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Changes in coral forest microbiomes predict the impact of marine heatwaves on habitat-forming species down to mesophotic depths

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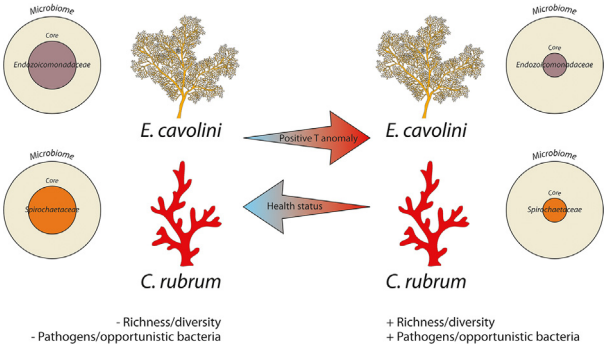
HIGHLIGHTS

- Coral-forest microbiomes are highly sensitive to thermal anomaly amplitude.
- Heatwaves increase the abundance and microbial diversity in coral forests.
- Heatwaves reduce dominant bacteria and increase opportunistic ones in corals.
- Microbiome shift is directly linked to heatwave not to coral necrosis.
- Microbiome alteration is a relevant indicator to predict coral-forest decline.

GRAPHICAL ABSTRACT



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ABSTRACT

Global warming is causing the increase in intensity and frequency of heatwaves, which are often associated with mass mortality events of marine organisms from shallow and mesophotic rocky habitats, including gorgonians and other sessile organisms. We investigated the microbiome responses of the gorgonians *Paramuricea clavata*, *Eunicella cavolini*, and the red coral *Corallium rubrum* to the episodic temperature anomalies detected in the North Western Mediterranean, during August 2011. Although the investigated corals showed no signs of visible necrosis, the abundance of associated Bacteria and Archaea increased with increasing seawater temperature, suggesting their temperature-dependent proliferation. Coral microbiomes were highly sensitive to thermal anomaly amplitude and exhibited increased bacterial diversity to greater thermal shifts. This effect was explained by the decline of dominant bacterial members and the increase of new, rare and opportunistic taxa, including pathogens, revealing a direct effect of heatwave-induced alteration of the microbiomes and not a secondary consequence of coral necrosis.

1. Introduction

Marine heatwaves are impacting marine ecosystems worldwide (Donovan et al., 2021; Frölicher et al., 2018; Smale et al., 2019) with negative effects on a wide range of organisms (from invertebrates, including corals, to marine fishes) (Frölicher and Laufkötter, 2018; Hobday

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et al., 2016) and habitats (Frölicher et al., 2018; Hobday et al., 2016). Since the 20th century, global warming has determined an increase in intensity, duration and frequency of marine heatwaves, which have doubled since 1982 (Frölicher et al., 2018; Oliver et al., 2018) and are predicted even to further increase in the future (Darmaraki et al., 2019; Frölicher and Laufkötter, 2018; Oliver et al., 2019).

Over the last 20 years, the Mediterranean Sea has experienced a dramatic increase in massive mortality events of marine organisms (mainly corals and sponges) (Cerrano et al., 2000; Garrabou et al., 2001; Huete-Stauffer et al., 2011; Jiménez et al., 2016). These events have been observed in shallow and mesophotic habitats (i.e., from 50 m to 200 m; (Cerrano et al., 2010)) and have been particularly intense in the North-Western Mediterranean, from Italian to Spanish coasts (Calvo et al., 2011; Di Camillo and Cerrano, 2015; Garrabou et al., 2009).

Several coral species, such as the gorgonians *Paramuricea clavata* (Risso, 1826), *Eunicella cavolini* (Koch, 1887), and the red coral *Corallium rubrum* (Linnaeus, 1758) have been shown to be among the most vulnerable species to such episodic climate-driven events (Garrabou et al., 2001; Huete-Stauffer et al., 2011; Linares et al., 2008). Since these organisms are ecosystem engineers, which play fundamental roles in rocky subtidal systems (e.g., as nursery areas and by promoting high biodiversity levels) (Angiolillo and Canese, 2018; Enrichetti et al., 2019), their loss is particularly dramatic for the diversity and functioning of hard-bottom habitats (Angiolillo and Canese, 2018; Gómez-Gras et al., 2021; Verdura et al., 2019).

Corals are holobionts, hosting microbial consortia, which include archaea, bacteria, fungi, dinoflagellates, and viruses (Ainsworth et al., 2010; Rosenberg et al., 2007; van Oppen and Blackall, 2019; Wood-Charlson et al., 2015). The coral microbiome provides key benefits to these animals, contributing not only to their overall physiological functions, but also to their health, by contrasting pathogen infections and conferring tolerance to stressful conditions (Krediet et al., 2013; Peixoto et al., 2017).

Although the biological mechanisms underlying marine mortality events are largely unknown, previous studies reported that coral mortality cannot be only due to thermal stress (Kersting et al., 2013; Rivetti et al., 2014), but also associated with pathogenic outbreaks (Rubio-Portillo et al., 2014; Vezzulli et al., 2010), which can cause the disruption of the delicate equilibria within the coral microbiota (Bourne et al., 2009; van Oppen and Blackall, 2019). The balance within the holobiont can indeed be altered by thermal stress, favouring the colonization of the coral tissue by microorganisms present in the surrounding water (Mouchka et al., 2010). At the same time, recent studies have also suggested that shifts in the microbiome composition of the reef corals can favour their adaptation to heat stress conditions (Ziegler et al., 2017) or reflect disease resistance (MacKnight et al., 2021).

Investigations conducted on gorgonian species in absence of thermal anomalies, reported that microbiomes of Mediterranean octocorals are rather stable over different seasons and do not change across water depth (Bayer et al., 2013; Hernandez-Agreda et al., 2017; van de Water et al., 2016, 2018a, 2018b, 2020). At the same time, a lower thermotolerance has been reported in deeper coral colonies (Torrents et al., 2008; Pivotto et al., 2015) although various ecological, physiological and genetic factors can modulate the response of different populations to marine heatwaves (Torrents et al., 2008). Therefore, investigating the response of microbiomes associated with corals to thermal stress can provide useful information on the tolerance of coral forests to temperature shifts and/or adaptation to ocean warming (Hackerott et al., 2021).

In the present study, we assessed the abundance, community structure (Bacteria and Archaea) and biodiversity of microbiomes of three octocoral species (*Paramuricea clavata*, *Eunicella cavolini*, and *Corallium rubrum*) collected in the North-Western Mediterranean Sea during a strong episodic heatwave event, which occurred in August 2011 (Rivetti et al., 2014). The investigated species are exposed to a gradient of decreasing temperatures with increasing depth. The availability of long-term information on temperature profiles offers the opportunity to investigate the potential impact of heatwaves on the microbiomes associated to coral forests (Bayer et al., 2013; van de Water et al., 2018a, 2018b, 2020) and to better understand the biological mechanisms determining their vulnerability to climate change.

2. Materials and methods

2.1. Study areas and sample collection

Coral samples were collected in the Marine Protected Area (MPA) of Portofino in the Ligurian Sea (North-Western Mediterranean; Fig. S1). Coral sampling was conducted on August 2011 in two different sites within the Portofino area: Punta dell'Indiano (44°17′56.77″ N, 9°10′59.56″ E) and Punta del Faro (44°17′41.02″ N, 9°13′31.30″ E; Fig. S1). Sampling of specimens of *P. clavata*, *E. cavolini*, and *C. rubrum* was performed along the natural occurring depth distribution (within the range: 15–70-m depth, Table S1) through the use of SCUBA TRIMIX technology (Cerrano et al., 2019). Specimens of *P. clavata*, *E. cavolini*, and *C. rubrum* were collected from colonies with no signs of tissue injuries or necrosis (Fig. S2a, c and e) even though some colonies near them started to have damaged tissue (Fig. S2b and d). In particular, a total of 54 coral fragments, 5 cm long ($n = 3$ sub-replicates for each colony), were collected from 3 coral colonies for each of the 3 species and from 2 depths, to ensure a robust experimental setup and investigate intra-specific and intra-colony differences, while avoiding excessive harvesting to minimize any potential impact on the local population of the target species. Fragments were transported in polyethylene tubes to the laboratory and stored at −80 °C until analysis.

2.2. Temperature data

At each site and sampling depth, temperature was measured by using a temperature probe (HOBO® Water Temp Pro v2). To assess the potential temporal fluctuations of seawater temperature in the two investigated sites of Portofino, we compared the data measured in August 2011 with the average values detected in August 1979 (from 0 to 80 m; Locarnini et al., 2018) and in August 1999 (from 0 to about 45-m depth), in August–September 1993, 1994–1998 and 1999 (at 0 and 35-m depth; Cerrano et al., 2000) in the same sites (Table S2).

