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Seasonal Stability in the Microbiomes of Temperate Gorgonians and the Red Coral *Corallium rubrum* Across the Mediterranean Sea

Jeroen A. J. M. van de Water[1] · Christian R. Voolstra[2] · Cecile Rottier[1] · Silvia Cocito[3] · Andrea Peirano[3] · Denis Allemand[1] · Christine Ferrier-Pagès[1]

Abstract Populations of key benthic habitat-forming octocoral species have declined significantly in the Mediterranean Sea due to mass mortality events caused by microbial disease outbreaks linked to high summer seawater temperatures. Recently, we showed that the microbial communities of these octocorals are relatively structured; however, our knowledge on the seasonal dynamics of these microbiomes is still limited. To investigate their seasonal stability, we collected four soft gorgonian species (*Eunicella singularis*, *Eunicella cavolini*, *Eunicella verrucosa* and *Leptogorgia sarmentosa*) and the precious red coral (*Corallium rubrum*) from two coastal locations with different terrestrial impact levels in the Mediterranean Sea, and used next-generation amplicon sequencing of the 16S rRNA gene. The microbiomes of all soft gorgonian species were dominated by the same 'core microbiome' bacteria belonging to the *Endozoicomonas* and the Cellvibrionales clade BD1-7, whereas the red coral microbiome was primarily composed of 'core' Spirochaetes, Oceanospirillales ME2 and Parcubacteria. The associations with these bacterial taxa were relatively consistent over time at each location for each octocoral species. However, differences in microbiome composition and seasonal dynamics were observed between locations and could primarily be attributed to locally variant bacteria. Overall, our data provide further evidence of the intricate symbiotic relationships that exist between Mediterranean octocorals and their associated microbes, which are ancient and highly conserved over both space and time, and suggest regulation of the microbiome composition by the host, depending on local conditions.

Keywords *Endozoicomonas* · Spirochaetes · Red coral · Gorgonian · 16S rRNA gene · Bacterial community · Holobiont · Evolution · Microbiome · Symbiosis

✉ Jeroen A. J. M. van de Water
jvdewater@centrescientifique.mc

[1] Centre Scientifique de Monaco, 8 Quai Antoine 1er, 98000 Monaco, Monaco

[2] Red Sea Research Center, Division of Biological and Environmental Science and Engineering (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia

[3] Marine Environment Research Centre, ENEA, La Spezia, Italy

Introduction

The 'holobiont' is a collective term for the inter-kingdom assemblages of host organisms and their microbial symbionts, including bacteria, archaea, viruses, fungi and algae. Bacterial symbioses have been recognized as essential for the health of eukaryotes and are integral to their adaptation and acclimation potential to environmental change [1, 2]. Increasing research efforts have endeavoured to identify the specific and obligate symbioses between prokaryotes and corals, among the thousands of bacterial phylotypes associated with individual coral colonies [3]. Recent studies have shown that the coral holobiont contains a 'core microbiome' [4, 5], consisting of microbes that are consistently found within a species, as well as microbes whose presence depends on local conditions, and transient microbial associates [6, 7]. While the functional roles of the cnidarian-associated microbiota are still largely unknown, they have been implicated in many services beneficial to the host, such as nitrogen fixation [8, 9], sulphur-cycling

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[10] and microbiome regulation through the secretion of antibiotics [11–14] and exclusion of pathogens by occupying available microbial niches [15, 16]. As such, it is essential for holobiont fitness to maintain a stable multi-functional microbial community. In contrast to the highly diverse microbiomes of tropical corals, Mediterranean octocorals have been shown to harbour highly structured bacterial assemblages dominated for >90% by only a few species [17, 18], making them ideal model organisms to study cnidarian-microbe interactions.

In the Mediterranean Sea, gorgonians are the most important habitat-forming species of benthic communities, contributing significantly to the structural complexity, biomass and biodiversity of these ecosystems [19]. The success of gorgonians can at least in part be attributed to the specific symbioses with bacteria, which were recently found to be relatively stable across spatial scales [7, 18]. In species belonging to the Gorgoniidae family, bacteria from the genus *Endozoicomonas* have been found to be the most prevalent [20–23], with some lesser contributions from clade BD1-7 [7]. *Endozoicomonas* have been found in numerous marine invertebrates and have been identified as crucial to the health of corals (expertly reviewed in [24]), with a loss of these bacteria having a severe negative impact on holobiont functioning. In tropical octocorals and deep-sea gorgonians, especially the precious red coral *Corallium rubrum*, Spirochaetes may be one of the (co-)dominant microbial associates [18, 25, 26], which may play roles in nitrogen and carbon fixation, as seen in the Spirochaete-termite symbiosis [27, 28]. The spatial stability of these bacteria-host associations, which may reside in the same habitat and location, suggests strong selection mechanisms employed by the holobiont of Mediterranean gorgonians. Indeed, gorgonian tissue extracts are known to interfere with bacterial quorum-sensing and have potent antimicrobial activity [29–32]. Local disturbances and elevated seawater temperatures have, however, been linked to disruptions in the microbiome of gorgonians [7, 33]. Recent mass mortality events caused by microbial disease outbreaks and thermal anomalies have resulted in significant population declines [34, 35]. This suggests that shifts in the gorgonian microbiome towards an 'unhealthy', dysbiotic state could occur on a seasonal scale, particularly during the summer months. Mediterranean octocorals experience large variations in seawater temperatures, from a minimum of 12 °C in winter to at least 23 °C in summer [36] and may experience significant river discharges, which brings sediment, turbidity and pollution, following rain in coastal areas, particularly in spring and autumn. It is therefore important to understand the seasonal variability in the gorgonian-associated bacterial assemblages or determine whether it is as stable as across spatial scales.

Here, we describe the temporal patterns in the microbiome of five closely (genera *Eunicella* and *Leptogorgia*) and distantly (genus *Corallium*) related octocorals from the Mediterranean Sea at a clear and turbid location. We show (1) that the microbiomes associated with octocorals are distinct from the microbial communities in the surrounding seawater and (2) that the highly structured bacterial assemblages are stable over time, but that local conditions may influence microbial community composition. Overall, our results provide new insights into the unique, specific associations between gorgonians and their microbial symbionts.

Material and Methods

Study Species and Sampling Regime

Octocoral samples were collected at 30–40 m depth near Cassis, France (43° 12′ N 05° 28′ E) and at 16–20 m depth on Dante Shoal near La Spezia, Italy (44° 01′ N 09° 50′ E) every 3 months (±1–2 weeks) between May 2014 and July 2015 (Fig. 1; Table 1). The La Spezia sampling location is characterized by high turbidity, which is highest in spring-summer and the lowest in fall-winter (total suspended solids concentration ranging between 1.61 and 2.65 mg L^{-1} [37]) and is the result of terrestrial runoff from the Magra River and sewage from the city of La Spezia, both of which get caught up in the westward Ligurian current [38]. In contrast, the sampling location near Cassis is not impacted by terrestrial runoff, as no river is present within a 60 km radius, and sewage input from the town of ~7000 inhabitants is limited, corresponding with low turbidity levels. While *Eunicella singularis*, *Eunicella verrucosa* and *Leptogorgia sarmentosa* were sampled at both locations; samples of *Corallium rubrum* and *Eunicella cavolini* were only obtained near Cassis as these species were not present at the La Spezia location. Fragments of five visually healthy colonies that did not show any macroscopic signs of epibiosis or partial mortality, were collected at each location and time point, with the exception of July 2015 when only three replicate fragments were collected at Cassis. Samples were rinsed twice with 0.2-μm filtered seawater to remove exogenous, loosely associated microorganisms and stored in ice-cold RNAlater (ThermoFisher Scientific) at 4 °C. In addition, three samples of 2 l of seawater each were collected next to the octocoral colonies and filtered sequentially through 8, 3 and 0.2-μm Whatman Nuclepore Track-Etched filters (Sigma-Aldrich), and the retentate was kept in RNAlater at 4 °C.

