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# Comparative Assessment of Mediterranean Gorgonian-Associated Microbial Communities Reveals Conserved Core and Locally Variant Bacteria

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**Abstract** Gorgonians are key habitat-forming species of Mediterranean benthic communities, but their populations have suffered from mass mortality events linked to high summer seawater temperatures and microbial disease. However, our knowledge on the diversity, dynamics and function of gorgonian-associated microbial communities is limited. Here, we analysed the spatial variability of the microbiomes of five sympatric gorgonian species (*Eunicella singularis*, *Eunicella cavolini*, *Eunicella verrucosa*, *Leptogorgia sarmentosa* and *Paramuricea clavata*), collected from the Mediterranean Sea over a scale of ~1100 km, using next-generation amplicon sequencing of the 16S rRNA gene. The microbiomes of all gorgonian species were generally dominated by members of the genus *Endozoicomonas*, which were at very low abundance in the surrounding seawater. Although the composition of the core microbiome (operational taxonomic units consistently present in a species) was found to be unique for each host species, significant overlap was observed. These spatially consistent associations between gorgonians and their core bacteria suggest intricate symbiotic relationships and regulation of the microbiome composition by

the host. At the same time, local variations in microbiome composition were observed. Functional predictive profiling indicated that these differences could be attributed to seawater pollution. Taken together, our data indicate that gorgonian-associated microbiomes are composed of spatially conserved bacteria (core microbiome members) and locally variant members, and that local pollution may influence these local associations, potentially impacting gorgonian health.

**Keywords** *Endozoicomonas* · Gorgonian · Coral · 16S rRNA gene · Bacterial community · Holobiont · Evolution

## Introduction

Gorgonians (Octocorallia, Anthozoa, Cnidaria) are among the most ubiquitous habit-forming species of benthic communities in the Mediterranean Sea contributing considerably to their biomass, structural complexity and energy flows [1–4] and influencing the diversity of numerous other organisms from continental shelves to deep-sea waters [5]. These habitats are reported to harbour a biodiversity that is equivalent to the diversity found on tropical coral reefs [3]. However, recent climate change-related thermal anomalies and disease outbreaks have resulted in mass mortality events and, hence, population declines [6, 7]. Diseases affecting gorgonian populations have initiated recent efforts to study gorgonian microbiomes. While some microbes have been implicated in disease development in gorgonians [7–10], other microbes likely engage in symbiotic relationships with these octocorals. To identify the microbial associates that are key to gorgonian health, it is important to assess the stability of the gorgonian-associated bacterial communities.

All macroorganisms, including gorgonians, form intricate inter-kingdom symbioses with a range of microbial

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symbionts, including bacteria, archaea, fungi and viruses. These assemblages have collectively been termed the ‘holobiont’. Currently, little is known about the function of gorgonian-associated microbes, and most of our knowledge on the Cnidarian-associated microbiota stems from research on tropical corals. In the coral holobiont [11, 12], bacteria provide several services beneficial to holobiont health, such as nitrogen fixation [13], sulphur-cycling [14], antibiotic production [15–18] and the exclusion of pathogens through occupation of available microbial niches [11, 19]. Maintaining a multi-functional microbial community is therefore essential to holobiont fitness. Host-microbiome specificity has indeed been suggested for hard and soft corals as their bacterial assemblages are distinct from those in seawater [20–23], and related coral species have relatively spatially conserved microbiomes, although they are distinct from those associated with unrelated, but sympatric, coral species [11, 24, 25].

Main bacterial associates of gorgonians belong to the phyla Bacteroidetes and Proteobacteria, particularly those from the classes Alphaproteobacteria and Gammaproteobacteria [26–28]. Species of the order Oceanospirillales (Gammaproteobacteria), especially from the genus *Endozoicomonas*, are commonly found in gorgonians [22, 29–32] and a wide range of other marine organisms [25, 33–40]. Although the function of these *Endozoicomonas* species is unknown, members of the order Oceanospirillales have been implicated in the breakdown of organic compounds [36] and nutrient cycling [14], but also as a pathogen in fish [41]. Interestingly, some deep-sea gorgonians and especially the red coral *Corallium rubrum* also engage in dominant symbioses with Spirochaetes, which have been suggested to play a role in nitrogen fixation [23, 42], but not with *Endozoicomonas*. Although the mechanisms employed by gorgonians to regulate their microbiome are still unknown, extracts of gorgonian holobionts have been shown to interfere with bacterial quorum sensing and possess antimicrobial activity [43–46]. This suggests active regulation of the microbiome by the gorgonian host and/or microbial members of the holobiont [47]. Despite these potential regulatory mechanisms, local anthropogenic disturbances have been implicated in the disruption of gorgonian microbiomes [30, 48] and mortality of temperate gorgonians has mostly been linked to seawater temperature anomalies that cause disease outbreaks [6–10]. While few studies have explored the microbiomes of gorgonian species, it remains to be investigated which bacteria are their stable associates, and therefore likely of importance to holobiont functioning. The differentiation of the core microbiome (i.e. bacterial species that are consistently present within a host organism’s microbiome) from the whole microbial community will allow the identification of those stable and consistent associates and aid in revealing their metabolic functions within the holobiont. To this end, we investigated the spatially conserved and variable bacterial communities associated with shallow Mediterranean

gorgonians across six locations, encompassing an area of 1100 km. In particular, we identified the core microbiome for each species, allowing the differentiation between stable and transient bacterial associates of gorgonians, and endeavoured to explain local variations in microbial communities using microbiome functionality analyses. Overall, our results provide insights into the unique associations between gorgonians and their resident microbes, and how local conditions may disrupt these interactions within the holobiont.