We also compared our data with the mean seawater temperatures of the Ligurian Sea (World Ocean Database, WOD18, http://www.nodc.noaa.gov) in the period 1955–2012 (Locarnini et al., 2018). Finally, we compared our values with data (mean, maximum and minimum values) of seawater temperature measured from surface down to 80 m in August from 1972 to 2018 in Estartit observatory of the North Western Mediterranean Sea (Salat et al., 2019, Table S2).

Thermal anomalies of Punta del Faro and Punta dell'indiano (ΔT) were calculated at different depths in August 2011 by comparison with data available in the literature and databases.

2.3. Determination of total prokaryotic abundance associated with the corals

Prokaryotic abundance associated with *P. clavata*, *E. cavolini* and *C. rubrum* was determined according to previous procedures (Danovaro, 2010; Luna et al., 2007), which were slightly modified by adding a step of ultrasound treatment as described below. Each coral fragment of about 1 cm^2 (ca. 50 mg) was crushed and subjected to ultrasound treatment (Branson Sonifier 2200, 60 W). Then samples were handled under laminar flow hood to avoid any contamination and washed twice with 5 mL of sterile artificial seawater pre-filtered through 0.2-μm-syringe filters (Whatman®, Anotop®, Merck KGaA, Darmstadt, Germany). Coral samples were crushed for 1 min with sterilized mortar and pestle and then vortexed for 1 min. They were sonicated three times for 1 min with intervals of 30 s during which tubes were manually shaken and left for 1 min to allow sedimentation of the coral material. Three aliquots of the supernatants from each coral fragment were then collected and diluted using sterile 0.02 μm pre-filtered seawater. For total prokaryotic counting, subsamples were fixed by adding 4 mL of 2% formalin, previously buffered with sodium tetraborate (20 g L^{-1}) and filtered through 0.2 μm pore size filters (black-

stained polycarbonate filters Whatman®, Nucleopore™, Merck KGaA, Darmstadt, Germany). The number of total prokaryotes was determined by epifluorescence microscopy (Zeiss Axioskop 2MOT, Jena, Germany). A minimum of 400 cells were counted per filter. Prokaryotic abundances were normalized to the area of the colony, in which each coral branch was approximated to a cylinder.

2.4. Catalyzed reporter deposition–fluorescence in-situ hybridization (CARD-FISH)

The abundance of Bacteria and Archaea was assessed by catalyzed reporter deposition–fluorescence in-situ hybridization (CARD-FISH; (Teira et al., 2004)). From each coral colony, one fragment of about 1 cm^2 (ca. 50 mg) was collected. After an initial fixation in 1% formalin diluted with 0.2-μm filtered seawater buffered with sodium tetraborate (20 g L^{-1}), coral fragments were crushed with sterile mortar and pestle, vortexed for 1 min and then sonicated 1 min for three times (Branson Sonifier 2200, 60 W). At the end of each sonication step, samples were manually shaken for 30 s. Then, samples were incubated for 1 h at room temperature, diluted, filtered onto 0.2-μm pore size nucleopore polycarbonate filters (Whatman® Nuclepore™; Merck KGaA, Darmstadt, Germany) under low vacuum (<100 mm Hg) and washed with 1% phosphate-buffered saline (PBS; 145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4; pH 7.6), and suspended in 1:1 solution of PBS: 96% ethanol. Samples were then dipped in low-gelling point agarose (0.2% (wt/vol) in Milli-Q water), dried on Petri dish at 37 °C, and dehydrated in 95% ethanol. Cell wall permeabilization was obtained by incubation at 37 °C with lysozyme for Bacteria or proteinase K for Archaea (Teira et al., 2004). After washing with Milli-Q and incubation in 0.01 M HCl (20 min at room temperature), filters were then washed again, dehydrated in 95% ethanol, dried and hybridized with oligonucleotide Horseradish Peroxidase (HRP)-labeled probes Eub-mix (Eub338, Eub338-II and Eub338-III) targeting Bacteria, and Arch915 targeting Archaea. The absence of nonspecific signals was checked using the NON-338 probe. Hybridization (35 °C for Bacteria, 46 °C for Archaea) was performed for 2 h. Filters were then washed in preheated buffer (3 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl and 0.01% *w/v*) and placed in PBS (added with 0.05% Triton X-100) and incubated at room temperature for 15 min. After removal of the buffer, samples were incubated for 30 min in the dark at 37 °C for Cy3-tyramide (PerkinElmer, Milan, Italy) signal amplification.

Samples were counted by epifluorescence microscopy (Zeiss Axioskop 2MOT, Jena, Germany; magnification ×1000) in green light excitation. For each filter, at least 20 optical fields containing at least a total of 400 cells were counted.

2.5. DNA extraction, amplification and high-throughput sequencing of 16S rRNA gene

DNA was isolated from *E. cavolini* and *C. rubrum* collected at different depths and subsequently used for 16S rRNA high-throughput sequencing analysis. Ca. 20 mg of coral tissue (wet weight) from each colony was scraped from the skeleton by using surgical disposable scalpels (Braun). Samples were homogenized with a sterile glass stick with 250 μL of 0.02 μm FSW in a sterile Eppendorf® tube. Coral slurries were centrifuged and total genomic DNA was extracted using Power Soil DNA Isolation kits according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Subsequently, DNA was quantified and amplified with primers Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013), which bind to the *E. coli* 16S rDNA gene at positions 340–784 and yield amplicons on the 400–500 bp range. To assess whether the extracted DNA was amplifiable, PCR analyses of DNA from coral colonies were carried out by means of the same primer pairs used for high-throughput sequencing.

The reaction was carried out on the Veriti™ 96-Well Thermal Cycler (Applied Biosystem, Monza, Italy) in a 25 μL final volume with 10 μL 5× PrimeSTAR® GXL PCR (Taqara Bio, Mountain View, US) reaction buffer, 1 μL dNTP mixture, 0.8 μL Taq (PrimeSTAR® GXL DNA Polymerase), 1 pmol of each oligo, 2 ng of template DNA and nuclease free water prefiltrated through a 0.02 μm pore size filter. The PCR program consisted of a denaturation step at 95 °C for 5 min, 31 cycles at 95 °C for 30 s, 53 °C for 30 s and 72 °C for 45 s and a final extension step at 72 °C for 5 min. Successful DNA amplification was verified by 1% agarose gel electrophoresis. Negative controls (an aliquot of PCR mix without template DNA) resulted in no visible amplification products, confirming that samples and processing techniques were essentially free of contamination.

The same primer pairs were used for high-throughput sequencing. Paired-end sequencing of 16S rRNA gene contained within the DNA extracted from *E. cavolini* was performed with Illumina MiSeq platform and the MiSeq reagent kit v.2 (Illumina, USA), while single-end sequencing of 16S rRNA gene from *C. rubrum* samples was performed with Roche Life Sciences 454 FLX+ platform. Previous investigations based on the use of the two different sequencing platforms demonstrated that they provide a comparable view of the microbial diversity (Luo et al., 2012).

2.6. Bioinformatic analyses

To allow us for a comprehensive and detailed comparison of the two datasets, leftover primer sequences were first removed from both sequencing runs through the *cutadapt* program (Martin, 2011). The DADA2 plugin for the QIIME2 pipeline (Bolyen et al., 2019) was then used to denoise separately 454 sequences (through the *denoise-pyro* option) and the forward-facing files of the MiSeq sequencing run (through the *denoise-single* option), truncating sequences in both sequencing runs at 250 bp. Reverse-facing files were not utilized.

Representative sequences (ASVs) and ASV tables from both denoising runs were then merged within QIIME2 (with the –p-overlap-method set to "sum" to sum relative abundance of identical ASVs in the two feature tables). After merging, ASVs were placed on a reference tree using the q2-fragment-insertion plugin within QIIME2, which supports the SEPP algorithm (Janssen et al., 2018), which allowed the removal of possibly spurious ASVs generated by the analysis and to better compare results from different sequencing platforms. A subset of the SILVA database (Quast et al., 2012) (release 132) was created through the extract-reads procedure within QIIME2: database sequences were trimmed to the region amplified by the primers used and truncated to 250 bp to reflect the ASV size chosen at the denoising step; sequences from the subsetted database was then used as an input for subsequent classification steps carried out on the merged representative sequences using the *classify-consensus-vsearch* tool within QIIME2, with standard parameters (Rognes et al., 2016). Taxonomic information was used to remove archaeal, eukaryotic, chloroplast and mitochondrial-related sequences before subsequent analyses. ASVs were aligned and the alignment used to create a rooted tree within QIIME2 using the implemented MAFFT and FastTree tools (Katoh and Standley, 2013).