DNA Extraction and 16S rRNA Gene Amplicon Library Preparation

To extract DNA from tissues and seawater filter retentate, we used the Genomic DNA buffer set and Genomic-tip 20/G columns (QIAGEN, Hilden, Germany) following the manufacturer's sample preparation and lysis protocol for tissues—

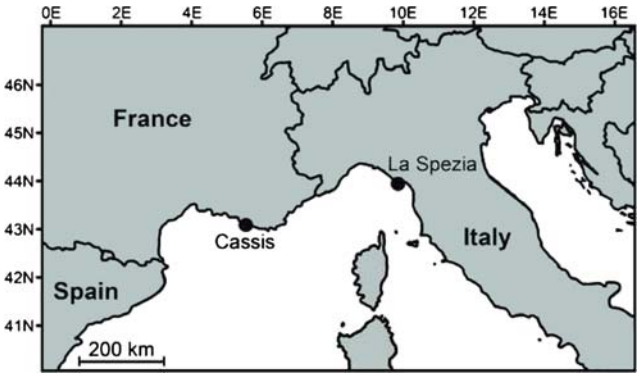

Fig. 1 Overview of the sampling locations. Gorgonians were collected from the Western Mediterranean Sea at two locations: Cassis in France and La Spezia in Italy

octocoral fragments and filters were placed directly in lysis buffer. For each soft gorgonian sample, 16S rDNA amplicon libraries were generated in triplicate using the Multiplex PCR kit (QIAGEN, Hilden, Germany) and the 784F/1061R primer set [39] in 30-μl reaction volumes containing 0.2 μM of each primer and 150 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 Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) and sequenced on the Illumina MiSeq platform.

To allow for direct comparisons with our previous study on spatial patterns in the *C. rubrum* microbiome, sequencing libraries of the red coral DNA samples were prepared with the 8F/334R primer set targeting the V1/V2 regions of the 16S rRNA gene [40] and were sequenced on the Illumina MiSeq platform at the Molecular Research LP laboratory (Shallowater, Texas, USA).

16S rRNA Gene Amplicon Data Analysis

The QIIME pipeline [41] was used for data processing. Paired-end MiSeq sequencing of the 176 samples of gorgonians generated 24,131,112 reads. Forward and reverse reads were merged using SeqPrep (https://github.com/jstjohn/SeqPrep), producing 9,539,086 joined reads (78.5%). Primer sequences were removed using extract_barcodes.py and the split_libraries.py script was used to remove low quality (Phred <20) sequences, reads <200 bp in length, and to assign each read to its respective sample. The resulting file was checked for chimeric sequences against the SILVA v123 database [42] using UCHIME [43]. The final quality-filtered sequence file contained 9,230,759 reads with a mean read length of 257 ± 8 base pairs. Operational taxonomic units (OTUs) were defined at the level of 97% similarity followed by taxonomy assignments against the SILVA reference database (version 123) using the UCLUST algorithm [44]. Singletons, unassigned OTUs, and OTUs classified as chloroplast or mitochondria were removed from the dataset. The OTU table was rarefied to 15,587 reads for comparisons across species, locations and time points. Alpha diversity metrics (total observed number of OTUs, predicted species (chao1), Simpson's evenness, Shannon-Wiener diversity and Fisher's alpha) were generated from OTU tables using the QIIME pipeline. In addition, the overall gorgonian core microbiome as well as the core microbiome of each species and the communities of locally stable bacterial associates were analysed. The complete dataset has been deposited in the NCBI Sequence Read Archive (SRA) with accession number SRP107866.

Table 1 Overview of the number of samples collected per species and of seawater at each location and sampling time point

		2014			2015	
Location	Species/seawater	Spring	Summer	Winter	Early spring	Late spring
Cassis	*Corallium rubrum*	2	5	5	5	3
	Eunicella cavolini	5	5	5	5	3
	Eunicella singularis	5	5	5	5	3
	Eunicella verrucosa	5	5	5	5	3
	Leptogorgia sarmentosa	5	5	5	5	3
	Seawater	3	3	3	3	3
La Spezia	*Eunicella singularis*	5	5	5	5	
	Eunicella verrucosa	5	5	5	5	
	Leptogorgia sarmentosa	5	5	5	5	
	Seawater	3	3	3	3	

Sequencing data from MR DNA (Shallowater, Texas, USA) was obtained as pre-merged and re-oriented sequence .fasta files and their associated quality .qual files. Data files contained 3,845,860 paired-end reads, which were quality filtered and chimera checked producing 3,280,553 reads with a mean length of 322 ± 15 base pairs. Quality filtering, OTU picking, taxonomy assignments and subsequent analyses were conducted as described above. This dataset has been deposited under accession number SRP107893 in the NCBI SRA.

Statistical Analysis

The phyloseq package [45] integrated in *R* was used to generate statistically relevant graphical presentations of the microbiome data obtained. To visualise differences between gorgonian-associated bacterial communities, non-metric dimensional scaling (nMDS) on Bray-Curtis dissimilarity matrices was performed. Ellipses representing the 95% confidence intervals were added to nMDS plots if >3 biological replicates were obtained. A heatmap was generated to present the average abundances of core microbiome OTUs within the holobiont of each gorgonian species at both locations over the different time points. Permutational analysis of variance (permANOVA) and pair-wise comparisons, with Monte Carlo simulations conducted in parallel, were performed under Type III partial sums of squares and 9999 permutations under the reduced model to test for differences in the beta diversity (based on the Bray-Curtis dissimilarity matrices) among bacterial communities over time at the two different locations for each gorgonian host species. To test for temporal variations in the microbial communities near Cassis, five sampling 'time' factor levels were considered and four levels at La Spezia. To assess differences in community diversity between locations at each sampling time point, comparisons were only performed for the four seasons, where samples had been collected at both locations. To provide an overall assessment of differences in the alpha diversity of the microbiomes, permANOVA and pair-wise comparisons were conducted (with the same settings as described above) based on a Euclidean distance matrix. The matrix was generated by considering all alpha diversity metrics as outcome variables, square root transformation of the data, and subsequent calculation of Euclidean distances between samples. All diversity analyses were conducted using PRIMER 6 and PERMANOVA+ (PRIMER-E Ltd) and differences were considered significant when $p < 0.05$. Differential abundance analysis was performed to investigate which OTUs were responsible for the observed differences in microbiome composition using negative binomial modelling (likelihood ratio tests to test for factors significantly impacting microbiome composition and Wald tests for pair-wise comparisons) using the DESeq2 package in *R* [46].

Nucleotide Sequence Accession Numbers

The sequences of OTUs belonging to the core microbiome and locally stable microbial associate communities found in this study were deposited in the GenBank database under accession numbers MF148323–MF148422.

Results

Assessment of the Bacterial Diversity in Soft Gorgonians

Consistent with earlier reports [7, 18], the composition of bacterial communities associated with soft gorgonians (Fig. S1A) was significantly different from bacterial communities in seawater at all time points at both sites, i.e. Cassis and La Spezia ($p < 0.03$; Suppl. File S1–Table S1 and S2). In general, the temporal stability/differences in the octocoral-associated microbiomes significantly depended on the host species and the sampling location (Supplementary File S1–Table S1). Microbiomes of the different gorgonians were overall species-specific, although no major differences were observed (I) between the microbiomes of *E. cavolini* and *E. singularis* at Cassis, (II) among any gorgonian species in the late spring of 2015 at Cassis and (III) between *E. singularis* and *E. verrucosa* in the summer of 2014 and the winter 2014/2015 at La Spezia (Suppl. File S1–Table S1 and S2). In addition, we found that the bacterial assemblages of *E. verrucosa* and *L. sarmentosa* differed significantly between the two locations at all time points, while such differences were observed for *E. singularis* only in the winter 2014/2015 and early spring of 2015 (Fig. 2; Suppl. File S1–Table S3). On temporal scales, however, the beta diversity of the microbiomes of all gorgonians was relatively stable at Cassis, with the only significant difference observed between the spring of 2014 and winter 2014/2015 in both *E. singularis* and *L. sarmentosa* (Fig. 2; Suppl. File S1–Table S4). In La Spezia, no significant temporal differences were observed in the microbiome diversity of *Eunicella* spp., but significant microbiome changes were observed in *L. sarmentosa* between spring sampling time points and the summer and winter time points (Fig. 2; Suppl. File S1–Table S4). In contrast to the differences in beta diversity, no major differences in alpha diversity were observed between gorgonian host species, but the alpha diversity in seawater was generally higher than in gorgonian microbiomes (Table 2; Suppl. File S1–Table S2).