## Materials and Methods

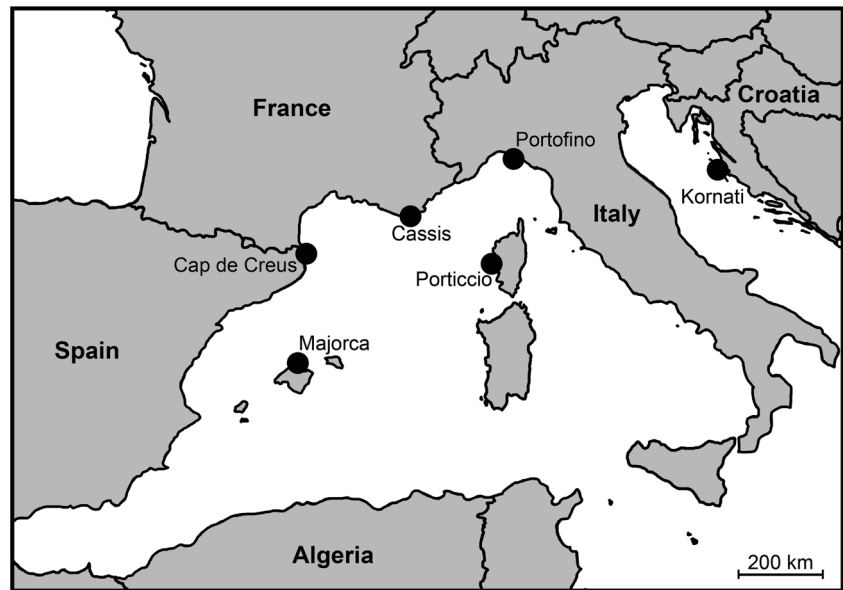
### Study Species and Spatial Sampling Across the Mediterranean Sea

Between April and June 2013, samples of gorgonians were collected at 30–40 m depth at six locations: Cap de Creus, Spain (42° 17.105′ N 3° 17.959′ E), Majorca island (39° 57.602′ N 3° 12.875′ E); Cassis, France (43° 12.311′ N 5° 28.962′ E); Porticcio, Corsica Island (41° 51.039′ N 8° 46.082′ E); Portofino, Italy (44° 18.162′ N 9° 13.075′ E) and Kornati National Park, Croatia (43° 48.287′ N 15° 16.527′ E) (Fig. 1). The seawater temperature at the time of sample collection ranged between 14 and 16 °C. For each species (*Eunicella cavolini*, *Eunicella singularis*, *Eunicella verrucosa*, *Leptogorgia sarmentosa*, *Paramuricea clavata*), samples were collected from three to six visually healthy colonies within a 100-m radius at each location considered for each particular species (Table 1). Colonies were considered healthy when no macroscopic signs of epibiosis or partial mortality were present. From each colony, one branch of approximately 6 cm was cut using scissors and placed in a sterile plastic bag containing ambient seawater. Each sample was rinsed twice with 0.2-µm filtered seawater to remove exogenous, loosely associated microorganisms and stored in ice-cold RNeasy lysis buffer (ThermoFisher Scientific) at 4 °C. At each location, 30 L of seawater was collected next to the gorgonian colonies sampled and filtered sequentially through 8, 3 and 0.2-µm Whatman Nuclepore Track-Etched filters (Sigma-Aldrich) and the 0.2-µm filter retentate was kept in RNeasy lysis buffer at 4 °C.

### DNA Extraction and 16S rRNA Gene Amplicon Library Preparation

Tissues and associated microbes were removed from the skeleton using an airbrush with 5 mL of 0.2 M EDTA, and filter retentates were bead beaten prior to DNA extraction. DNA was extracted from tissues and seawater 0.2-µm filter retentate using the Genomic DNA Buffer Set and Genomic-tip 20/G columns (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. For each sample, 16S rDNA amplicon libraries were generated in triplicate using the Multiplex PCR

**Fig. 1** Overview of the sampling locations. Gorgonians were collected from the Western Mediterranean Sea at six locations spanning a distance of 1100 km: Cap de Creus and Majorca in Spain, Cassis and Porticcio in France, Portofino in Italy and Kornati National Park in Croatia



kit (QIAGEN, Hilden, Germany) and the 784F/1061R primer set [49] in 30- $\mu$ l reaction volumes containing 0.2  $\mu$ M of each primer and 30 ng of template DNA. The PCR protocol consisted of an initial denaturation step of 95 °C for 15 min followed by 30 amplification cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s and extension at 72 °C for 40 s) and a final extension step of 10 min at 72 °C. PCR products were cleaned using the PureLink PCR Purification Kit (Invitrogen, Carlsbad CA, USA) and subsequently quantified using a Qbit fluorometer (Invitrogen, Carlsbad CA, USA) and ran on 1 % agarose electrophoresis gel to confirm purity. PCR product triplicates were pooled in equal quantity and sent to the KAUST BioScience Core Laboratory (Thuwal, Saudi Arabia). Libraries were generated using the GS FLX Titanium emPCR Kit (Roche, Branford, CT) and sequenced on the 454 GS FLX system with Titanium FLX chemistry.

### 16S rRNA Gene Amplicon Data Analysis

Pyrosequencing produced 345,643 reads and the QIIME pipeline [50] was used for data processing. The quality (.qual)

**Table 1** Overview of the number of samples collected per gorgonian species per location

Location	<i>Eunicella cavolini</i>	<i>Eunicella singularis</i>	<i>Eunicella verrucosa</i>	<i>Leptogorgia sarmentosa</i>	<i>Paramuricea clavata</i>
Cap de Creus	—	3	—	3	—
Cassis	3	3	—	—	—
Croatia	6	5	—	—	4
Majorca	3	3	—	3	—
Porticcio	3	3	—	—	—
Portofino	3	3	3	3	—

and reads (.fasta) files were filtered using the split\_libraries.py script to remove low quality (Phred < 25) sequences, reads <200 or >500 bp in length, primers and barcodes, and to assign each read to its respective sample. The resulting file containing 204,556 reads was checked for chimeric sequences against the curated SILVA reference database (version 119) [51] using UCHIME [52], but none were identified. Operational taxonomic units (OTUs) were defined at the level of 97 % similarity followed by taxonomy assignments against the SILVA reference database (version 119) using the UCLUST algorithm [53]. Singletons, unassigned OTUs, and OTUs classified as chloroplast or mitochondria were removed from the dataset. Overall microbiome (rarefied to 992 reads for each sample) and core microbiomes of each species (OTUs present in 100 % of the samples) at each sampling location (rarefied to 592 reads for each sample) were determined. Alpha diversity metrics (total observed number of OTUs, predicted species (chao1), Shannon-Weiner diversity and Fisher's alpha) and beta diversity UniFrac distance matrices were generated from OTU tables using the QIIME pipeline. The complete dataset has been deposited in the NCBI Sequence Read Archive (SRA) with accession number SRP071003.