For alpha- and beta-diversity computations, the dataset was first normalized by rarefying at 6000 sequences using the scaling with ranked subsampling (SRS) approach (Beule and Karlovsky, 2020). The rarefied table was utilized to generate alpha-diversity indices (i.e. ASV richness, Pielou's evenness, Shannon index, dominance and percentage of singletons on the total richness).

To assess the impact of heatwaves on coral-forest microbiomes we compared our results with two datasets from *C. rubrum* and *E. cavolini* colonies not subjected to thermal anomalies available in literature (Corinaldesi et al., 2021; van de Water et al., 2020). In particular, raw sequences of the microbiomes of *E. cavolini*, collected at mesophotic depth in November 2016 and May 2017 (PRJNA506661; van de Water et al., 2020), and raw sequences of microbiomes of *C. rubrum* collected at 35 m-depth in March 2017 (Corinaldesi et al., 2021) were used for a comparison. These datasets were selected as they were obtained from colonies of the same sampling area (Portofino) and based on the use of the same primers, allowing us to make reliable comparisons.

All these datasets were cleaned and denoised using the *denoise-single* option as above before merging with samples from the species investigated in

the present study. Feature tables and sequences, obtained from the comparison between these microbiomes, were merged.

2.7. Statistical analyses

To test for differences in the investigated variables between different coral colonies, species and water depths, permutational analysis of variance (PERMANOVA) was conducted and the tests were based on matrices of Euclidean distance (Anderson, 2001). Differences among colonies, species and water depths of prokaryotic, bacterial and archaeal abundances were analysed applying different sampling designs. To assess whether the prokaryotic, bacterial and archaeal abundances significantly changed among colonies of each species and among different species, we applied a two-way PERMANOVA test considering colonies (3 levels: colony 1, 2 and 3) and species (3 levels: *P. clavata*, *E. cavolini* and *C. rubrum*) as fixed and orthogonal factors within the PRIMER-E 7 program. To assess whether the prokaryotic, bacterial and archaeal abundances changed among species and water depths, we applied a two-way PERMANOVA test considering species (3 levels: *P. clavata*, *E. cavolini* and *C. rubrum*) and water depths (3 levels: 15 m, 35 m and 70 m) as fixed and orthogonal factors. When significant effects of the considered factors were observed, pair-wise tests were also carried out to ascertain in which colonies, species or water depths the significant differences were observed. To determine whether the prokaryotic, bacterial and archaeal abundances were influenced by water temperature and/or depth, we carried out linear regression analyses using the GGPubr package (Kassambara and Kassambara, 2020) in R (v.4.0.5) and verified the effect of depth on temperature as potential covariate by means of one-way ANCOVA test performed through the *rstatix* package.

Statistical analyses on the microbial assemblage structure were carried out in R (v.4.0.5). In particular, the average contribution of taxa to the assemblages was calculated using the *simper* function provided by *vegan* (Dixon, 2003) with 999 permutations and tests for the statistical significance of differences in alpha-diversity analyses were carried out using the *compare_means* function provided by *GGPubr* (Kassambara and Kassambara, 2020) to carry out ANOVA tests.

Beta-dispersion analysis was carried through the *betadisper* function provided by *vegan* to evaluate the distance from centroids within each sample category, and an ANOVA test with a post-hoc Tukey test was carried out on the beta-dispersion analysis results (within R) to assess the significance of such distances between sample categories.

3. Results

3.1. Seawater temperature

Temperature values measured during sampling at each site and depth are reported in Table S1. As expected, values of temperatures decreased significantly with increasing water depth ($n = 42$, $F = 35{,}26$ $P < 0.001$).

The comparison between the temperature values measured at the two sites of the present study, in August 2011 with historical data (Cerrano et al., 2000; Locarnini et al., 2018; Salat et al., 2019) is reported in Table S2 and in Fig. 1A–C. In particular, the comparisons with the average values reported at Punta del Faro and Punta dell'Indiano in August of the years 1979, 1993, 1994–1998 and 1999, in the Ligurian Sea in August from 1955 to 2012, and in the NW Mediterranean from 1972 to 2018 are illustrated in Fig. 1A, B and C, respectively.

The comparison of temperatures measured at Punta del Faro in August 2011 revealed even higher values than those reported in August 1999 when a large-scale mass mortality was observed (Cerrano et al., 2000; Fig. 1A). In August 2011, temperatures were also higher than the monthly average values reported for both the Ligurian Sea and the NW Mediterranean (Fig. 1B and C). At these depths, positive thermal anomalies observed at Punta del Faro were higher than those measured at Punta dell'Indiano (Table 1). In particular, at Punta del Faro a positive thermal anomaly greater than 3 °C was recorded down to 70 m-depth (Table 1).

3.2. Total prokaryotic abundance associated with corals

Prokaryotic abundances associated with *P. clavata* (from 2.07 ± 0.74 to $4.03 \pm 1.32 \times 10^7$ cells cm^{-2} of coral surface) did not change significantly among the three colonies collected at the same depth and between different depths (Fig. 2; Tables S3–S4–S5).

In both *E. cavolini* and *C. rubrum*, no significant differences were observed when prokaryotic abundances were compared among different colonies at a given depth. The only exception was reported for *C. rubrum* collected at 70-m depth (PERMANOVA, $p < 0.05$, Table S4).

On the other hand, prokaryotic abundances were significantly higher at shallower depths than at greater depths for both *E. cavolini* (6.45 ± 1.27 vs. $1.05 \pm 0.29 \times 10^7$ cells cm^{-2} at 15 and 35-m depth, respectively; PERMANOVA, $p = 0.001$) and *C. rubrum* ($5.43 \pm 2.09 \times 10^6$ vs. $1.15 \pm 0.67 \times 10^6$ cells cm^{-2} at 30 and 70-m depth, respectively; PERMANOVA $p < 0.05$, Table S5).

Overall, the highest prokaryotic abundances were observed in *E. cavolini* collected at 15-m depth and the lowest in *C. rubrum* at 70-m depth (*t*-test $p < 0.01$; Table S6). At 40-m depth, total prokaryotic abundance of *P. clavata* was significantly higher than in *E. cavolini* and *C. rubrum* (t-test $p < 0.01$, Table S6). Similarly, at 70-m depth, prokaryotic abundance of *P. clavata* was also significantly higher than in *C. rubrum* (PERMANOVA, $p = 0.001$; Table S7). Statistical analyses revealed that prokaryotic abundances increased significantly with increasing temperature ($n = 39$; $F = 22.573$, $p < 0.001$, Fig. 3A) even after checking for water depth as a potential covariate (Table S8).

3.3. Bacteria and Archaea associated with corals

CARD-FISH analyses conducted on all coral species (Fig. 4A–C) revealed that the abundance of Bacteria was significantly higher than that of Archaea (PERMANOVA, $p < 0.05$; Table S9).

Bacteria accounted, on average, for ca. 61% (from 56% in *E. cavolini* at 35-m depth to 66% in *C. rubrum* at 70-m depth) of the total prokaryotic abundance (determined as the sum of bacterial and archaeal abundance). In *E. cavolini*, both bacterial and archaeal abundances were significantly higher at 15-m depth ($4.05 \pm 0.65 \times 10^6$ cells cm^{-2} and $2.45 \pm 0.69 \times 10^6$ cells cm^{-2}, respectively) than at 35-m depth ($8.16 \pm 0.13 \times 10^5$ bacterial cells cm^{-2} and $6.27 \pm 1.16 \times 10^5$ archaeal cells cm^{-2}; PERMANOVA $p < 0.001$ for both Bacteria and Archaea; Table S10). The same pattern was observed for *C. rubrum* (on average: $1.23 \pm 0.45 \times 10^6$ bacterial cells cm^{-2} and $7.50 \pm 0.29 \times 10^5$ archaeal cells cm^{-2}; PERMANOVA, $p < 0.05$ and $p < 0.001$, respectively Table S10). Conversely, in *P. clavata* only bacterial abundances (on average: $2.51 \pm 0.49 \times 10^6$ cells cm^{-2}) were significantly different at the two depths (PERMANOVA, $p < 0.05$ Table S10), while archaeal abundances (on average: $1.65 \pm 0.52 \times 10^6$ cells cm^{-2}) were not (PERMANOVA, $p = 0.08$; Table S10).

Relationships between temperature and bacterial and archaeal abundances were always significant and positive, also after controlling for depth as a covariate ($n = 51$, $F = 20.242$ for bacteria and $F = 11.517$ for archaea, $p < 0.001$; see Fig. 3B–C and Table S8).