Composition of the Gorgonian Microbiome

The microbiome of all soft gorgonians was largely dominated by Gammaproteobacteria and Alphaproteobacteria (Suppl. File S 2). Bacteria belonging to other taxonomic classes, including Mollicutes, Flavobacteriia, Spirochaetes, Cytophagia and Sphingobacteria contributed to a lesser extend to the

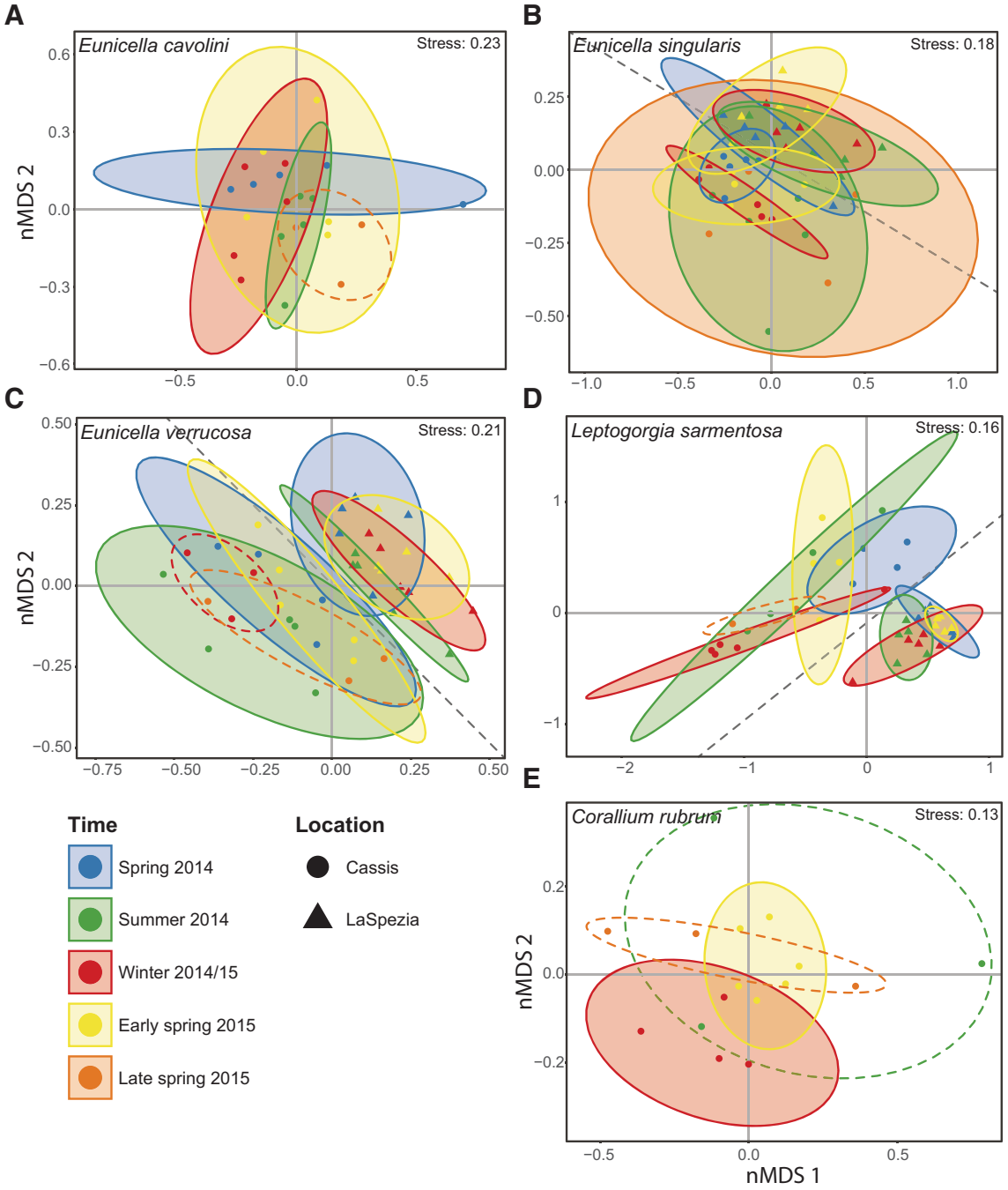

Fig. 2 Seasonal patterns in microbial diversity associated with Mediterranean octocorals. **a**–**e** Beta diversity of the microbiome is presented in a non-metric dimensional scaling plot based on Bray-Curtis dissimilarity matrices for different octocoral species **a** *Eunicella cavolini*, **b** *E. singularis*, **c** *E. verrucosa*, **d** *Leptogorgia sarmentosa* and **e** *Corallium rubrum*. Locations are indicated with *symbols*, and *time points* with colour. *Ellipses* with *solid lines* indicate the 95% confidence interval. *Ellipses* with *dotted lines* were added for illustrative purpose as sample replicate number was ≤3

gorgonian microbiome, but their abundances did change depending on host species and time of year (Suppl. File S2). The main Gammaproteobacteria found associated with all gorgonians were bacteria belonging to the genus *Endozoicomonas* (order Oceanospirillales) and the genus 'clade BD1-7' (order Cellvibrionales) (Fig. 3). Analysis of the core microbiome (OTUs present in 100% of the samples of a species), which dominated the microbiome of all gorgonians, found that three OTUs (*Endozoicomonas* OTU47380 and OTU69149; *BD1*-7 OTU20874) were (1) consistently associated with all gorgonian host species across space and time, (2) the main contributors to the gorgonian microbiome and (3) were identical to core microbes previously identified (accession number KU738805, KU738801 and KU738792 [7], respectively)

Table 2 Alpha diversity metrics (mean ± SEM) for samples of five gorgonian species collected at the two different locations throughout the year