### Statistical Analysis

To visualise differences between gorgonian-associated and seawater bacterial communities, principal coordinate analysis (PCoA) was performed on unweighted UniFrac distance matrices. Permutational multivariate analysis of variance (PERMANOVA) and pair-wise comparisons were performed under Type III partial sums of squares and 9999 permutations under the reduced model to test for differences

among bacterial communities at different locations and gorgonian host species. In addition, PERMDISP analyses were used to assess the homogeneity of multivariate dispersions. In case the number of unique permutations was limited (>8800), Monte Carlo (MC) simulations were performed in parallel. Differences in alpha diversity, based on the Shannon-Wiener Index, were determined using one-way analyses of variance. Differences were considered significant at  $p < 0.05$ . All beta diversity analyses were conducted using PRIMER 6 and PERMANOVA+ (PRIMER-E Ltd), and differences in alpha diversity were analysed using S-PLUS 8.0 (Insightful Corp.).

### Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)

Using the quality-filtered sequence file, closed reference OTU picking was conducted at the level of 97 % similarity followed by taxonomy assignments against the GreenGenes reference database (version gg\_13\_5) using the UCLUST algorithm [53] in QIIME. From the resulting OTU table, derived from the 277-bp 16S rRNA amplicon dataset, metagenomes were predicted using PICRUSt [54], ensuring that only samples containing more than 475 reads after 16S gene copy number normalisation were included. Predicted metagenomes were subsequently analysed for differences in functional microbiome profiles based on KEGG pathways among samples with the software package Statistical Analysis of Metagenomic Profiles (STAMP; version 2.1.3) [55], using analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test and Storey FDR multiple test correction. Differences were considered significant at  $p < 0.05$ .

### Phylogenetic Analyses of Gorgonian-Associated Microbes

The QIIME pipeline [50] was used to identify the most representative sequence of each *Endozoicomonas* OTU. An initial phylogenetic tree was built based on the published full-length 16S rDNA sequences available from GenBank, using the maximum parsimony approach in MEGA6 [56], and the representative sequences from this study were added to build the final tree, based on a 300-bp alignment matrix. The bootstrap consensus tree was inferred from 1000 replicates.

### Nucleotide Sequence Accession Numbers

The sequences of the core microbiome OTUs found in this study were deposited in the GenBank database under accession numbers KU738786–KU738806.

## Results

### Overall Bacterial Diversity on Gorgonians

The bacterial communities associated with the gorgonians were distinct from [Pseudo- $F_{(1, 56)} = 6.2, p < 0.001$ , 9854 unique permutations] (Figure S1) and less diverse [Shannon-Wiener Index:  $F_{(1, 56)} = 40.9, p < 0.01$ ] (Table 2) than those in seawater. Overall, no significant differences in the beta diversity of the overall gorgonian-associated bacterial communities were observed among sampling locations, which was likely due to high within-site variation [PERMDISP:  $F_{(5, 46)} = 6.98, p < 0.01$ ] between samples of the *Eunicella* spp. collected near Cap de Creus, Cassis, Croatia and Porticcio, henceforth called (A) locations, and Portofino and Majorca, hereafter called (B) locations, and an unbalanced sampling design [57]. Bacterial communities on *Eunicella* species at (A) locations were dominated by members of the order Oceanospirillales ( $93.1 \pm 7.3$  %), particularly from the genus *Endozoicomonas*; however, those at (B) locations appeared more diverse containing large numbers of bacteria belonging to a range of different orders, including Burkholderiales, Mycoplasmatales, Spirochaetales and Vibrionales (Figs. 2 and S2; Table 2). The diversity of *Endozoicomonas* was also significantly reduced in *Eunicella* spp. at (B) locations compared to those at (A) locations (*E. singularis* [ $F_{(1, 18)} = 35.8, p < 0.01$ ], *E. cavolini* [ $F_{(1, 15)} = 26.0, p < 0.01$ ]) (Fig. 3a). In *L. sarmentosa*, however, the community diversity was variable among all the locations sampled, while *P. clavata* collected in Croatia had a distinct microbiome despite being dominated by *Endozoicomonas* (Figures S2). Overall, the diversity of bacterial OTUs associated with gorgonians was relatively low, ranging from 21 OTUs in *P. clavata* to 60 and 64 in *E. singularis* and *E. cavolini*, respectively, and 109 in *L. sarmentosa*.

### Core Microbiomes of Mediterranean Gorgonians

To understand the underlying causes of the differences observed, we also analysed the contributions and composition of the core microbiomes of each species. Core microbiomes (OTUs present in 100 % of the samples of each gorgonian species) provided major contributions to the microbiome of *E. cavolini* ( $83.3 \pm 4.7$  %) and *E. singularis* ( $82.7 \pm 15.0$  %) at (A) locations but significantly less ( $43.3 \pm 0.3$  %, [ $F_{(1, 18)} = 27.3, p < 0.01$ ], and  $26.1 \pm 19.7$  % [ $F_{(1, 15)} = 15.5, p < 0.01$ ]), respectively, at (B) locations (Fig. 4). In *L. sarmentosa*, however, no major differences in the contribution of the core microbiomes, which represented only 17.4 % ( $\pm 5.3$  %) of the total bacterial community, were found between the locations [ $F_{(1, 7)} = 0.2, p = 0.67$ ] (Fig. 4).

The core microbiomes of all gorgonians were dominated by members of the order Oceanospirillales, in particular those belonging to the genus *Endozoicomonas* (Figs. 2 and S2). The core



**Table 2** Overview of alpha diversity metrics (average  $\pm$  standard error of the mean) of the gorgonian-associated microbiomes

Species	Location	Simpson's Evenness	Shannon-Wiener index	OTUs microbiome	Locally stable microbial associates	OTUs core microbiome
<i>Eunicella cavolini</i>	Cassis	0.029 $\pm$ 0.002	1.71 $\pm$ 0.20	57 $\pm$ 6	30	5
	Croatia	0.045 $\pm$ 0.017	2.29 $\pm$ 0.89	59 $\pm$ 13	15	
	Majorca	0.064 $\pm$ 0.045	3.49 $\pm$ 0.43	86 $\pm$ 7	40	
	Porticcio	0.035 $\pm$ 0.006	2.10 $\pm$ 0.34	58 $\pm$ 4	23	
	Portofino	0.060 $\pm$ 0.020	2.73 $\pm$ 0.49	57 $\pm$ 10	18	
<i>Eunicella singularis</i>	Cap de Creus	0.034 $\pm$ 0.004	1.38 $\pm$ 0.12	44 $\pm$ 7	15	5
	Cassis	0.037 $\pm$ 0.010	1.80 $\pm$ 0.39	49 $\pm$ 13	21	
	Croatia	0.032 $\pm$ 0.006	1.66 $\pm$ 0.72	52 $\pm$ 13	17	
	Majorca	0.064 $\pm$ 0.018	2.77 $\pm$ 0.70	56 $\pm$ 17	25	
	Porticcio	0.031 $\pm$ 0.005	1.48 $\pm$ 0.08	50 $\pm$ 9	19	
	Portofino	0.071 $\pm$ 0.054	4.22 $\pm$ 1.82	133 $\pm$ 58	18	
<i>Eunicella verrucosa</i>	Portofino	0.115 $\pm$ 0.012	4.82 $\pm$ 0.62	116 $\pm$ 31	37	
<i>Leptogorgia sarmentosa</i>	Cap de Creus	0.055 $\pm$ 0.009	3.43 $\pm$ 1.07	91 $\pm$ 42	17	4
	Majorca	0.038 $\pm$ 0.024	2.36 $\pm$ 0.33	70 $\pm$ 9	19	
	Portofino	0.052 $\pm$ 0.006	2.83 $\pm$ 0.52	64 $\pm$ 11	19	
<i>Paramuricea clavata</i>	Croatia	0.095 $\pm$ 0.016	1.18 $\pm$ 0.20	17 $\pm$ 2	13	
Seawater		0.211 $\pm$ 0.029	5.87 $\pm$ 0.35	142 $\pm$ 25		