3.4. Microbiome taxonomic composition

The number of sequences obtained from *C. rubrum* samples ranged from 10,318 to 96,320, whereas in *E. cavolini* they ranged from 110,424 to 552,703. After denoising, non-chimeric sequences from *C. rubrum* ranged from 7130 to 66,913, whereas in *E. cavolini* these ranged from 29,552 to 140,596 (Table S11). In all *C. rubrum* and *E. cavolini* samples, rarefaction curves reached or were close to reaching the plateau selecting 6000 reads as sequencing depth (Fig. S3).

The 16S rRNA metabarcoding analysis in *C. rubrum* and *E. cavolini* revealed that ASV richness and Shannon index were significantly lower in *C. rubrum* (343 ± 292 ASVs and 5.26 ± 2.48, respectively) than in *E. cavolini* (1654 ± 511 ASVs and 8.85 ± 0.85, respectively;

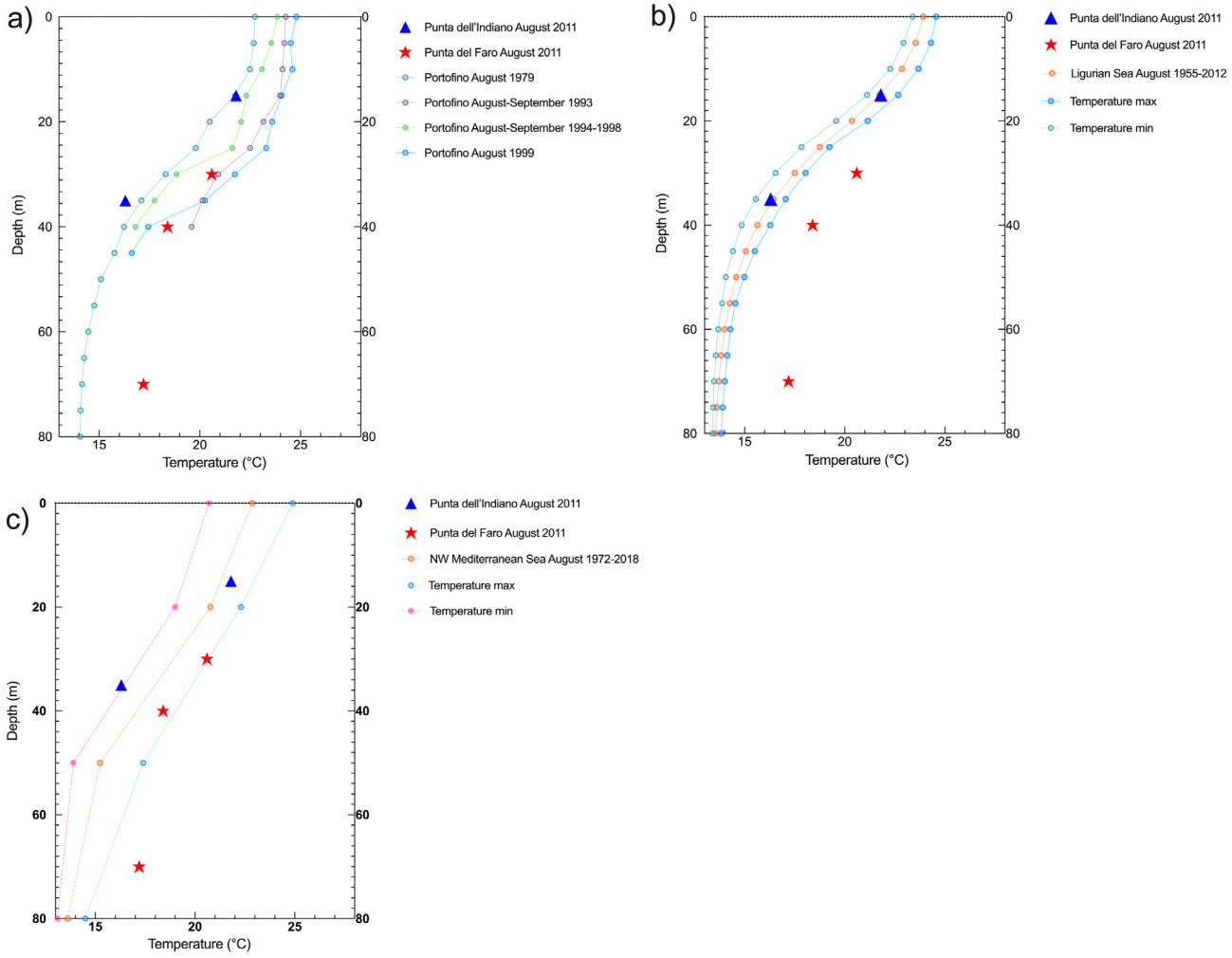

Fig. 1. Time series of temperature values along the seawater vertical profile in the Ligurian and NW Mediterranean Seas. Vertical profile of seawater temperatures in the Portofino area measured on the day of sampling carried out in August 2011 compared with mean values recorded in August 1979 (Locarnini et al., 2018), August–September 1993, 1994–1998 and August 1999 (Cerrano et al., 2000) (A). Vertical profile of mean, maximum and minimum values of seawater temperature measured in August from 1955 to 2012 in the Ligurian Sea (http://www.nodc.noaa.gov) (B). Mean, maximum and minimum values of seawater temperature along the depth gradient in August from 1972 to 2018 measured at the Estartit observatory in the North Western Mediterranean Sea (Salat et al., 2019) (C).

p-value < 0.05; ANOVA, Table S12). No statistical differences among samples belonging to the two species were found for the evenness index.

In *C. rubrum* and *E. cavolini*, opposite patterns in ASV richness were found although the differences were not significant. Shannon index and evenness were significantly higher (p-value < 0.05) in *C. rubrum* samples at 70 m than at 30-m depth (7.35 ± 0.97 vs 3.18 ± 0.37 for the Shannon index and 0.83 ± 0.01 vs 0.43 ± 0.04 for evenness, respectively, Fig. S4a–c, Table S13). An opposite pattern was found in *E. cavolini* at the different depths, but with no statistical differences.

Table 1
Positive anomalies of seawater temperatures in the Ligurian Sea in August 2011. Ranges of positive anomalies of the temperatures recorded in Punta dell'Indiano e Punta del Faro in August 2011 compared to the monthly average values recorded in the Ligurian Sea from 1955 to 2012 (http://www.nodc.noaa.gov, Cerrano et al., 2000, Locarnini et al., 2018).

Sampling sites	Depth (m)	Positive temperature anomaly
Punta dell'Indiano	15	0.1–0.7
Punta dell'Indiano	35	0.0–0.7
Punta del Faro	30	1.8–4.1
Punta del Faro	40	0.2–3.6
Punta del Faro	70	3.1–3.7

The main bacterial taxa present in *C. rubrum* at 30-m depth belonged to *Spirochaetaceae* (on average 46% of the bacterial assemblage), followed by *Immundisolibacteraceae* (on average, 20%), *Marinobacteraceae* (on average 10%) and *Enterobacteriaceae* (on average 5%; Fig. 5; Supplementary Materials). Unclassified Bacteria accounted for ca. 1% of the total bacterial assemblage.

The main bacterial families present in the *C. rubrum* at 70-m depth were *Enterobacteriaceae* (on average ca. 12%), followed by *Pseudomonadaceae* (on average 9%) and Actinobacteria (both on average, 7%). Unclassified Bacteria accounted for ca. 10% of the assemblage, while rare taxa for ca. 23%. The most abundant microbial taxa associated with *E. cavolini* were *Endozoicomonadaceae* (11% and 20% at 15-m and 35-m depth, respectively), uncultured *Actinomarinales* (on average 8%) and *Rhodobacteraceae* (7% at 15 m and 4% at 35 m; Fig. 5). Groups contributing less than 1% to the assemblage accounted for ca. 40–44%. SIMPER analysis revealed that the dissimilarity of coral microbiomes among all of the colonies within the same species (without considering the effect of depth) ranged, on average, from 19% in *E. cavolini* to ca. 79% in *C. rubrum*. The microbiome composition in different colonies of *C. rubrum* or *E. cavolini* at the same depth was similar for more than 60%. The beta-diversity between *C. rubrum* samples collected at 30-m and 70-m depth was 79%, with *Spirochaetacea*e, *Immundisolibacteraceae*, rare taxa, *Marinobacteraceae*, unclassified Bacteria, Actinobacteria and *Enterobacteriaceae* contributing