			Alpha diversity metric				
Species	Location	Time point	chao1	Shannon–Wiener index	Simpson's evenness	Fisher's alpha	Unique OTUs
E. cavolini	Cassis	Spring 2014	1088 ± 84	1.71 ± 0.46	0.01 ± 0.00	54.9 ± 17.2	302 ± 75
		Summer 2014	769 ± 89	2.11 ± 0.34	0.01 ± 0.00	44.8 ± 4.5	261 ± 22
		Winter 2014/2015	715 ± 92	1.21 ± 0.05	0.01 ± 0.00	31.6 ± 2.7	195 ± 14
		Early spring 2015	876 ± 54	2.11 ± 0.47	0.01 ± 0.00	48.0 ± 9.5	275 ± 46
		Late spring 2015	836 ± 93	2.56 ± 0.37	0.01 ± 0.00	56.3 ± 8.4	315 ± 39
E. verrucosa	Cassis	Spring 2014	841 ± 105	2.33 ± 0.56	0.01 ± 0.00	70.9 ± 15.8	377 ± 70
		Summer 2014	746 ± 117	1.44 ± 0.39	0.00 ± 0.00	67.7 ± 14.6	362 ± 66
		Winter 2014/2015	497 ± 40	0.76 ± 0.28	0.01 ± 0.00	37.5 ± 3.6	226 ± 18
		Early spring 2015	871 ± 115	1.97 ± 0.50	0.00 ± 0.00	80.8 ± 17.2	418 ± 72
		Late spring 2015	952 ± 244	2.32 ± 1.18	0.01 ± 0.00	102.5 ± 34.4	502 ± 147
	La Spezia	Spring 2014	884 ± 176	3.97 ± 0.50	0.01 ± 0.00	113.0 ± 27.1	546 ± 104
		Summer 2014	1071 ± 128	4.53 ± 0.62	0.01 ± 0.00	145.2 ± 17.2	677 ± 63
		Winter 2014/2015	1276 ± 74	4.73 ± 0.59	0.01 ± 0.00	174.8 ± 15.3	784 ± 54
		Early spring 2015	1060 ± 88	3.45 ± 0.33	0.01 ± 0.00	104.1 ± 9.5	521 ± 38
L. sarmentosa	Cassis	Spring 2014	848 ± 39	3.36 ± 0.27	0.01 ± 0.00	75.4 ± 9.1	401 ± 40
		Summer 2014	1334 ± 493	3.64 ± 0.87	0.01 ± 0.00	190.4 ± 83.2	789 ± 278
		Winter 2014/2015	2591 ± 521	5.91 ± 1.18	0.01 ± 0.01	476.0 ± 118.2	1605 ± 334
		Early spring 2015	1140 ± 175	3.25 ± 0.32	0.01 ± 0.00	109.2 ± 16.6	539 ± 66
		Late spring 2015	2146 ± 550	5.35 ± 1.78	0.01 ± 0.01	338.8 ± 124.7	1282 ± 361
	La Spezia	Spring 2014	699 ± 48	2.58 ± 0.11	0.01 ± 0.00	49.3 ± 3.5	283 ± 17
		Summer 2014	973 ± 60	3.23 ± 0.21	0.01 ± 0.00	78.9 ± 4.3	417 ± 18
		Winter 2014/2015	896 ± 109	3.30 ± 0.18	0.02 ± 0.00	67.7 ± 12.9	364 ± 57
		Early spring 2015	717 ± 29	2.73 ± 0.12	0.01 ± 0.00	51.0 ± 2.9	292 ± 14
E. singularis	Cassis	Spring 2014	826 ± 99	1.88 ± 0.22	0.01 ± 0.00	42.6 ± 4.6	251 ± 22
		Summer 2014	805 ± 171	2.87 ± 0.33	0.01 ± 0.00	65.4 ± 15.4	351 ± 70
		Winter 2014/2015	758 ± 62	1.96 ± 0.17	0.01 ± 0.00	45.8 ± 8.4	264 ± 41
		Early spring 2015	772 ± 128	2.03 ± 0.25	0.01 ± 0.00	51.6 ± 14.5	290 ± 65
		Late spring 2015	808 ± 121	3.01 ± 0.95	0.01 ± 0.00	74.3 ± 28.3	383 ± 119
	La Spezia	Spring 2014	982 ± 162	2.02 ± 0.46	0.01 ± 0.00	63.0 ± 20.6	336 ± 88
		Summer 2014	1146 ± 121	4.16 ± 0.76	0.01 ± 0.00	117.0 ± 25.4	561 ± 102
		Winter 2014/2015	1072 ± 146	3.11 ± 0.74	0.01 ± 0.00	86.5 ± 23.9	438 ± 94
		Early spring 2015	859 ± 67	1.95 ± 0.22	0.01 ± 0.00	48.3 ± 7.4	277 ± 35
Seawater	Cassis	Spring 2014	2122 ± 124	6.30 ± 0.03	0.03 ± 0.00	225.1 ± 12.4	956 ± 40
		Summer 2014	2172 ± 140	6.70 ± 0.05	0.04 ± 0.00	225.2 ± 18.4	956 ± 61
		Winter 2014/2015	1371 ± 66	7.07 ± 0.01	0.08 ± 0.00	171.7 ± 5.7	776 ± 20
		Early spring 2015	1717 ± 49	6.68 ± 0.05	0.05 ± 0.01	182.6 ± 4.5	814 ± 16
		Late spring 2015	2327 ± 166	6.77 ± 0.16	0.04 ± 0.00	239.5 ± 28.2	1001 ± 89
	La Spezia	Spring 2014	1238 ± 105	6.38 ± 0.17	0.04 ± 0.00	151.9 ± 16.7	703 ± 60
		Summer 2014	1726 ± 185	6.43 ± 0.06	0.04 ± 0.00	186.9 ± 14.6	828 ± 50
		Winter 2014/2015	1633 ± 358	6.99 ± 0.03	0.06 ± 0.01	208.4 ± 34.8	896 ± 116
		Early spring 2015	2021 ± 238	6.64 ± 0.13	0.03 ± 0.00	235.4 ± 23.8	988 ± 78
C. rubrum	Cassis	Summer 2014	1660 ± 80	2.32 ± 0.18	0.01 ± 0.00	98.3 ± 2.9	608 ± 15
		Winter 2014/2015	1575 ± 121	2.68 ± 0.10	0.01 ± 0.00	94.6 ± 7.0	588 ± 36
		Early spring 2015	1563 ± 70	2.86 ± 0.08	0.01 ± 0.00	92.2 ± 4.0	575 ± 21
		Late spring 2015	1496 ± 122	2.62 ± 0.16	0.01 ± 0.00	89.4 ± 3.8	561 ± 20
Seawater	Cassis	Summer 2014	2931 ± 468	6.65 ± 0.06	0.01 ± 0.00	327.8 ± 66.5	1627 ± 266
		Winter 2014/2015	2595 ± 68	6.98 ± 0.08	0.01 ± 0.00	335.6 ± 19.3	1664 ± 77
		Early spring 2015	3629 ± 5	6.71 ± 0.08	0.01 ± 0.00	441.5 ± 2.2	2069 ± 8
		Late spring 2015	3647 ± 343	6.67 ± 0.07	0.01 ± 0.00	430.2 ± 25.1	2026 ± 93

(Suppl. File S3). Interestingly, the abundances of these three OTUs seemed to vary significantly among host species and appear to depend on sampling location (Fig. 4; Suppl. File S3). Each host species also harboured a few additional low abundant core microbes (e.g. *Endozoicomonas* OTU53279 and OTU75183 in *E. cavolini* and *E. singularis*; OTU79173 in *L. sarmentosa*) (Fig. 4; Suppl. File 3). We also analysed which OTUs were consistently present within one host species in at least one sampling location (locally stable microbial associates [7]) to identify bacteria that may contribute to local adaptation, and found that those were in fact commonly found at low abundances in most (but not all) samples from several species at both locations (Fig. 4; Suppl. File S3). Overall, this shows that there is significant overlap in the (core) microbiome (here set conservatively at an OTU presence in 100% of samples of a species) membership among