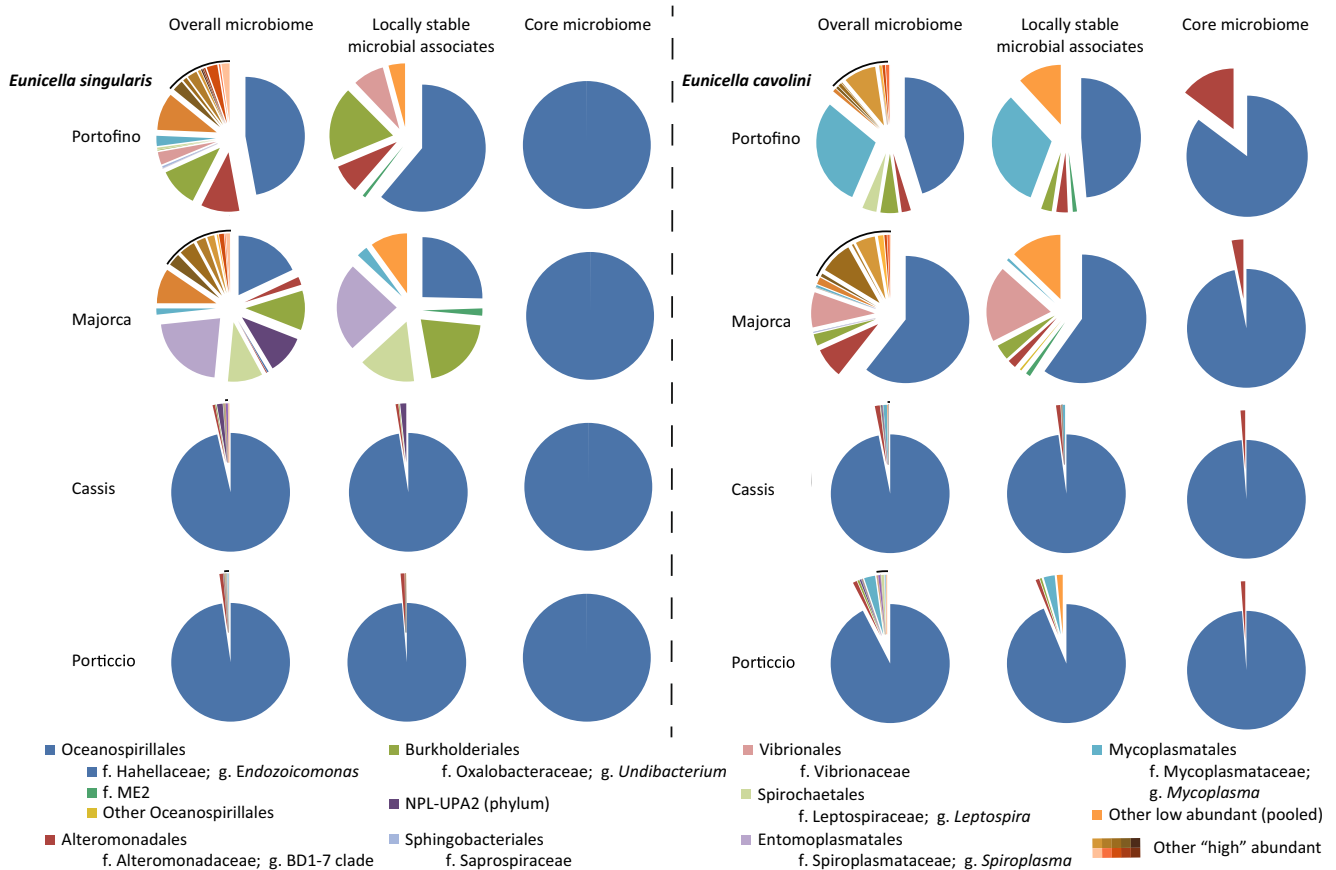
microbiomes of *E. cavolini* and *E. singularis* were composed of four *Endozoicomonas* OTUs (KU738801, KU738805, KU738791, KU738804), along with a low abundant *Endozoicomonas* OTU (KU738806) in *E. singularis* and an OTU belonging to the Alteromonadales clade BD1-7 (KU738792) in *E. cavolini* (Table S1). All core microbiome *Endozoicomonas* OTUs were highly related to OTUs previously found in *E. cavolini* [22] and the tropical gorgonian *Gorgonia ventalina* [28] (Fig. 5, Table S2). In particular, *Endozoicomonas* KU738801 was abundantly associated with *Eunicella* species representing 63.1 % ( $\pm 9.4$  %) and 72.0 % ( $\pm 14.9$  %) of the overall bacterial community in *E. singularis* and *E. cavolini*, respectively, in (A) locations, while in (B) locations, its reduced abundance (*E. singularis* [ $F_{(1, 18)} = 27.6, p < 0.01$ ], *E. cavolini* [ $F_{(1, 15)} = 9.6, p < 0.01$ ]) resulted from the overall reduction in core microbiome contributions (Fig. 4, Table S1). *E. verrucosa* also harboured the four shared *Endozoicomonas* OTUs (with KU738801 as the most abundant, although relatively low at 18.0 % ( $\pm 13.6$  %)), but no core microbiome could be established as it was only collected from (B) location Portofino (Figure S1, Table S1). *L. sarmentosa* communities showed little consistency among locations and only a minor core microbiome (25.3  $\pm$  5.4 % of overall community) could be discerned (Fig. 4). Members of its core microbiome were one *Undibacterium* OTU (1.8  $\pm$  0.3 %; KU738798) and three *Endozoicomonas* OTUs (KU738801, KU738805, KU738794). KU738801 was, however, significantly more abundant on *L. sarmentosa* collected at the (A) location Cap de Creus compared with the (B) locations (Table S1). Most core microbiome OTUs (KU738801, KU738805, KU738804,

KU738792, KU738798) were also detected in seawater but at significantly lower relative abundances compared with the gorgonian-associated microbiomes at (A) locations (Table S1).