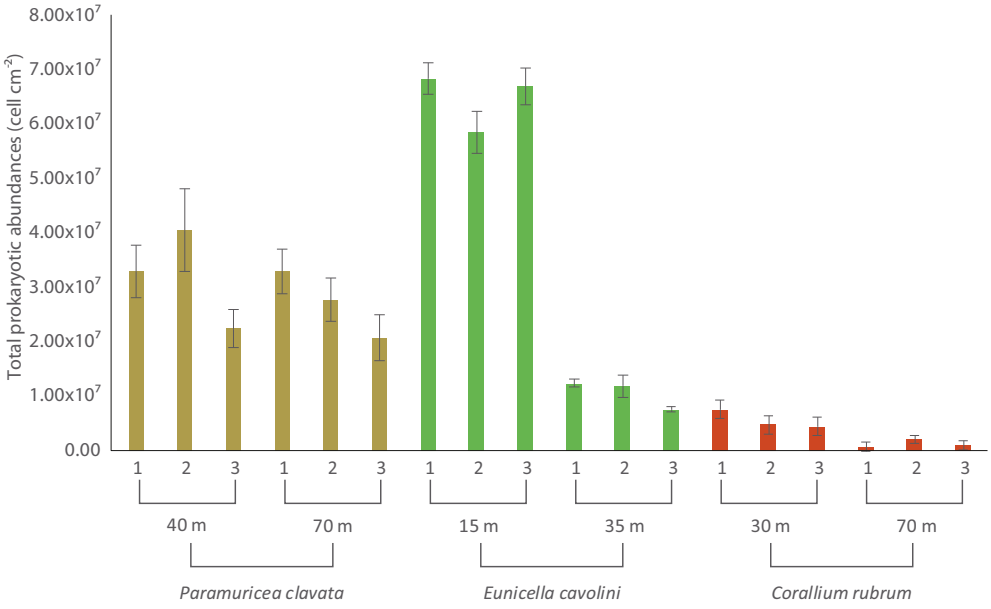

Fig. 2. Total prokaryotic abundance associated with *P. clavata*, *E. cavolini*, *C. rubrum*. Total prokaryotic abundance in three different colonies of *P. clavata* (40 m and 70 m), *E. cavolini* (15 m and 35 m), *C. rubrum* (30 m and 70 m). Data are reported as mean ± standard deviations of each colony analysed ($n = 3$).

up to ca. 72% of the dissimilarity; the beta-diversity between samples of *E. cavolini* collected at 15-m and 35-m depth was 19% (with *Endozoicomonadaceae*, *Rhodobacteraceae* and other rare taxa contributing up to 70% of the dissimilarity).

The beta-diversity between *C. rubrum* and *E. cavolini* samples at similar depths (30–35 m) was 91%, with *Spirochaetaceae*, rare taxa and *Immundisolibacteraceae* contributing up to ca. 56% of the dissimilarity.

Comparative analyses between our data during the heatwave with those, already published, from samples not subjected to thermal anomalies (see Materials and methods for details) showed significantly higher ASV richness (1654 ± 510 vs. 43 ± 26 for *E. cavolini* and 343 ± 292 vs 26 ± 2 for *C. rubrum*, p-value < 0.05), evenness (0.83 ± 0.08 vs. 0.30 ± 0.07 for *E. cavolini* and 0.63 ± 0.23 vs. 0.41 ± 0.05 for *C. rubrum*) and Shannon index (8.85 ± 0.85 vs. 1.65 ± 0.73 for *E. cavolini* and 5.26 ± 2.48 vs. 1.93 ± 0.27 for *C. rubrum*) in our samples. Six taxa contributed for >80% to the differences between colonies of *E. cavolini* not subjected to thermal anomaly and those collected in the present study: in particular, members of the *Endozoicomonadaceae* were responsible for ca. 47% of the variation (91% in the samples not subjected to thermal anomaly, 11% in the colonies at 15-m depth and 20% in the colonies at 35-m depth; Fig. 6A–C and Table S14).

For *C. rubrum* samples, 10 taxa contributed for >80% to the differences, with *Spirochaetaceae* accounting for ca. 30% of the variation (61% in the samples not subjected to thermal anomaly, 46% in colonies at 30 m and 4% in colonies at 70 m; Fig. 6B–D and Table S15).

Beta-dispersion analysis revealed that distance to group centroids was significantly higher in both species during the thermal anomaly compared to samples in absence of heatwave (ANOVA, overall p-value < 0.05,

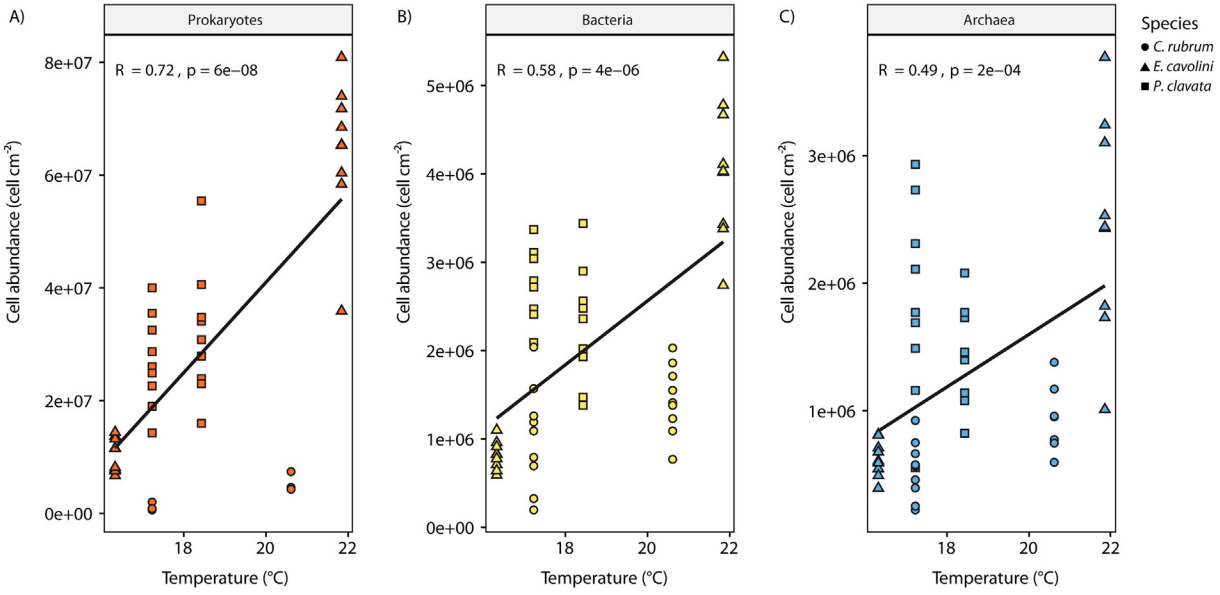

Fig. 3. Relationships between coral-associated bacteria, archaea, and prokaryotes and temperature. Relationship between temperature and total prokaryotic (A), bacterial (B) and archaeal (C) abundances associated with the different coral species collected at different depths. For each relationship, r and p-values are reported. Values associated with each coral species are indicated as different shapes.