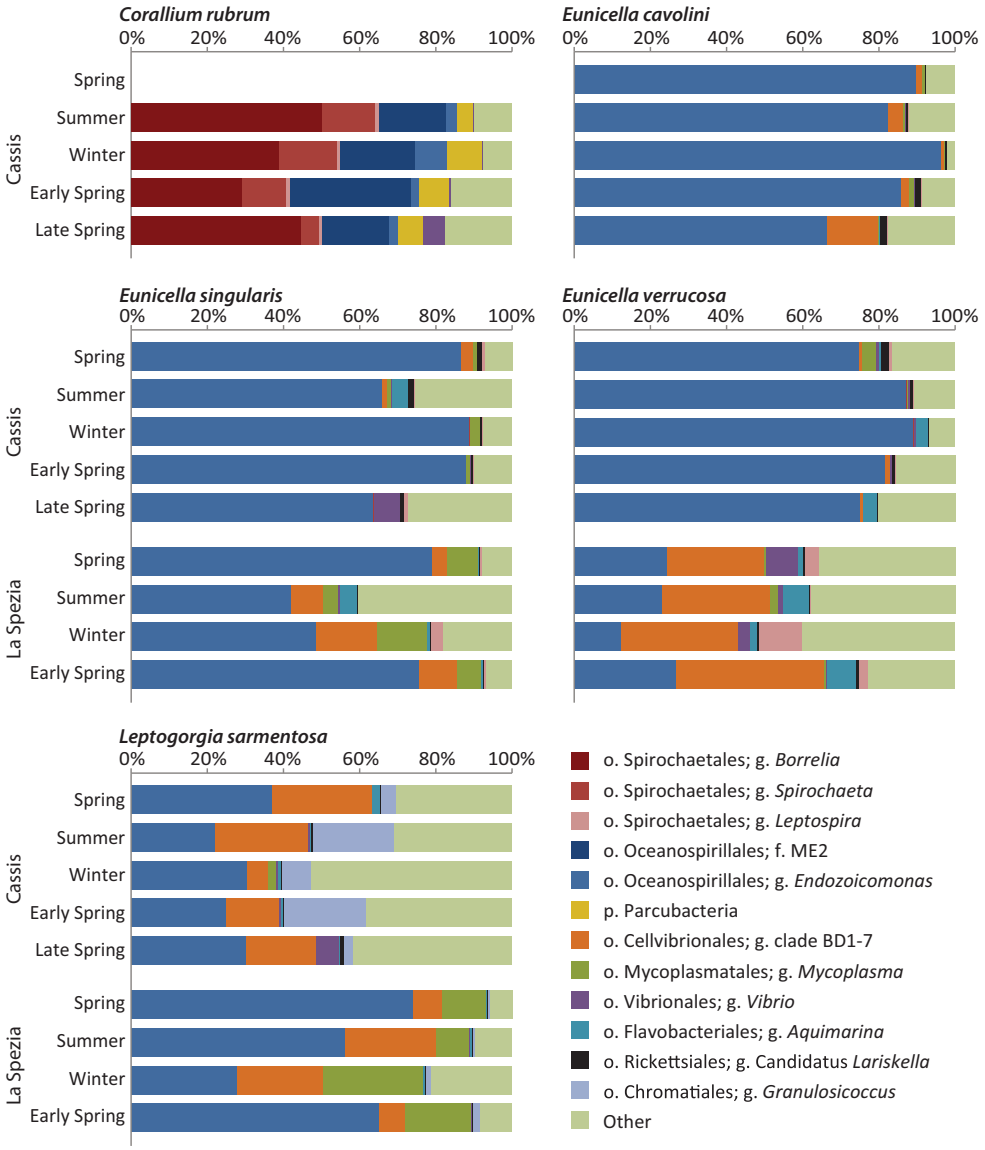

Fig. 3 Overview of the composition of the bacterial community associated with Mediterranean octocorals at two locations on seasonal scales. Microbiome community composition is presented at the genus level (or lowest possible taxonomic level (p. phylum; o. order; f. family; g. genus)) for *Corallium rubrum*, *Eunicella cavolini*, *E. singularis*, *E. verrucosa* and *Leptogorgia sarmentosa*. The contribution of each taxon is indicated in percentages (%)

Mediterranean gorgonians. Examples of locally stable microbial associates of gorgonians belonged to the genera *Mycoplasma*, *Vibrio* (incl. 100% match to the coral pathogen *V. shiloi*) and *Leptospira* as well as some not clearly defined candidate genera (*Lariskella*, *Hepatoplasma*, *Fritschea*) and members of the Chromatiales-family Granulosicoccus and the order Flavobacterales (Fig. 3; Suppl. File S3).

Temporal Changes in Gorgonian Microbiome Composition

Despite its general dominance, the contributions of the core microbiomes changed over time in all gorgonian host species, but no consistent patterns were observed (Suppl. File S3). Specifically, the contributions of *E. cavolini* and *E. singularis* core microbiomes were reduced in summer and late spring in Cassis, but only in summer in La Spezia. In *E. verrucosa*, the changes were minor, although the core microbiome was more abundant in Cassis compared to La Spezia. The opposite was observed, however, in *L. sarmentosa*, which also showed reduced core microbiome contributions in winter. When also considering the locally stable microbial associates, we found that this particularly affected the microbiome of *L. sarmentosa* (Suppl. File S3).

At Cassis, in-depth analysis of temporal changes in the microbiomes using differential OTU abundance analysis, showed very few changes in the relative amount of specific OTUs in *Eunicella* spp. among time points (Suppl. File S4), which was consistent with the patterns in beta diversity. The most notable difference was the significant increase in the OTU corresponding to *Vibrio shiloi* (OTU64664) in the bacterial community of *E. singularis* in the late spring of 2015 compared to the other time points (Figs. 3 and 4; Suppl. File S4). A similar, but non-significant, trend in this OTU's presence was also observed in *L. sarmentosa* (Fig. 4). Comparisons of the *L. sarmentosa*

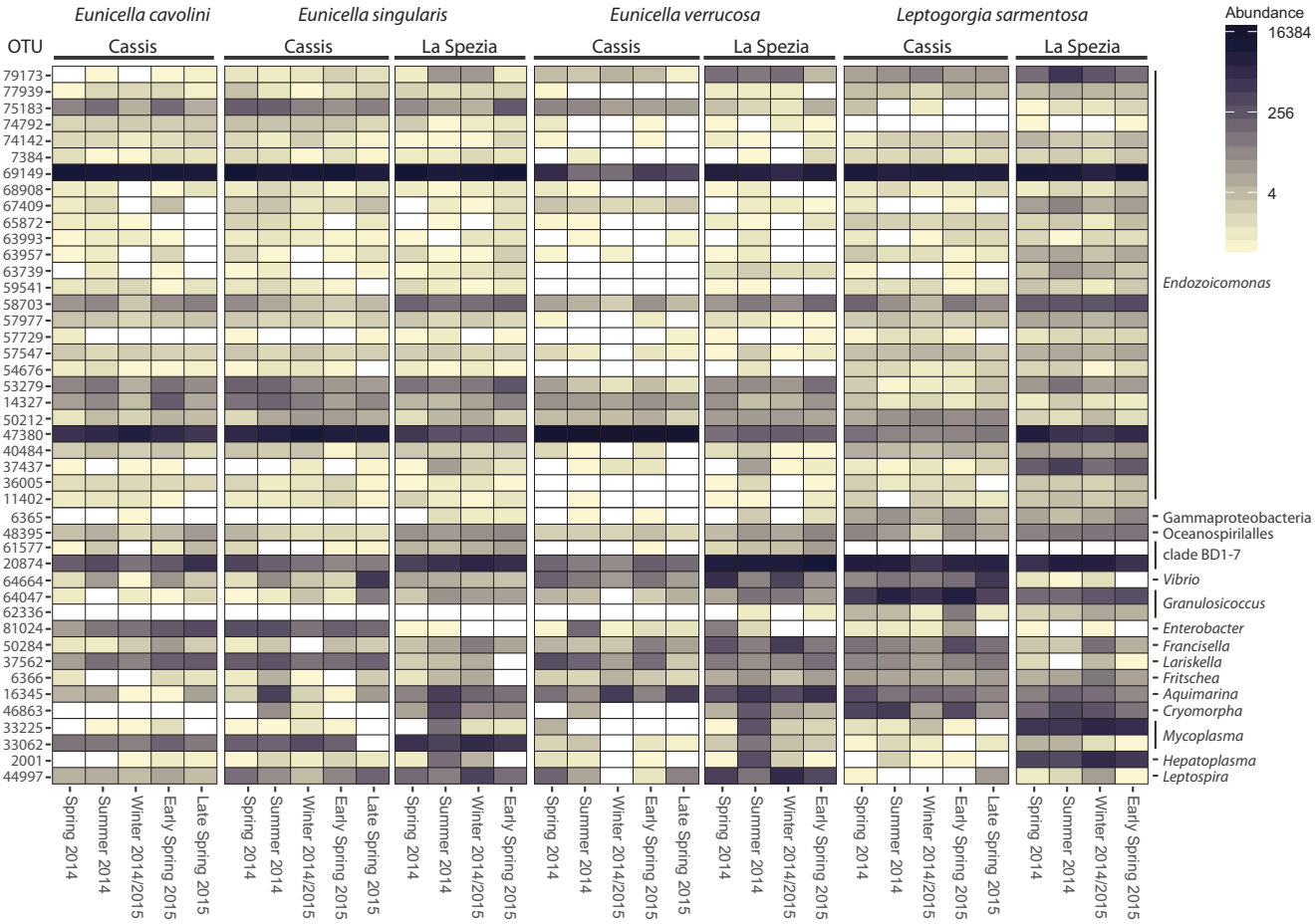

Fig. 4 Seasonal patterns in abundance of core and locally variant microbes of Mediterranean soft gorgonians. Heatmap presents the abundance of OTUs considered as part of the core microbiome and locally stable microbial associates of the gorgonians *Eunicella cavolini*, *E. singularis*, *E. verrucosa*, *Leptogorgia sarmentosa* at two different locations—Cassis and La Spezia. The taxonomy of each OTU is given at the genus level (or lowest possible taxonomic level)