*Endozoicomonas* also dominated the microbial community of *P. clavata* (74.8  $\pm$  36.4 %; Figure S2), but the core community, which comprised 98.8 % ( $\pm 0.3$  %) of the overall microbiome (Fig. 4), had a significantly different membership from those of the other gorgonian species analysed (Figure S2). While *P. clavata* did harbour OTUs KU738801 and KU738791 as well, these were very low abundant (KU738801: 0.9 % ( $\pm 0.1$  %); and KU738791 0.05 % ( $\pm 0.01$  %) of the overall community). Instead, the *P. clavata* core microbiome contained seven *Endozoicomonas* OTUs (KU738795, KU738790, KU738796, KU738797, KU738799, KU738802, KU738803) unique for this gorgonian species, which formed a distinct phylogenetic group (Fig. 5, Table S2) although still closely related to *Endozoicomonas* found on *Eunicella* species. While most of these were low abundant, KU738802 represented 71.1 % ( $\pm 20.4$  %) of the overall community (Table S1). In addition, five representatives of the phylum NPL-UPA2 (KU738786, KU738787, KU738788, KU738789, KU738793) were consistently associated with *P. clavata* in Croatia, together composing 25.0 % ( $\pm 36.2$  %) of the overall community.

### Local Differences in Microbiome Associations

We also analysed which bacteria were consistently associated with the gorgonian species *E. singularis*, *E. cavolini* and



**Fig. 2** Overview of the composition of the overall microbiome and the community of locally stable microbial associates and the core microbiomes associated with *Eunicella cavolini* and *E. singularis* at (A) locations Cassis and Porticcio and (B) locations Portofino and Majorca. The contribution of each taxon per (core) microbiome is indicated in percentages (%). Contributions of taxa to the overall community are

presented at the order level, and contributions of taxa to the community of locally stable microbial associates and the core microbiomes at the family (f.)/genus (g.) level. Taxa are listed sequentially in a *clockwise direction*, starting with Oceanospirillales/*Endozoicomonas*. Data for other species and locations are provided in Figure S2

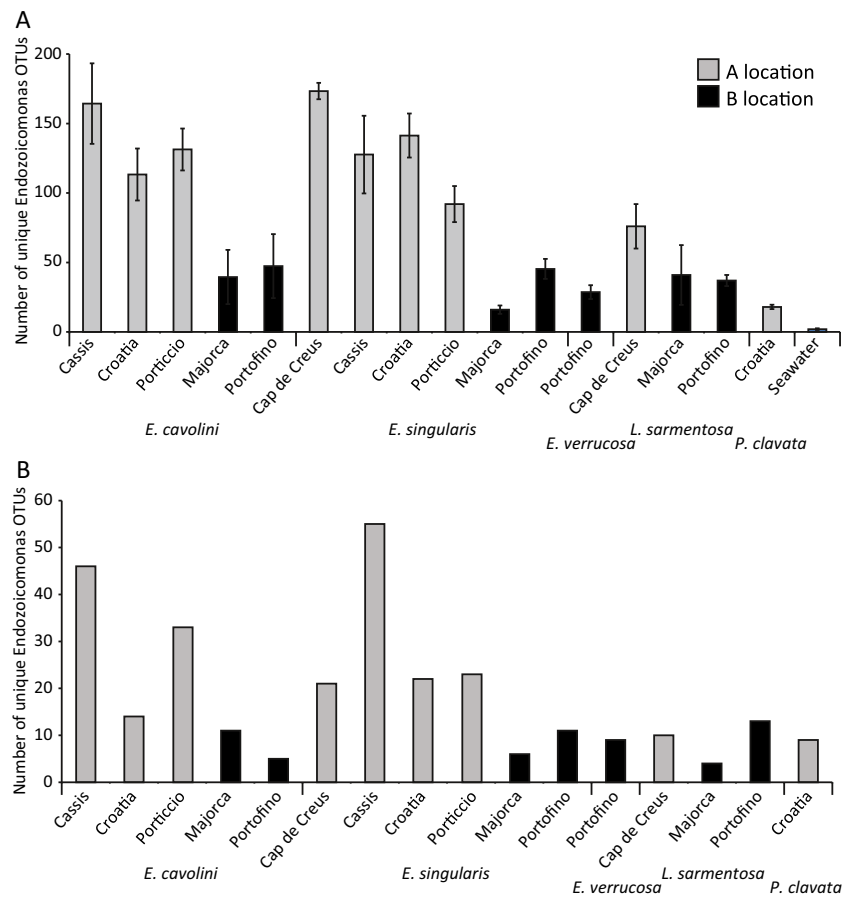
*L. sarmentosa* at the different sampling locations, in addition to the core microbiome members. In general, we found differences in the communities of these locally stable bacterial associates among all locations for the same gorgonian species and among nearly all species within the same location (Table S3). However, in *Eunicella* species at (A) locations, the vast majority of locally stable bacterial associates were members of the core microbiome (Fig. 4). In fact, only 1.5 % ( $\pm 1.8$  %) of the bacteria stably associated with the gorgonians at each location were represented by other bacterial taxa than those OTUs belonging to the core microbiome and other members of the order Oceanospirillales (Figs. 2 and S2). At (B) locations, however, relatively high numbers of bacteria belonging to the genera *Undibacterium*, *Leptospira*, *Mycoplasma*, *Spiroplasma* and the family Vibrionaceae were found along with members of other low-abundant taxa (Figs. 2 and S2), while the diversity in *Endozoicomonas* appeared to be reduced (Fig. 3b). In *L. sarmentosa*, the microbiome was dominated by bacteria that were locally consistently associated with this gorgonian but were not part of

the core microbiome (Fig. 4); hence, significant differences in the membership of the locally stable bacterial associates were found among all sampling locations (Table S3). OTUs belonging to the Oceanospirillales family ME2 contributed significantly to the microbial community of *L. sarmentosa* near Majorca, while in Portofino, OTUs from the genera *Mycoplasma* and the Alteromonadaceae clade BD1-7 and OTUs belonging to the family Saprospiraceae were found consistently associated with this gorgonian (Figure S2). At Cap de Creus, however, the Oceanospirillales family ME2 and OTUs assigned to the genus *Mycoplasma* were the main groups of bacteria consistently associated with this gorgonian in addition to the core microbes.