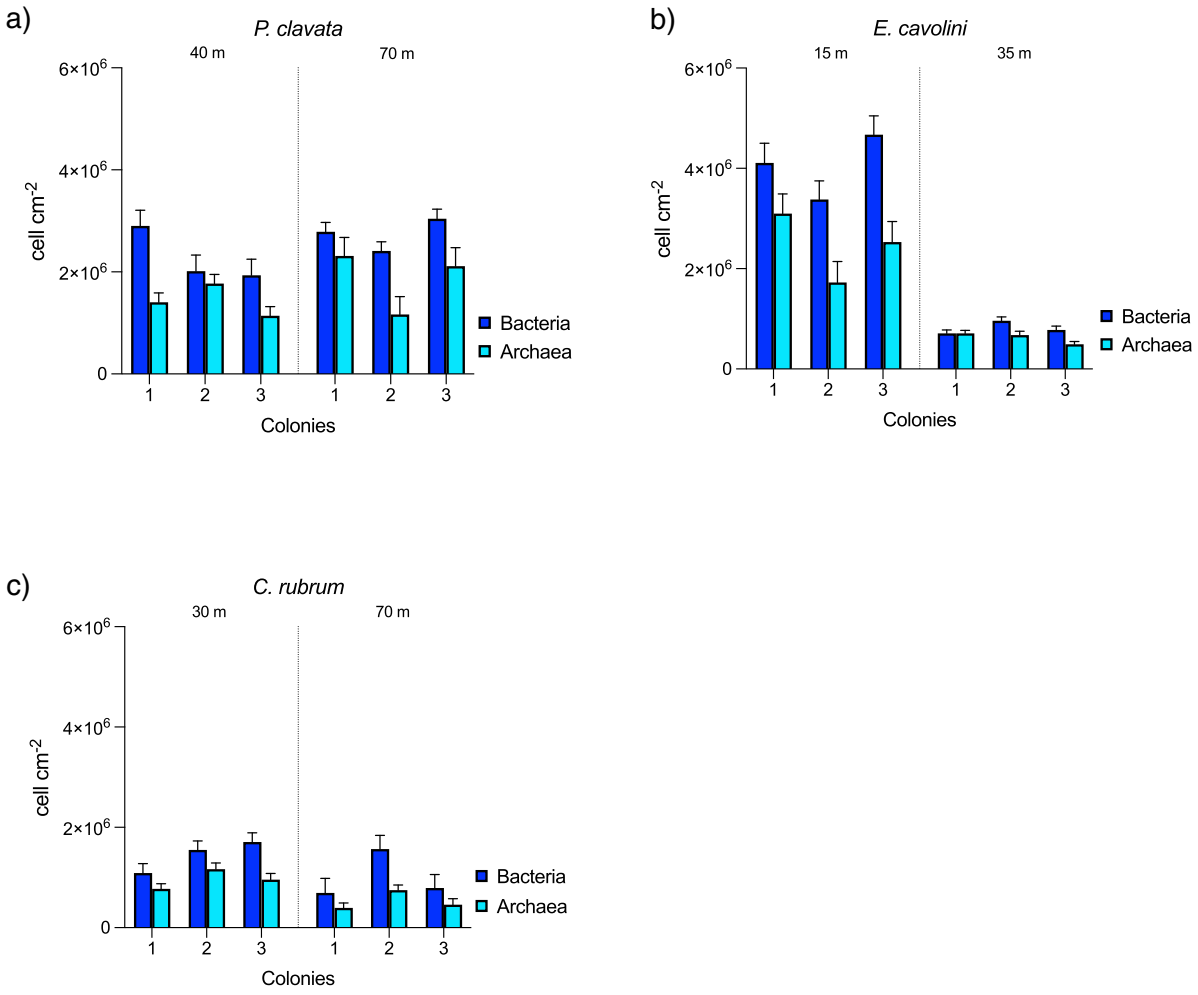

Fig. 4. Bacteria and Archaea associated with *P. clavata*, *E. cavolini*, *C. rubrum*. Bacterial and archaeal abundances across the three coral species collected at the different water depths: (A) *P. clavata* (at 40 m and 70 m depth), *E. cavolini* (at 15 m and 35 m depth) and *C. rubrum* (at 30 m and 70 m depth).

Fig. S5a). In particular, during the heatwave in the *C. rubrum* samples at 30-m depth the distance to median was 0.22 and for samples at 70-m depth was 0.33, whereas in the samples in absence of anomaly the distance to median was 0.05. For *E. cavolini*, the distance to median for samples at 15-m depth was 0.26 and at 35-m depth was 0.30, whereas for samples not subjected to thermal anomaly was 0.07.

The microbiomes of the *C. rubrum* colonies unaffected by the thermal anomaly showed an average dissimilarity of 35% with the colonies at 30-m depth, and of 96% with the colonies at 70 m. The microbiomes of *E. cavolini* colonies not subjected to thermal anomaly were, on average, dissimilar for 89% with colonies at 15-m depth and for 80% with colonies at 35-m depth (Fig. S5b).

4. Discussion

4.1. The impact of heatwave on the seawater temperatures of the NW Mediterranean Sea

Massive mortalities of gorgonian corals and other organisms have been documented in the NW Mediterranean Sea since 1986 (Bavestrello et al., 1994; Bavestrello and Boero, 1986). In the summer of 1999, a massive mortality event associated with thermal anomalies completely changed the seascapes of the Ligurian Sea from the surface down to 40-m depth (Cerrano et al., 2000). The Mediterranean Sea is undergoing a significant seawater warming, with an increasing frequency of summer temperature peaks (Marbà et al., 2015). Temperatures measured in 2011 in the present study suggest the progressive warming of the Ligurian Sea occurring over the last four decades (Bianchi et al., 2019), and that seawater warming is not homogeneous in the entire NW Mediterranean (Rivetti et al., 2014). We also observed an evident positive thermal anomaly in one of the two sites investigated (Punta del Faro), even at higher depths, down to 70 m, where such a high temperature shift had never been reported before. In the other site (i.e., Punta dell'Indiano), the positive thermal anomaly was instead slighter.

4.2. Microbiome response to temperature changes

There is evidence that microbiomes of octocorals, including *C. rubrum*, keep their composition rather stable in the different seasons and along the bathymetric gradient (Bayer et al., 2013; Hernandez-Agreda et al., 2017; van de Water et al., 2016, 2018a, 2018b, 2020). At the same time, other findings suggest that all microbial components are sensitive to shifts in temperatures and that changes in the coral-microbial association may favour coral disease (Vanwonterghem and Webster, 2020; Webster et al., 2016). In particular, prolonged high temperatures (i.e., >18 °C) at depths of 30 m and beyond, have been reported to induce physiological stress in gorgonians, causing tissue necrosis and the rise of pathogens (including *Vibrio* spp.), which can lead to mass mortality (Bally and Garrabou, 2007). Furthermore, experimental investigations revealed that in *C. rubrum* and *E. cavolini*, the colonies located at deeper depths showed a lower tolerance to thermal anomalies than shallower ones, and that the risk of necrosis increases more rapidly in deeper corals exposed to temperature increases

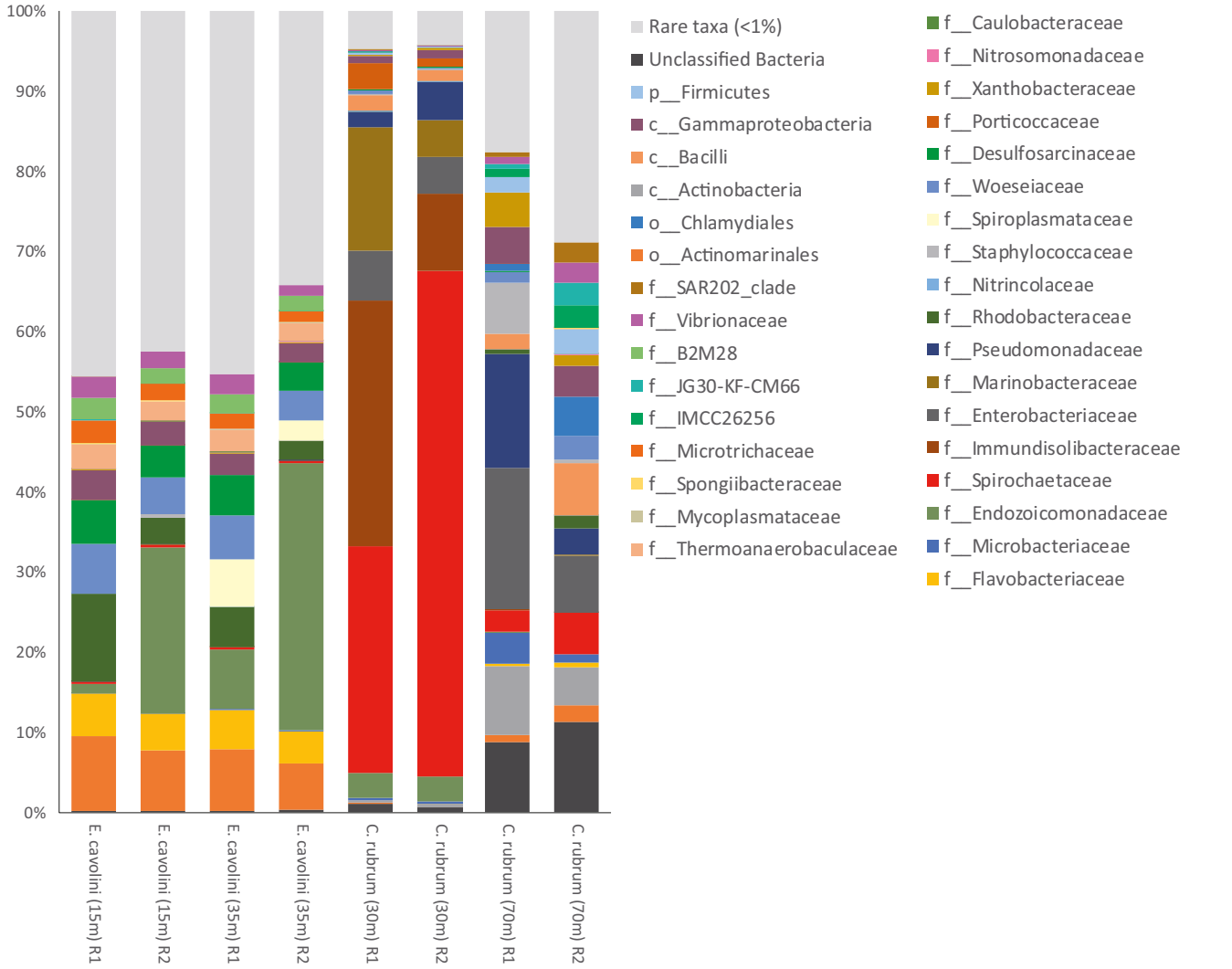

Fig. 5. Taxonomic composition of coral microbiomes. Barplot showing the taxonomic composition of the bacterial assemblages associated with corals at the family level in terms of sequence contribution to each bacterial family. Taxa contributing less than 1% were summed and indicated as "rare taxa (<1%)" Taxa names are preceded by a letter according to the maximum depth of taxonomic assignment: "p" for phylum, "c" for class, "o" for order and "f" for family.

