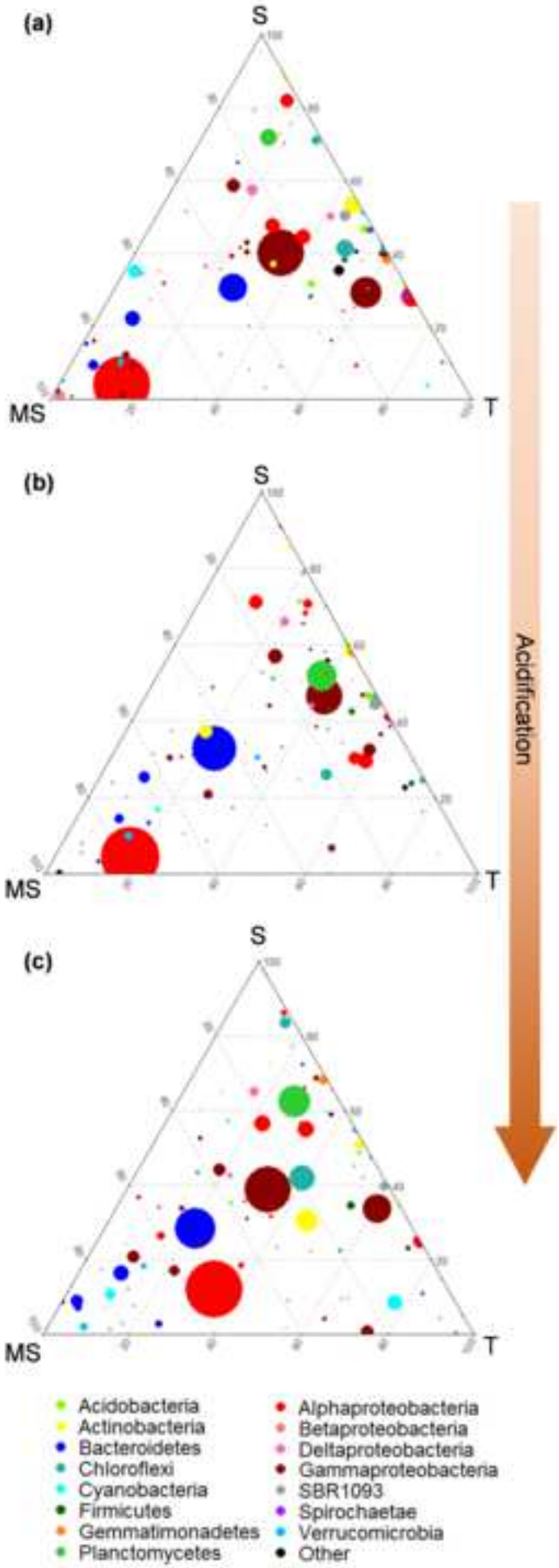

Figure 4

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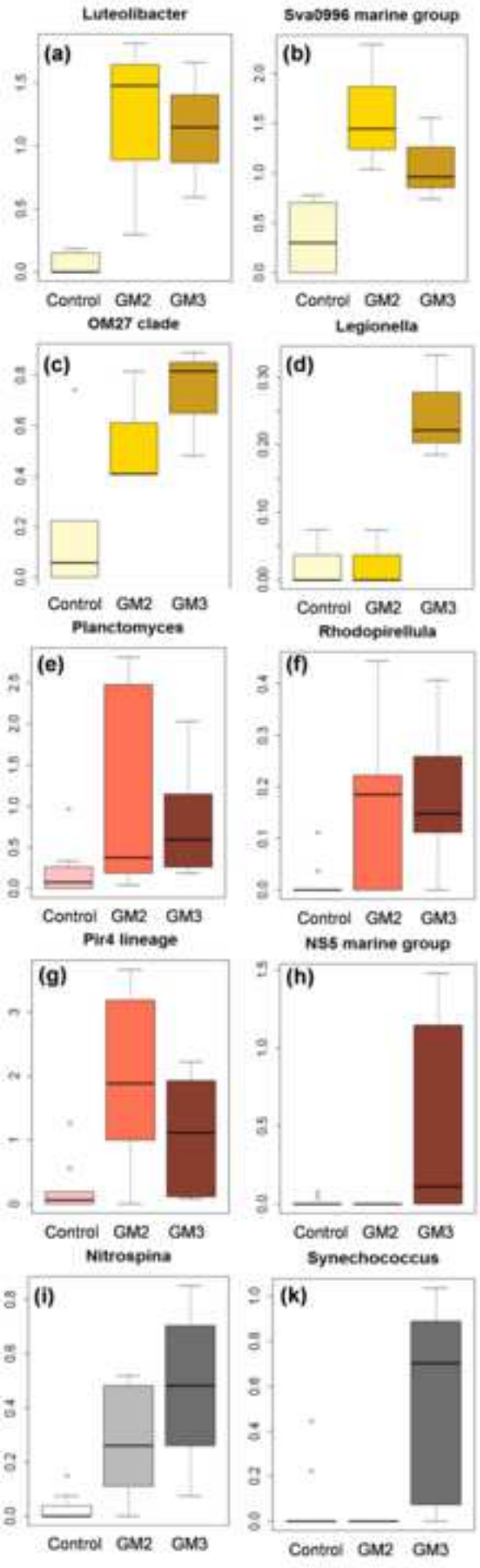

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Declaration of interests

☒ 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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