### Local Changes in Microbiome Functionality

To understand the potential implications of the shifts observed in *Eunicella*-associated bacterial communities among (A) and (B) locations for gorgonian health, we employed PICRUST analysis on *E. singularis*, for which

**Fig. 3** Diversity of *Endozoicomonas* in **a** the overall bacterial community and **b** the locally stable microbial associates on gorgonians at different locations and in the seawater

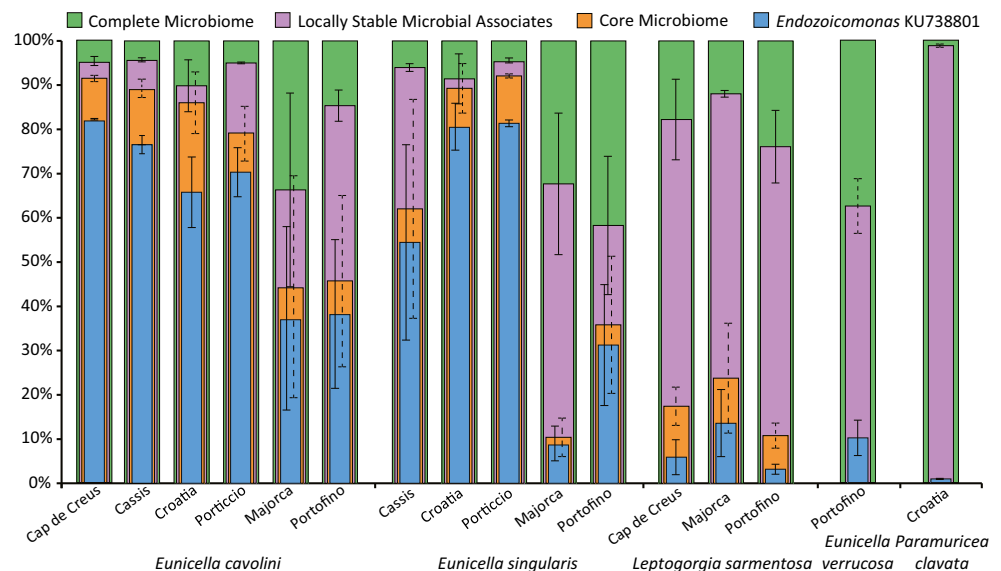


we had the most complete comparative dataset. Overall, we found a significant increase in the number of genes related to (1) the degradation of xenobiotics; (2) the production of antimicrobial compounds and (3) energy, amino acid, lipid, vitamin and carbohydrate metabolism (Table S4).

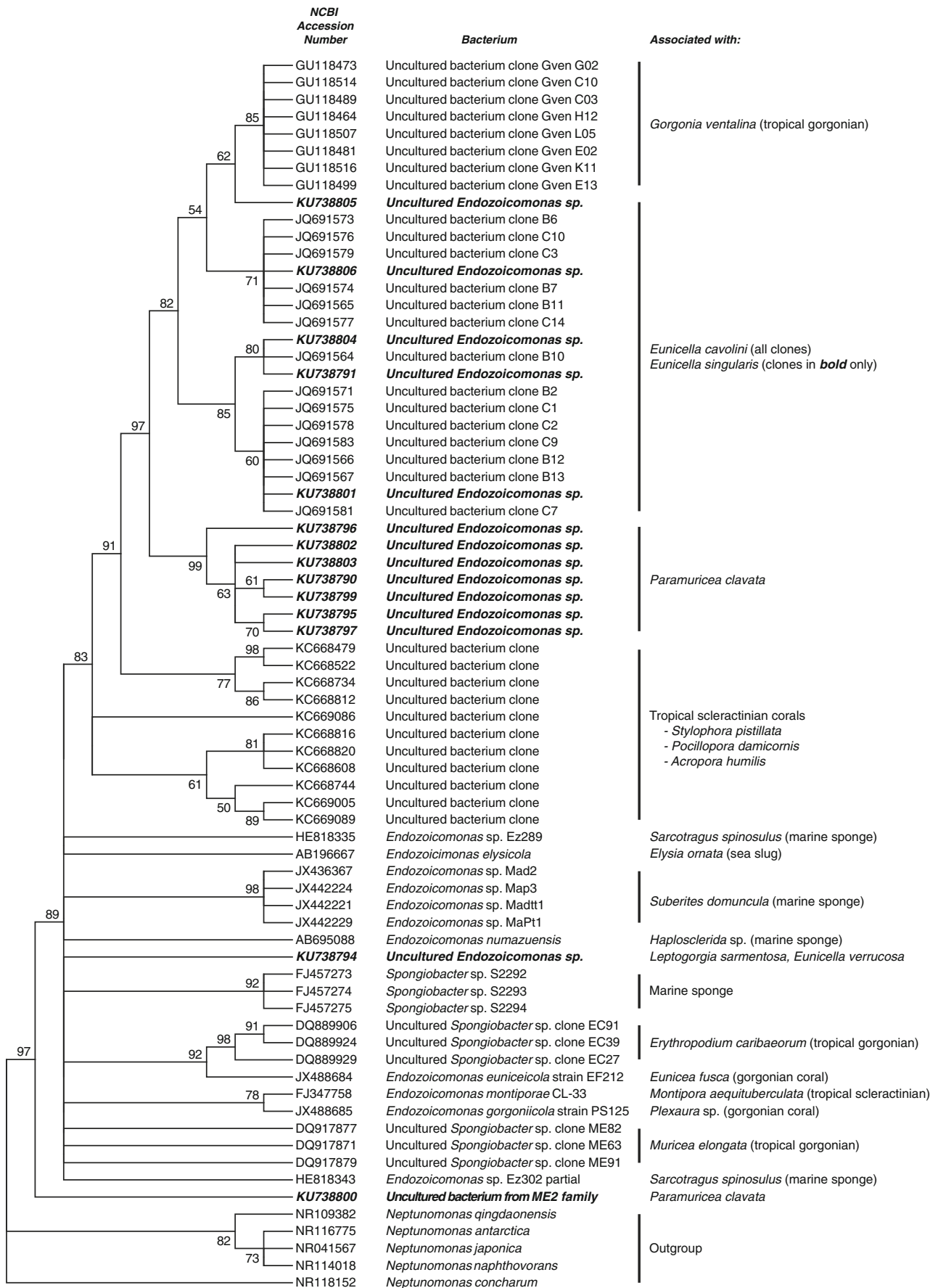
## Discussion

In this study, we show that the majority of Mediterranean gorgonian species, sampled on a large spatial scale at shallow depths, possess a microbiome of limited diversity compared with tropical scleractinian corals, and which is dominated by