(Pivotto et al., 2015; Torrents et al., 2008). However, previous studies have not yet elucidated the biological mechanisms determining mass mortality events in gorgonians, as it remains unclear whether the rise of pathogens is a consequence or the cause of necrosis and mortality.

Our study provides the first quantitative results on the microbiomes of *P. clavata*, *E. cavolini* and *C. rubrum* collected during the heatwave of 2011. Such corals did not show any sign of visible necrosis or disease, although an initial impact on other coral colonies was already evident (Fig. S2).

The positive relationship between prokaryotic abundance associated with corals and seawater temperatures suggests that the proliferation of prokaryotes is a temperature-dependent phenomenon. CARD-FISH analysis, provided additional evidence that both bacterial and archaeal abundances increased along with seawater warming, although the proliferation of prokaryotes was primarily due to the increase of Bacteria (representing >56% of the sum of both Bacteria and Archaea) (Fig. 3).

Current knowledge on the role of Archaea in coral microbiomes is extremely limited; available results from tropical scleractinian corals, such as *Pocillopora damicornis* (Garren and Azam, 2012) and from the mucus of *Porites lobata* (Garren and Azam, 2010) indicate the presence of Archaea, but they do not clarify if they are present in the coral tissue behaving as diazotrophs (Lesser et al., 2018) or are associated to coral mucus and then adsorbed on the coral surface (Wild et al., 2004). In some cases, Archaea have been shown to support their hosts by either carrying out ammonia oxidation or denitrification, depending on oxygen availability within the mucus layer (Siboni et al., 2008). The relevance of Archaea in our samples (ca. 40% vs. 5–20% in ambient seawater or sediments (Danovaro et al., 2015; Karner et al., 2001)) allows us to hypothesize that they can play an important functional role in the octocorals.

4.3. Heatwave impact on microbiome diversity

Coral-microbiome composition and diversity changed significantly among the different coral species investigated, as also observed in conditions without thermal anomaly (Robertson et al., 2016; van de Water et al., 2018b). Changes in terms of microbial taxa associated with different coral species were expected, also due to their differences in terms of habitat, trophic strategy and bathymetric distribution (McCauley et al., 2020; Ziegler et al., 2019). However, previous findings suggested that environmental changes have limited effects on the microbiome composition of colonies of the same coral species, as revealed by the studies conducted on the gorgonian *E. cavolini* and on the red coral (*C. rubrum*), in which the alpha-diversity of bacteria in their microbiomes remained stable over different seasons and at different depths (Bayer et al., 2013; van de Water et al., 2018a, 2018b, 2020). Our results partially contrast with previous findings on the same coral species since bacterial alpha-diversity of the

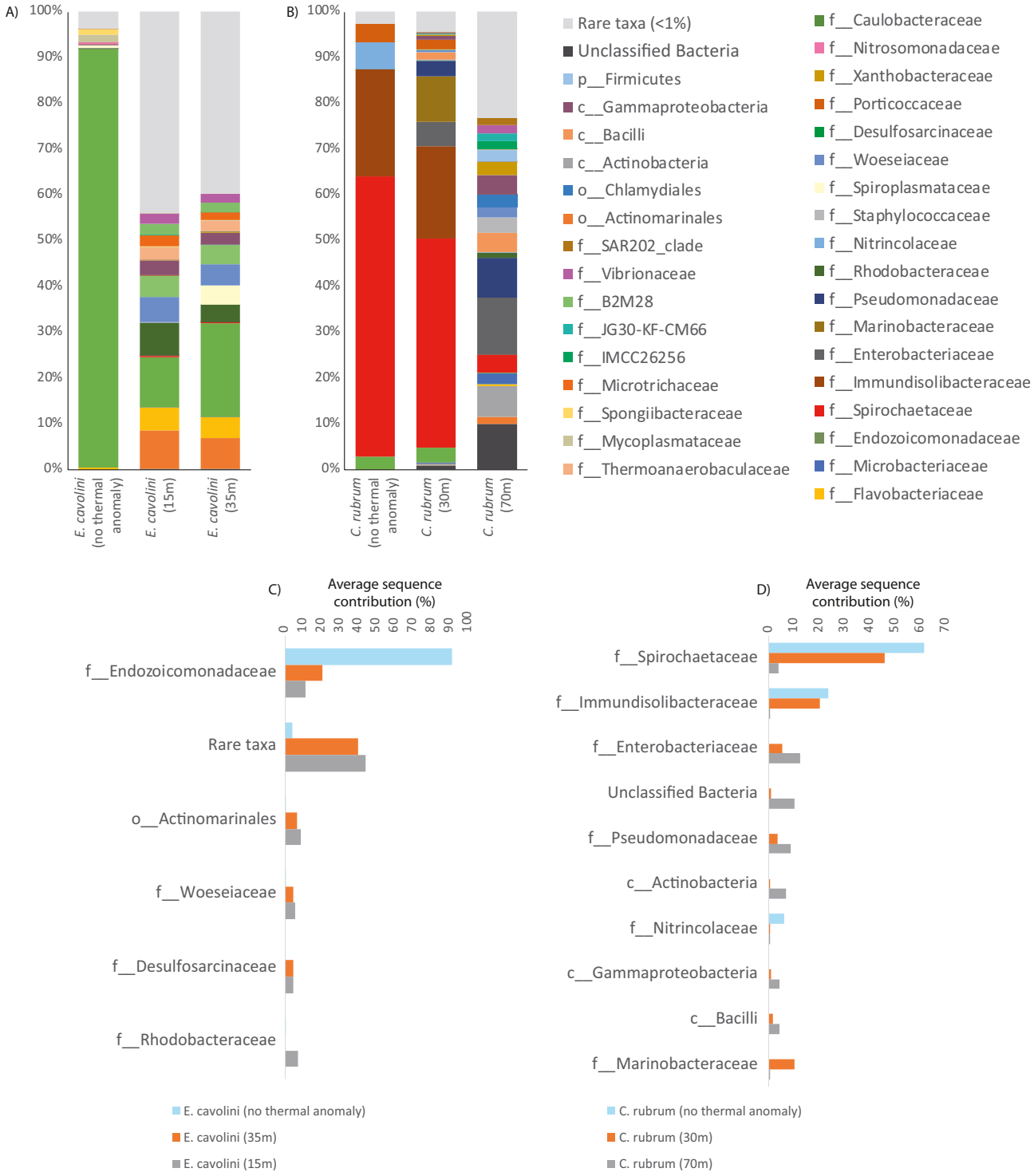

Fig. 6. Changes of microbiome composition under different water temperature conditions. (A–B) Barplots showing the average sequence contribution (as a percentage on 6000 sequences) of bacterial families contributing at least for 1% to the total assemblage to the microbiomes of different *E. cavolini* and *C. rubrum* colonies subjected to temperature anomalies (15 m and 35 m and 30 m and 70 m, respectively) and in conditions without thermal anomaly. Data of bacterial families associated with *E. cavolini* and *C. rubrum* colonies without thermal anomaly were obtained from Van de Water et al., 2020 and Corinaldesi et al., 2021. (C–D) Barplots representing bacterial taxa with significantly different abundances in both *E. cavolini* (C) and *C. rubrum* (D) subjected to thermal anomaly and without thermal anomaly conditions. Taxa contributing to 80% of the variation were considered.

microbiomes of *C. rubrum*, was higher in the deepest corals (i.e., at 70-m depth) than in the shallower ones (i.e. 30-m depth), whereas *E. cavolini* microbiomes did not change between the two different depths (i.e. at 15 and 35 m). Such patterns were consistent with those of the average amplitude of the thermal shift both at Punta del Faro and in Punta dell'Indiano suggesting that octocoral microbiomes are highly sensitive to positive temperature anomalies and in particular to the amplitude of the thermal shift.

The analysis of the taxonomic composition of the coral microbiomes indicated that *E. cavolini* was dominated by the *Endozoicomonadaceae* family.