microbiome among all time points revealed some changes in the relative numbers of some generally low abundant OTUs, particularly between spring and winter, explaining the change in beta diversity observed. Between spring 2014 and winter 2014/2015, 62 OTUs were found to be differentially abundant, and 22 OTUs between the winter 2014/2015 and early spring 2015 (Suppl. File S4). The main patterns observed were (1) a decreased abundance of five OTUs commonly associated with gorgonians (Oceanospirillales (OTU48395), clade BD1-7 (OTU58703), Granulosicoccus (OTU62336), *Francisella* (OTU50284) and JTB225 (OTU21753) in winter and (2) an increase in OTUs that are related to the genera *Caedibacter*, *Francisella* and likely *Coxiella* in spring (Fig. 4; Suppl. File S4).

Although no seasonal changes in microbiome beta diversity was observed in *Eunicella* spp. in La Spezia, numerous OTUs were differentially abundant between seasons (Fig. 4; Suppl. File S4). Most notably, the abundances of common *Mycoplasma* and *Hepatoplasma* OTUs were higher in summer, despite an apparent decrease in *Mycoplasma* abundance (Fig. 3), while the common *Leptospira* (OTU44997) was reduced in abundance at this time. Similarly, we observed an increase in abundance of a *Vibrio* OTU (OTU49555) in summer, despite a reduction in the number of a common *Vibrio* OTU (OTU64664). In addition, the numbers of various Enterobacterales species were found to be significantly increased in spring compared with summer and winter. Despite the observed differences in beta diversity in the *L. sarmentosa* microbiome between spring on the one hand and summer and winter on the other hand at La Spezia, the number of differentially abundant OTUs was rather limited. In fact, the main drivers were the core *Endozoicomonas* and Cellvibrionales OTUs. Of particular interest was the lower abundance of *Endozoicomonas* OTU79173 and 'clade BD1-7' OTU20874 versus the increased numbers of *Endozoicomonas* OTU47380 and OTU69149 during spring time.

Spatial Differences in Gorgonian Microbiome Composition

Differential OTU abundance analysis showed that there were also major differences in the microbiomes of gorgonians

between Cassis and La Spezia, corroborating the beta diversity results. However, the differences in the *E. verrucosa*-associated bacterial community (109 differentially abundant OTUs) could primarily be attributed to a 123-fold reduced abundance of the generally most dominant *Endozoicomonas* in Mediterranean gorgonians (OTU47380) and an increase in the relative numbers of other common microbial associates, such as 'clade BD1-7' (OTU20874), *Endozoicomonas* (OTU69149), *Leptospira* (OTU44997) and *Aquimarina* (OTU16345) as well as numerous low abundant OTUs, including various other *Endozoicomonas* and Cellvibrionales OTUs plus multiple Legionellales and Enterobacterales (Fig. 4; Suppl. Files 3 and 4). Similar spatial differences were observed in the microbiome of *E. singularis* (42 differentially abundant OTUs), with the particular notion that there was a major increase in the abundance of *Mycoplasma* (OTU33062) at La Spezia compared with Cassis (Fig. 4; Suppl. Files 3 and 4). *L. sarmentosa* (528 differentially abundant OTUs) also showed a high abundance of the two Mollicutes genera *Mycoplasma*, although these were different OTUs (most dominant OTU23384 and OTU33225), and *Hepatoplasma* (OTU2001) at La Spezia (Fig. 4; Suppl. Files 3 and 4). In contrast to *Eunicella* spp., the microbiome of *L. sarmentosa* contained relatively higher numbers of the main *Endozoicomonas* OTUs (OTU47380 and OTU69149) at La Spezia, while OTUs identified as *Vibrio* and Chromatiales were significantly more abundant in Cassis (Fig. 4; Suppl. Files 3 and 4).

Composition and Temporal Patterns of the Microbiome of the Red Coral *Corallium rubrum*

The diversity of the microbiome of *C. rubrum* was significantly different from seawater bacterial communities (1B, Table 2, Suppl. File S1–Table S1) at all time points (Suppl. File S1–Table S2). However, no significant changes in alpha and beta diversity were observed in the red coral's microbiome on a temporal scale (Fig. 2; Suppl. File S1–Table S4). Members of the classes Spirochaetes (order Spirochaetales) and Gammaproteobacteria (order Oceanospirillales) and the phylum Parcubacteria dominated the bacterial assemblages of *C. rubrum* (Fig. 3; Suppl. File S2). In-depth analysis revealed that 44 OTUs made up the core microbiome, of which only 7 represented 86–95% of the overall bacterial community (Suppl. File S3). These 7 OTUs were 1 Parcubacteria OTU, 3 Spirochaetales OTUs (1 Leptospiraceae, 2 Spirochaetaceae) and 3 Oceanospirillales (1 *Endozoicomonas*, 1 ME2 and 1 unclassified). All other core OTUs were very low abundant (<1%), but included 6 *Endozoicomonas* OTUs, 7 ME2 OTUs and 3 Spirochaetaceae OTUs as well as 1 Cellvibrionales BD1-7 OTU and 2 Propionibacterium OTUs (Suppl. File S3).

While no temporal changes in overall microbiome diversity were observed, few OTUs showed changes in abundance between seasons (Fig. 5; Suppl. File S4); particularly several Pseudoalteromonadaceae OTUs were more abundant during summer. Core microbes were generally stable in abundance over time, for example no Parcubacteria and Spirochaetales core OTU was found differentially abundant between seasons. However, some core Gammaproteobacteria did change in relative abundance, including the most abundant *Endozoicomonas* (OTU 30320), which was more abundant in winter compared to early spring, and the most abundant ME2 (OTU20697), which was more abundant in early spring compared to summer, and could explain the difference in ME2 and *Endozoicomonas* abundances observed at these time points (Figs. 3 and 5). In addition, a low abundant unclassified Gammaproteobacteria (OTU 4533) was generally found at higher abundance in spring than in winter or summer. All other OTUs found differentially abundant between seasons were primarily very low abundant Gammaproteobacteria that are not part of the core microbiome.

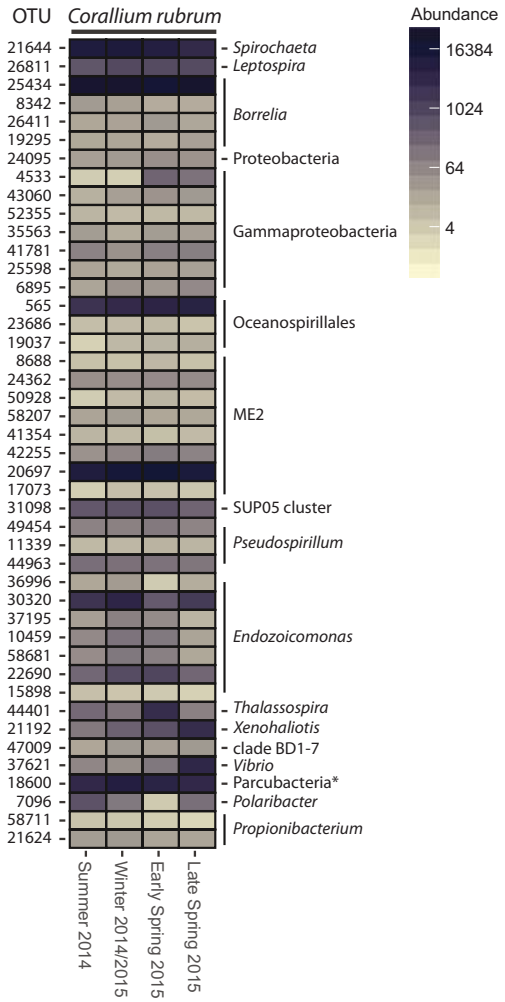

Fig. 5 Seasonal patterns in abundance of core microbes of the red coral *Corallium rubrum*. Heatmap presents the standardized abundance of core OTUs in the bacterial community associated with *C. rubrum* at Cassis on seasonal scales. The taxonomy of each OTU is given at the genus level (or lowest possible taxonomic level)