**Fig. 4** Contributions of core microbiome to the overall bacterial community. Percentage contribution of *Endozoicomonas* KU738801 (blue), the core microbiome (orange) and the locally stable microbial associates (violet) to the overall bacterial community (green) associated with the gorgonian species at different locations within the western Mediterranean Sea. No core microbiome could be discerned for those species collected at only one location







**Fig. 5** Endozoicomonas phylogeny. Reconstruction of the phylogenetic tree of *Endozoicomonas* based on the 16S ribosomal rRNA gene using a maximum parsimony model. Percentages of 1000 bootstrap replicates are indicated next to the tree nodes if they are >50 %. Sequences from the present study are in **bold** and *italics*. The tree is rooted using sequences from the genus *Neptunomonas* (order of Oceanospirillales) as the outgroup

bacteria from the genus *Endozoicomonas*, in undisturbed locations. In particular, the core microbiome of *Eunicella* gorgonians is highly structured, consisting of four common OTUs and making up 90 % of the associated microbiome. In contrast, the bacterial assemblages of *Leptogorgia sarmentosa* appeared to lack a general structure, while *Paramuricea clavata* harbours an *Endozoicomonas*-dominated bacterial community distinct from those found on *Eunicella* species, even when present next to one another. While the gorgonian-associated microbiomes were generally stable, shifts were observed in locations affected by disturbances (likely the influx of contaminated freshwater), suggesting that these gorgonian holobionts may be adversely impacted or adapted to local conditions.

### The Core Microbiome of Mediterranean Gorgonians

Our study is a first attempt to elucidate the core microbiome of Mediterranean gorgonians, allowing the identification of their stable and consistent microbial associates and potentially revealing the functions these microbes provide within the holobiont. While we show that the core microbiome of each gorgonian species is largely dominated by bacteria from the genus *Endozoicomonas* and that it is relatively stable over a large geographical range, each sympatric species' core microbiome had its own unique combination of OTUs. Interestingly, however, we also observed that there was significant overlap among the core microbiomes as all gorgonians analysed (regardless of species and location) shared at least one core OTU. This was particularly true between more closely related gorgonians, as *Eunicella* species (family Gorgoniidae) had significantly more overlap with one another than with other species (*Leptogorgia*) within this family; microbiomes associated with Gorgoniidae were more divergent from those associated with species from the family Paramuricidae (*Paramuricea*). This overlap of major core microbiome members shows the apparent importance of these specific phylotypes for the health of these sympatric temperate gorgonians. This is, however, in stark contrast with previous reports on scleractinian corals, where each coral species was found to be associated with closely related but unique *Endozoicomonas* phylotypes [33, 58].

The microbiome of *P. clavata*, *E. singularis* and *E. cavolini* were highly structured and dominated by the core microbiome. The most significant contribution to the microbiome of *Eunicella* species was made by OTU

KU738801, which showed 100 % similarity with a previously observed highly dominant *Endozoicomonas* in *E. cavolini* [22]. However, in *P. clavata*, this OTU was very low abundant and its primary role appeared to have been taken over by KU738802. In fact, the majority of the *Endozoicomonas* in the *P. clavata* core microbiome were unique to this gorgonian. This contrast in the microbiomes of different gorgonian taxonomic families indicates evolutionary divergence in the symbiotic association. However, it remains to be seen whether these microbiomes are also functionally distinct or that there is functional overlap.

Although the function of *Endozoicomonas* bacteria is still largely unknown, their high abundance and diversity in gorgonians [22, 29–32] and other marine organisms [25, 33–40] over space and time in this and other studies emphasizes that they are important symbionts. In fact, selection for a stable *Endozoicomonas*-dominated bacterial community by Mediterranean gorgonians was demonstrated in a temporal study of the microbiome of *P. clavata* [59], detecting no *Endozoicomonas* spp. associated with this gorgonian during an environmental disturbance but demonstrating a return to an *Endozoicomonas*-dominated microbiome once the disturbance had subsided. Recent studies indicate that these bacteria may play roles in the digestion of complex molecules [36] and nutrient cycling [14] in scleractinian corals. In addition, it has been hypothesized that *Endozoicomonas* may be a key “architect microbe” that structures the microbiome [60], potentially through the use of antibiotics [14, 61] and the production of biofilm that allows other microbes to settle [34]. Even though the diversity of *Endozoicomonas* species associated with the gorgonians was high (669 of all 1512 OTUs), the preference of gorgonians, and some scleractinian corals [33], to be consistently associated with only a few of those phylotypes (13 OTUs; 0.86 %) is striking and suggests that these phylotypes are particularly beneficial to host health. While each OTU may have a specific role within the microbiome, the low abundance and rare associations of the vast majority of these OTUs raise doubts about their functional role in host-microbe symbiosis. One potential explanation might be that gorgonians maintain a diverse pool of low-abundance *Endozoicomonas* species with similar functions as the members of their core microbiome but different tolerance levels to environmental factors. This could allow the gorgonian to shuffle the membership of its microbiome in response to changing conditions that may compromise core microbe function and the physiological needs of the holobiont, without losing functionality. This principle would be similar to ‘symbiont shuffling’ observed in scleractinian corals [62], where corals, which generally harbour one dominant algal endosymbiont, actively regulate another algal symbiont type, which already exists in the coral tissues at low abundance, to become dominant in order to maintain its physiological requirements following or during disturbances to increase the stress tolerance of the holobiont.

In addition to Oceanospirillales, the core microbiome of the Mediterranean gorgonians analysed consisted of several other OTUs. *E. cavolini* harboured a member of clade BD1-7 of the Alteromonadaceae that had previously also been observed in this gorgonian [22], while *L. sarmentosa* and *P. clavata* consistently formed an association with an *Undibacterium* and members of the phylum NPL-UPA2, respectively. Although no functions for these bacteria are currently known, the consistent association of these microbes or their close relatives with gorgonians also suggest strong evolutionary symbiotic relationships.