Members of this family have been previously reported as the dominant components in coral colonies collected from ca. 25 to 60-m depth in several regions of the Mediterranean (Bayer et al., 2013; van de Water et al., 2017, 2020). The *Endozoicomonadaceae* family is typically associated with several coral species across a wide variety of oceanic regions (Neave et al., 2017a, 2017b), and it is considered a stable component of the *E. cavolini*'s microbiome (van de Water et al., 2018a, 2018b). Bacteria belonging to *Endozoicomonadaceae* have key roles since they can contribute to sulfur cycling, protection from pathogens and involvement in nitrogen cycling, protein and carbohydrate transport (Pantos et al., 2015; Raina et al., 2009; van de Water et al., 2017). Previous studies on tropical scleractinians corals suggested that *Endozoicomonadaceae* remain largely unchanged even under stressed conditions (Ziegler et al., 2019). However, in our study the contribution of this family to the microbiome dropped to negligible values (<1%) during the heatwave of August 2011, allowing us to hypothesize a high sensitivity of this bacterial family to temperature shifts.

We also found that in *E. cavolini*, during the heatwave, *Cellvibrionales* provided a negligible contribution to the microbiomes, unlike what has been previously reported for gorgonians, including *E. cavolini* (van de Water et al., 2018a, 2018b). These bacteria are considered key components in these corals being aerobic, mesophilic, neutrophilic and chemoorganotrophic and able to use complex carbohydrates and steroids as substrates (Holert et al., 2018; Spring et al., 2015). Therefore, the decrease in the quantitative relevance of this family reinforces the hypothesis of a microbiome alteration due to the marine heatwave of August 2011.

A consistent fraction of the microbiomes of *E. cavolini* was also characterized by the presence of *Actinomarinales* and *Rhodobacteraceae*. The former are members of bacterioplankton either in temperate and tropical waters (Mizuno et al., 2015; Weber and Apprill, 2020), and are reported here for the first time in association with Mediterranean corals, whereas *Rhodobacteraceae* have been previously reported in association with either temperate and tropical corals (La Rivière et al., 2015; Luo et al., 2021) and include fast-growing opportunistic species that proliferate in stressful conditions, such as during thermal stress (Cárdenas et al., 2012; Pootakham et al., 2019; van de Water et al., 2016; Welsh et al., 2017). In our study, the highest contribution of *Rhodobacteraceae* was found in corals from shallow depths, which were subjected to the highest temperature, confirming their sensitivity to thermal shifts.

The thermal anomaly caused even more important changes in the microbiomes of *C. rubrum* than in the gorgonian *E. cavolini*. In particular, in the microbiomes of the deepest red-coral colonies, the contributions of *Spirochaetaceae*, commonly present in the octocoral holobionts (van de Water et al., 2016, 2017), collapsed (from 90 to 10%, in unstressed vs stressed colonies; Fig. 6B) (van de Water et al., 2018b). *Spirochaetales* are typically associated with healthy red-coral colonies, and characterized by large stability among seasons (Corinaldesi et al., 2021; van de Water et al., 2016, 2018a, 2018b,). Therefore, their significant decrease at 70-m depth, where the average temperature anomaly was the most relevant (Table 1), provides evidence of an impact on the coral microbiome composition. At the same time, other bacterial members including *Enterobacteriaceae* and *Pseudomonadaceae* significantly increased (Fig. 6D). The genus *Pseudomonas* has been repeatedly associated with octocoral holobionts (Brück et al., 2007), with some taxa of this genus behaving as opportunistic pathogens (Chu and Vollmer, 2016; Grottoli et al., 2018). This finding supports the hypothesis that the health of red corals was compromised during the heatwave event occurred in August 2011. Similarly, *Enterobacteriaceae* have been reported in diseased tropical corals (and their mucus (Beatty et al., 2019; Beleneva et al., 2005; Daniels et al., 2015)), allowing us to hypothesize that, also in red corals, these bacteria can be causative agents of disease (Bourne et al., 2016), ultimately leading to coral mortality (Garrabou et al., 2019).

In the colonies of *C. rubrum* at 70-m depth, the contribution of bacterial taxa belonging to the family *Vibrionaceae*, although very low (<2%), was, on average, up to 1 order of magnitude higher than in those at 30-m depth while in *E. cavolini*, the *Vibrionaceae* contribution at 15 m and 35-m depth was very similar. Previous studies have showed a relationship between members of the genus *Vibrio* and global warming (Vezzulli et al., 2012) and have suggested that *Vibrio* infections can trigger mass mortality events in Mediterranean Sea (Martin et al., 2002; Vezzulli et al., 2010). However, as high abundances of *Vibrionaceae* have been reported in already necrotized gorgonians and at temperature > 22–23 °C (Martin et al., 2002), the low quantitative relevance of such a bacterial family in the present study, may be due to the fact that corals were not yet affected by necrosis. At the same time, the increase in the contribution of this family, in colonies subject to a wider temperature shift, can be an early warning sign that their health is deteriorating.

We exclude that the shifts in the microbiome composition observed in the present study were due to sample starvation and/or to artefacts or sample contamination (Morris et al., 2019; Vanwonterghem and Webster, 2020; Vezzulli et al., 2010), because coral samples were collected in a MPA, far from pollution sources and in conditions of adequate food availability (Doglioli et al., 2004; Misic et al., 2011). In addition, coral samples were manipulated in sterile conditions using the most appropriate protocols to avoid any external microbiological contamination (Danovaro, 2010). This conclusion is also supported by the lack, in our samples, of any human pathogen within the family *Enterobacteriaceae* (which can include *Serratia marcescens*, *Escherichia coli*, *Enterococcus faecalis*).

The microbiomes of the red-coral colonies living at 70-m depth showed a clear increase in the relevance of unassigned Bacteria (including those belonging to Gammaproteobacteria) and of other rare bacterial taxa, which are responsible for the high bacterial richness and diversity observed. The unknown identity of these taxa does not allow us to infer on their functional role in the coral microbiome, but they might represent an adaptation to stressful conditions, and even protect the corals from opportunistic and pathogenic taxa, as previously reported for tropical corals (Glasl et al., 2016; Reshef et al., 2006). At the same time, we cannot exclude that some of these unassigned and rare taxa are specifically adapted and selected for living with the red coral at such depths.

The comparison of our microbiomes with those available in literature for the same species and the same site in periods of non-anomalous temperatures (Corinaldesi et al., 2021; van de Water et al., 2020), revealed the presence of a much higher alpha- and beta-bacterial diversity during the heatwave. Such a shift in the microbiome diversity of *E. cavolini* and *C. rubrum* during thermal anomaly was consistently due to the decrease of *Endozoicomonadaceae* and *Spirochaetaceae* (i.e. considered the respective core taxa), and to the increase of other rare taxa (van de Water et al., 2016, 2018a, 2018b). However, the impact of heatwaves on microbiomes reported here was obtained only through a post hoc analysis. In addition, the patterns of similarity between such microbiomes were not consistent across depths and sites, therefore we cannot exclude that other factors, such as the different genotypes and local adaptation of the microbial taxa, the degree of flexibility of the holobiont structure, specific acclimatization processes and thermotolerance to different depths (Neave et al., 2017a, 2017b; van de Water et al., 2020) could have promoted the presence of specific rare taxa, thus contributing to influence the whole holobiont.

5. Conclusions

Our data suggest that the thermal anomaly observed in 2011 likely had a major impact on coral microbiomes down to 70-m depth. The temperature anomaly caused a shift towards a new adaptive state of the holobiont by increasing the abundance of Bacteria and Archaea and alpha and beta-bacterial diversity. At the same time, the relevance of the dominant taxa decreased, thus allowing the proliferation of potential pathogens and a large fraction of unknown and rare taxa. The increase of opportunistic and pathogenic bacteria during the thermal anomaly can be considered an early warning indicator of the worsening of the health conditions of the corals, before than any necrosis can be detected. This study provides new insights into the diversity and composition of coral microbiomes and their sensitivity to thermal anomalies, and suggests that bacterial pathogens are a direct effect of the microbiome alteration and not the secondary consequence of coral necrosis.

The predicted increase in the intensity and frequency of heatwaves across seas and oceans caused by climate change (Smale et al., 2019), along with the high sensitivity of coral holobionts to temperature shifts, suggest that coral forests are under threat, in either shallow and mesophotic habitats.

CRediT authorship contribution statement

Ci. Co., Ca. Ce., A.D. and R.D. conceived the study. Ca. Ce. provided samples for the study. S.V. and M.T. drafted the first version of the manuscript. M.T. performed bioinformatic analyses on microbiomes. C.C., S.V., M.T., A.D., S.C. contributed to data elaboration and interpretation. All authors contributed to manuscript preparation and to the final version.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153701.

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