Discussion

In this study, we show that the microbiomes of five Mediterranean octocorals are highly structured, being dominated by their core microbes, and are relatively stable over time. The microbiomes of all soft gorgonian species showed significant overlap in bacterial associates, and were composed primarily of the same *Endozoicomonas* and Cellvibrionales BD1-7 bacteria. The red coral microbiome was dominated by Spirochaetales, the Oceanospirillales family ME2 and a member of the Parcubacteria, while *Endozoicomonas* played a relatively minor role. The temporal dynamics in the abundances of the gorgonian-associated bacteria appeared to largely depend on the location they lived at; however, it is unclear whether these are adverse impacts on holobiont physiology or adaptation to local conditions.

Core and Locally Stable Bacteria in the Microbiome of Mediterranean Soft Gorgonians

Consistent with our previous study on the spatial stability of the microbiomes of Mediterranean soft gorgonians [7], we show that the bacterial communities of *Eunicella* spp. and *Leptogorgia sarmentosa* are highly dominated by only a few bacterial species, particularly from the Oceanospirillales genus *Endozoicomonas* and the Cellvibrionales clade BD1-7. The order Cellvibrionales was proposed only relatively recent [47], and Alteromonadales clade BD1-7 bacteria found as core microbes in *Eunicella cavolini* [7] have now been assigned to this new order. In our current study, using higher sequencing depth than before, we actually found that this bacterium is consistently associated with all gorgonians of the Gorgoniidae family in the Mediterranean Sea, suggesting that it is crucial for the health of these holobionts. Although the function of these bacteria is currently still unknown, genetic studies have suggested that BD1-7 bacteria are oligotrophs and may have the potential to use alternative energy sources for mixotrophic growth, such as light for the generation of ATP via proteorhodopsin proton pump [48, 49].

The most dominant bacterial associates of all gorgonians from the Gorgoniidae family belonged to the genus *Endozoicomonas*. In fact, all species assessed in this study possessed the same main *Endozoicomonas* OTUs in their bacterial community, showing their particular importance for gorgonian holobiont functioning. These bacteria are often found in the microbiome of corals, gorgonians and other marine invertebrates, and are considered crucial for host health [23, 33, 50–54], via their involvement in microbiome structuring, through secretion of antibiotic and quorum-sensing compounds [3, 55–57], and nutrient cycling and acquisition [10, 20, 55, 58–61]. The relative stability of the association and abundance of each OTU show that local environmental changes had relatively little impact and suggest that they may be protectively harboured within the tissues of the host, as found in tropical corals and anemones [3, 62]. Whether the *Endozoicomonas* species found in our study are indeed present in large aggregates in the host tissues and which functions they perform, is currently under investigation.

Interestingly, we also found that one OTU matching the coral pathogen *Vibrio shiloi* was commonly present on the gorgonians. *Vibrio* species have previously been implicated in disease in both tropical and Mediterranean scleractinians and gorgonians. For example, strains of *V. coralliilyticus* have been identified as the causative agent of disease in the Mediterranean gorgonian *Paramuricea clavata* [34, 35] and in tropical corals [63, 64], while *V. shiloi* has been implicated in bacteria-induced bleaching in *Oculina patagonica* [65]. Recently, it was reported that *V. shiloi* was only found in disease lesions and is generally not associated with *O. patagonica*, and together with *V. coralliilyticus* synergistically causes disease [66]. However, we found *V. shiloi* to be consistently present with all gorgonians year-round at low abundances, although in the late spring of 2015 its abundance showed an increasing trend. As all gorgonians were visually healthy at the time of sampling, this suggests that this bacterium may be an opportunistic pathogen and only cause disease when the environmental conditions increase its virulence and the integrity of the holobiont is compromised.

Another interesting association was found between all Gorgoniidae species and OTUs of the Mollicutes genera *Mycoplasma* and *Hepatoplasma*; however, their abundance was particularly high in *L. sarmentosa* and *E. singularis* in La Spezia. Interestingly, the most abundant *Mycoplasma* OTU in *L. sarmentosa* was different from the one found at high abundance in *E. singularis*, suggesting host preference. While *Mycoplasma* are generally considered to be intracellular parasites of plants and animals, species associated with gorgonians [67–69] and the cold-water coral *Lophelia pertusa* [70, 71] have been suggested to be commensals or symbionts. In *L. pertusa*, for example, the *Mycoplasma* bacteria were found extracellularly adjacent to the coral's spirocysts, which lead to the hypothesis that they benefit from hemolymph components leaking from prey captured by the host, without affecting host health [71]. *Hepatoplasma* bacteria were relatively rare in Cassis, but more common at La Spezia, particularly in association with *L. sarmentosa*. Sequences related to *Hepatoplasma* have previously been found in various cnidarians, including *Nematostella vectensis* [72] and gorgonians [67]. Although the origin and function of these bacteria are unknown, extensive studies on *Hepatoplasma* in terrestrial isopods suggest they are ectosymbionts [73, 74]. While *Hepatoplasma* could thus be a symbiont of anthozoans aiding in nutrient metabolism of the holobiont, it cannot be excluded that this bacterium originates from the planktonic arthropods that gorgonians prey on.

We also observed that the gorgonians commonly interacted with Granulosicoccus, *Lariskella*, and *Fritschea* bacteria. While currently nothing is known about the role of these bacteria in cnidarian biology, they have previously been found in these

animals. For example, our Candidatus *Lariskella* OTU shared 100% homology with a Rickettsiales bacterium found in the freshwater *Hydra* [75], and members of this candidate genus have generally be considered endosymbionts in arthropods hosts [76]. The Chlamydiae Candidatus *Fritschea* has been discovered in tropical corals [50, 77], a jellyfish (Viver et al. unpublished) and other marine invertebrates [78, 79]. Members of the family Granulosicoccus (order Chromatiales) are often found associated with algae and seagrass, but 16S sequences matching 99 and 100% with the common Granulosicoccus OTUs in our study have recently been detected in *Eunicella cavolini* [21] and various tropical scleractinian corals [50, 77], respectively.

Temporal and Spatial Stability of the Gorgonian Microbiome

While the gorgonian-associated microbiomes were generally composed of the same core microbial species and locally stable microbial associates, our main objective was to investigate the stability of these associations over time. Seasonal differences in bacterial communities have been reported for several scleractinian and gorgonian coral species at different geographical scales [80–83]. Although observed in the Mediterranean cold-water coral *Madrepora oculata* [84] and the gorgonian *P. clavata* [85], the temporally stable interactions between Mediterranean gorgonians and their microbial associates are remarkable, particularly because of major differences in seawater temperature and rainfall between the sampling months (Table 3), and suggest tight microbiome regulation by these host animals. Even on the OTU level, relatively few changes were observed, which mostly involved low abundant bacteria that did not belong to the core or common microbiome. The only exception was *L. sarmentosa* at La Spezia in which the microbiome underwent significant modifications in composition over time, but which were primarily driven by a re-shuffling in the abundances of the main core microbes and the locally stable microbial associates. Overall, this indicates that the *L. sarmentosa* holobiont may be more flexible and able to adapt to the environmental conditions. Significant shifts in the bacterial communities of the gorgonians and at the OTU level mostly took place between spring time points on the one hand and summer and winter time points on the other. As environmental conditions rapidly change during spring, this may be the most likely factor responsible for the observed microbiome shifts.