### Local Differences in Gorgonian-Associated Microbiomes

Our spatial survey of gorgonian-associated bacterial communities showed that the microbiomes of *E. singularis*, *E. cavolini* and *L. sarmentosa* contained bacteria that were specifically associated with the gorgonians at each location. Overall, the microbiome of the *Eunicella* species in areas with limited to no human impacts was relatively consistent across an 1100-km distance, suggesting each species has strong affinities for a specific microbiome structure. However, local conditions near Portofino and Majorca appeared to cause an alteration in the microbial diversity. Disruptions of the balance between the main bacterial associates and the host animal have previously been observed in a range of scleractinian corals that are diseased [63], under stress [58, 60, 64, 65] or due to natural variability [66]. Several studies have linked these changes in coral-associated microbiomes to local environmental conditions, which may impair the coral's immune system [67] and thereby its ability to regulate the microbiome. For example, high nutrient availability has been shown to cause an increase in the abundance of denitrifying microbes [61, 68] and change the reef microbiome [69], which suggests local adaptation of the microbiome and supporting the probiotic hypothesis [70]. The most likely disturbing factor in Majorca and Portofino was an influx of freshwater containing land-based pollutants and other human impacts. For example, a submarine groundwater discharge was found to be present near the Majorca sampling site ([71]; Covadonga Orejas Saco del Valle, personal observation), and heavy rainfall occurred in the Portofino area during the sampling period (personal observation; [www.ilmeteo.it](http://www.ilmeteo.it)). In addition, the Portofino area experiences high pressure from diving tourism and sport fishing [48], which has been related to increased disease incidence in tropical corals [72, 73], as well as sediment runoff and untreated wastewaters from human impact areas and two river systems [74, 75]. The gorgonian-associated microbial communities at these locations harboured several bacteria that support this explanation: for example, *Undibacterium*, commonly found in freshwater [76, 77]; members of the Saprospiraceae, typical of eutrophic environments [60], and sewage bacteria belonging to the Enterobacteriaceae were present. In addition, increased turbidity and sedimentation at

Portofino (personal observation: CSM dive team) may have contributed to shifts in the gorgonian microbiomes [30, 78]. Based on the functional profiling of the *Eunicella singularis*-associated bacterial communities, the enrichment of genes involved in the degradation of xenobiotics in Portofino and Majorca suggest that the seawater may have been polluted. This increased abundance of bacteria capable of degrading xenobiotics may indicate adaptation of the microbiome to local conditions to deal with potentially toxic compounds. However, the increased metabolism and the amplified production of antimicrobial compounds in the microbiome could reduce the symbiotic nutrient distribution and disrupt microbiome structure further, respectively, pointing to impaired holobiont functionality.

While *Endozoicomonas* are the main constituents of the Mediterranean gorgonian microbiomes, their abundance and diversity was in particular affected by local conditions. Stable bacterial symbionts in scleractinian corals are generally rare and likely occupy host-constructed niches [79] that are less sensitive to the surrounding environment [64], but even the abundance of these stable symbionts was significantly impacted in our study. The loss of important *Endozoicomonas* symbionts, potentially involved in microbiome-structuring [60], antibiotic secretion [61] and nutrient cycling [14, 36], may have significant implications for host health and could contribute to disease development [48]. While it is still unclear what the effect of the shift in microbiome might be on gorgonian health, a reduced capability of the natural host microbiome to prevent the establishment of pathogens could explain why the microbiomes of gorgonians at the disturbed sites near Majorca and Portofino contained several potential pathogenic bacteria. For example, several *Vibrionaceae* OTUs were found on some gorgonians at these locations in our study and the *Vibrio* species have previously been implicated in disease outbreaks and mass mortality events of gorgonians [9, 10, 80, 81]. In addition, *Spiroplasma eriocheiris*, a known pathogen of the Chinese mitten crab [82], was found on *E. singularis* at Majorca. Overall, this suggests that the gorgonians did not possess a microbiome adapted to the local conditions, but rather that a disturbance caused a shift in the bacterial community that could have detrimental, but yet macroscopically invisible, effects on gorgonian health.

Compared to the *Eunicella* species, the microbiome of *L. sarmentosa* was significantly different at the disturbed sites and no increase in diversity was observed as is characteristic for disturbances. *Eunicella*-associated microbiomes were largely dominated by the core microbiome and stable on a spatial scale, while the core microbiome played a relatively minor role in *L. sarmentosa*. This could suggest that *L. sarmentosa* has a more flexible microbiome that allows for adjustments to local conditions and phenotypic plasticity [66], whereas the stable *Eunicella* microbiomes might limit the potential of these gorgonians for local adaptation.

## The Holobiont Model

One of the main questions in the field of coral microbial ecology is how the coral-associated microbial community composition is regulated. Recent studies on scleractinian corals have suggested that regulation of the bacterial community takes place on the individual biont level [64, 83], where the coral host shapes the bacterial community based on its metabolism [84] and the use of antimicrobial compounds, but local environmental conditions then select for its membership. Two theories have been formulated concerning the establishment and maintenance of the holobiont host-microbe communities: the holobiont model [11, 12] and the hologenome theory of evolution [85]. Although both models incorporate the assumptions of the formation of symbiotic associations among microbes and hosts, which can be influenced by environmental stress, the main difference is that the hologenome theory includes heredity, both directly, through vertical transmission from parent to offspring, and indirectly, through the release of microbes by the parents into the environment which can be subsequently acquired by their offspring. Transmission of bacteria between generations (i.e. heredity) requires that (1) these bacteria are ubiquitous and (2) there are strong co-evolutionary, or phyllosymbiotic, signals [86]. Here, we observed that the microbiomes of related gorgonians were more similar than those of more distantly related species, suggesting host animal specificity [11, 25] and evolutionary selection. While we did observe several bacterial associates consistently present on a spatial scale in each gorgonian species analysed (i.e. ubiquity, core microbiome), most of these bacteria, however, did not show phyllosymbiotic signals as they were not exclusive to a single gorgonian species. This lack of heredity, based on a highly conserved core microbiome among different gorgonian species, thus favours the holobiont model.

In conclusion, our data show that shallow Mediterranean gorgonians of the genus *Eunicella* possess a structured (core) microbiome dominated by *Endozoicomonas*, which is spatially stable but can be influenced by local conditions. In contrast, other gorgonians, such as *Leptogorgia sarmentosa*, have a less defined core microbiome. As no adverse health effects on gorgonian hosts had been observed, such changes could suggest adaptation to (temporal) differences in local environments, supported by the holobiont model.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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