Although the gorgonian-associated microbiomes were generally stable over time, we did observe major differences between the two sampling sites. The Cassis and La Spezia locations are characterized by very different environmental conditions: The water at the Cassis location is relatively clear and unaffected by terrestrial input, while the La Spezia location is impacted by river and sewage influxes resulting in high levels of turbidity and pollution. These dissimilarities in the local environment may explain the differences observed here in the gorgonian-associated bacterial communities. Recently, we reported that the gorgonian microbiome is relatively stable on spatial scales, but that local disturbances may indeed impact the bacterial community associated with these animals [7]. In this study, however, our results indicate that the extent of the impacts on the microbiome appears to be highly species-dependent, but also raise questions as to what may constitute a 'healthy' gorgonian microbiome. For example, the abundance of core and common microbiome members was significantly lower in *E. verrucosa* near La Spezia compared with Cassis; particularly the most abundant *Endozoicomonas* OTU47380. Reduction in *Endozoicomonas* abundance in corals is often characteristic of stress [51, 52, 55, 77, 86] and combined with the increased diversity observed suggests that the *E. verrucosa* microbiome was negatively impacted at this turbid location. Surprisingly, however, the bacterial community associated with *L. sarmentosa* showed the opposite pattern, being significantly more dominated by core and common bacterial associates at La Spezia, which indicates that this holobiont may in fact have been healthier under the same conditions. These contrasting signatures in the bacterial communities of these two gorgonians from the Gorgoniidae family suggest that these species may still have different habitat preferences despite living sympatrically close to muddy bottoms [87]. As some core bacteria, such as *Endozoicomonas* OTU47380, were reduced in abundance in *E. verrucosa*, but showed higher abundance in *L. sarmentosa* at La Spezia, the environmental conditions probably did not affect the viability of these microbes and provides further support for host control of the microbiome. Which factors are responsible for these differences in bacterial assemblages, however, remains to be investigated. Overall, our data shows that the microbiome of

Table 3 Environmental conditions at the sampling locations

		2014			2015	
Location	Species/seawater	Spring	Summer	Winter	Early spring	Late spring
Cassis	Seawater temperature (°C)	15.9	21.3	17.5	13.9	17.8
	Rainfall (mm)	32.1	90.8	191.6	62.0	90.6
La Spezia	Seawater temperature (°C)	15.9	22.7	13.8	14.1	
	Rainfall (mm)	32.0	41.1	94.2	43.2	

the Mediterranean soft gorgonians is relatively stable over time and under control of the host, but that local conditions shape its associated bacterial communities.

Microbiome of the Red Coral *Corallium rubrum*

The microbiome of the red coral was highly distinct from any known cnidarian-associated bacterial community, including the Mediterranean soft gorgonians. Spirochaetales (genera Borrelia, Spirochaeta and Leptospira) were the most dominant bacteria, followed by the Oceanospirillales (ME2 family, an unknown Oceanospirillales and *Endozoicomonas*) and an OTU belonging to the relatively unknown phylum Parcubacteria (previously classified as Candidate Division OD1) [18]. The core microbiome consisted of 7 main OTUs, representing 86.4–94.5% of the microbiome, in addition to 37 low abundant (<1%) OTUs. This highly structured microbiome of *C. rubrum* is consistent with our study on the spatial stability of the red coral microbiome throughout the Mediterranean Sea [18] and suggests that the microbiome is not only stable on spatial scales, but also over time (>2 years). Indeed, we did not find any significant temporal changes within the red coral-associated bacterial community in this study. Overall, our results indicate that the red coral holobiont possesses strong bacterial selection mechanisms to maintain a healthy microbiome. Octocorals are known to produce secondary metabolites with antimicrobial activity which may be used by the host to regulate its microbiome [29–32]; however, members of the coral-associated microbiome may also competitively prevent non-symbiotic residents from entering the bacterial community through nutrient competition and antibiotic production.

While the exact function of the bacteria within the *C. rubrum* microbiome is still unknown, the consistent association of the main OTUs on temporal and spatial scales provides strong evidence for symbiotic relationships. Spirochaetes were found to be relatively dominant bacteria associated with some octocorals only recently [18, 25, 26], although they have been observed at low abundance on scleractinian corals before [4, 70, 88, 89]. While the order Spirochaetales harbours multiple pathogens, some species are known endosymbionts [90–92] involved in digestion as well as carbon and nitrogen fixation into bioavailable nutrients for the hosts [27, 28]. The Spirochaetales associated with the red coral may provide similar functions to the holobiont as previously suggested and may also be implicated in regulation of the microbiome [18]. The Parcubacteria are largely unknown bacteria that have primarily been found living in anoxic conditions [93], and recent genomic investigations have shown that they have severely reduced metabolic capabilities [94]. For example, they are incapable of producing co-factors, nucleotides, amino acids and fatty acids. This lack of biosynthesis machinery suggests a highly specialised life-style, likely of symbiotic nature [94]. It remains, however, unclear what services Parcubacteria provide to their host. The other main group of bacteria associated with the red coral are members of the order Oceanospirillales. *Endozoicomonas* are probably the best studied coral-associated bacteria, but appear to play only minor roles in the *C. rubrum* holobiont. Members of the relatively unknown ME2 family may, however, fulfil the same function in the red coral as the *Endozoicomonas* in other corals whose microbiome is dominated by bacteria from this genus.

Co-evolution of the Gorgonian-Associated Microbiome

To explain the regulation of microbiome composition within the holobiont, two theories have been postulated: the holobiont model [15] and the hologenome theory of evolution [95]. Although both theories state that the host shapes its microbiome based on metabolism and the use of antimicrobials but that local conditions can impact its membership, we previously argued that the holobiont model applies better to the Mediterranean gorgonian holobionts [7]. Our main argument was that the hologenome theory of evolution includes heredity (i.e. transmission of bacteria between generations), which requires that (1) these symbionts are ubiquitous and (2) there are strong phylosymbiotic signals, but that such phylosymbiotic signals were absent in the gorgonian-associated microbiomes due to the significant overlap in ubiquitous bacteria (core and common microbial associates; which are in at least all gorgonians from the Gorgoniidae family mostly the same). Taken together, our studies (here and [7, 18]) covering six octocoral species from two taxonomic sub-orders, three families and four genera (Fig. 6) show that there is significant divergence in microbiome composition along host phylogenetic lines. However, these co-evolutionary signals appear to end at the family level as gorgonian species from different genera within the same family show significant overlap in microbiome composition at the bacterial species level (97% identity similarity). As such, these ancient host-microbe associations have been highly conserved through evolutionary time, but differences between the microbiomes of the Gorgoniidae species may still exist at the bacterial strain level. Further investigations at the strain level of the various ubiquitous microbes could provide us with a higher resolution on phylosymbiotic signals in the gorgonian holobionts and may show strain specificity among the various

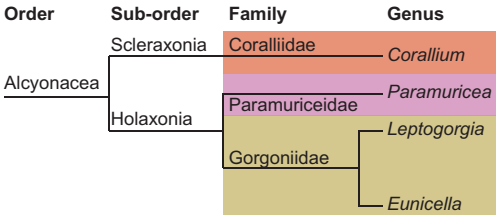

Fig. 6 Schematic overview of the taxonomy of the Mediterranean octocorals whose microbiome was analysed here and in our previous studies [7, 18]. The *different colours* identify taxa harbouring distinct core microbiomes. Taxa grouped by colour, e.g. genera within the Gorgoniidae, show significant overlap in (core) microbiome composition

gorgonian holobionts. However, our current data provides additional support for the holobiont model.

Concluding Remarks

In this study, we provide an in-depth analysis of the bacterial community of five Mediterranean gorgonians from two different taxonomic sub-orders on temporal scales. Overall, we show that their highly structured microbiomes are relatively stable over time, but are likely adjusted to local environmental conditions, supported by the holobiont model. The strong and specific associations of the host with up to 7 dominant bacterial species and ~40 common low abundant associates show tight regulation of the holobiont membership. Divergence in microbiome composition was, however, clear along distant phylogenetic lines, although there is significant overlap among species belonging to the same family. The physiological contributions of these microbial symbionts to host health remain to be elucidated.

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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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