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Microbiome environmental shifts differ between two co-occurring octocoral hosts

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ABSTRACT: Corals harbor a complex suite of beneficial microbial symbionts. Shuffling the composition of these symbionts could, in theory, help the host withstand rapidly emerging or geographically varying stressors without requiring genetic change to the coral itself. The relative impact of host qualities on microbiome (bacteria) composition should provide insight into the extent that shifting microbial symbionts can play in resilience to environmental disturbance on reefs. We sought to assess the differences in microbiome composition across a large spatial scale (between Puerto Rico and Bonaire; >700 km apart) and in response to localized anthropogenic impacts in 2 co-occurring Caribbean soft corals (*Eunicea flexuosa* and *Gorgonia ventalina*) with well-documented differing degrees of population genetic differentiation. Host species was the strongest determinant of microbiome composition, with between-hosts distinction due largely to differences in the abundant genera *Endozoicomonas* and *Mycoplasma*. Interestingly, the microbiome of the more genetically subdivided *G. ventalina* showed stronger differentiation between islands and in response to local anthropogenic impacts than the less subdivided *E. flexuosa*. For both hosts, anthropogenic impacts on microbiome composition were stronger in Bonaire. Again, *Endozoicomonas* was responsible for much of the differentiation between and within islands and included host- and island-specific sequence variants. The level of intra-species microbiome variation mirrored the known geographic differentiation of their hosts, a trend that is consistent in the literature for 8 other coral species. Thus, while potentially adaptive bacteria may shuffle in response to environmental changes, our findings suggest that most changes to microbiomes are likely constrained by host genetics.

KEY WORDS: Anthropogenic impact · Coral · Symbiosis · Microbial communities · Genetic differentiation

1. INTRODUCTION

As studies of the microbial associates of eukaryotes have moved beyond the descriptive, focus has shifted to assessing sources of intraspecific variation in microbiomes (all microbial taxa in a defined environment) among hosts, specifically in response to environmental differences. At one extreme, some members of the microbiome may strictly co-evolve with their hosts, becoming, in essence, a heritable entity (Bordenstein & Theis 2015, Theis et al. 2016, Rosenberg & Zilber-Rosenberg 2018) that remains stable in the face of environmental change. By contrast, environmental shifts may trigger the host to take up bacterial symbionts that maintain or improve host performance and move into and out of the microbiome in tandem with environmental change (Hansen & Moran 2011, Webster & Reusch 2017, Ziegler et al. 2017, Rocca et al. 2019, Mueller et al. 2020, Osman et al. 2020). While many symbioses will fall somewhere between these extremes, determining the degree to which hosts and their microbiomes co-evolve or shift and reassemble is critical for understanding both the evolution of host

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traits and their capacity to adapt to changing conditions.

Corals are an especially interesting case for studying the spectrum of microbiome flexibility. The generation times of many large corals run to decades or even centuries, hindering adaption to a fast-changing environment. Corals also harbor a diverse array of microbial symbionts (bacteria, archaea, fungi, and algae) in high abundance. In contrast to their long-lived hosts, these microbes can respond quickly to environmental changes (over hours, days, or weeks; Kline et al. 2006, Ziegler et al. 2017, Caughman et al. 2021, Haydon et al. 2021). Such rapid acclimation may enhance the ability of the host coral to survive rapid ecological changes (Bourne et al. 2016). This adaptability may allow corals to persist through acute stressful conditions (Reshef et al. 2006, Bordenstein & Theis 2015, Bourne et al. 2016, Santoro et al. 2021). To understand how symbionts can assist the adaptation of their coral hosts, we must elucidate the capacity for changes to the microbiome across species, among populations, and in response to stressors.

The stability in one member of the coral microbiome has already been explored for many of these variables: symbiotic micro-algae (Family Symbiodiniaceae). These micro-algal symbionts are highly diverse, with species-specific and generalist genera that can be found in corals and other hosts (Baker 2003, LaJeunesse et al. 2018). Corals can transmit their symbiotic micro-algae to their offspring either by vertical transmission, as often seen in brooders, or by horizontal transmission, used by most broadcast spawners (Baker 2003, van Oppen 2004, Coffroth & Santos 2005). Genetically differentiated populations of corals often harbor location-specific Symbiodiniaceae genotypes (LaJeunesse 2002, Santos et al. 2003, Bongaerts et al. 2010). By contrast, corals with little such genetic subdivision may house genetically identical Symbiodiniaceae over large distances (100s to 1000s of km) (Loh et al. 2001, Barneah et al. 2004, Prada et al. 2014). More generally, modes of symbiont transmission and levels of host genetic subdivision are often correlated with the genetic structure of the micro-algal endosymbionts (LaJeunesse et al. 2003, 2004b, Stat et al. 2008, Finney et al. 2010), echoing patterns seen in mammals (Moeller et al. 2017) and insects (Wang et al. 2020). Furthermore, under stressful conditions, some corals can actively swap their Symbiodiniaceae for better-suited algal phylotypes (Baker 2003, Berkelmans & van Oppen 2006). Whether these patterns of host-specificity and flexibility with environmental changes also hold true for the prokaryotic members of the microbiome is not known.

Microbiomes (henceforth used to refer to the prokaryotic community unless otherwise noted) in corals often vary in similarity with the phylogenetic closeness of their hosts (Sunagawa et al. 2010, Bourne et al. 2016, Chu & Vollmer 2016, van Oppen & Blackall 2019). Host-specificity is strong for microbiomes from coral tissue and skeleton, while those from mucus are more variable (Pollock et al. 2018). The stability of microbiome composition across time is less consistent, with some coral microbiomes showing high seasonal or annual variability (Kimes et al. 2013, van de Water et al. 2018) and others remaining stable for years (Littman et al. 2009, Lema et al. 2014a, McCauley et al. 2016).

Despite these aspects of species-specificity, the microbial communities associated with some corals still respond to local environmental conditions. Some such communities have unique signatures at different locations consistent with local adaptation (Hong et al. 2009, Pantos et al. 2015, Hernandez-Agreda et al. 2016, Dunphy et al. 2019). In addition, many coral species exhibit altered microbial compositions when stressed by heat (Vega Thurber et al. 2009, Ziegler et al. 2017), pH (Vega Thurber et al. 2009, Grottoli et al. 2018), or disease (Roder et al. 2014, Meyer et al. 2016). However, such microbiome plasticity is not universal among host species or even among genotypes of the same host (Cárdenas et al. 2012, Kimes et al. 2013, Lema et al. 2014a,b, Wright et al. 2017, Grottoli et al. 2018, Rachmawati 2018). Why the microbiomes of different coral hosts respond to environmental changes so differently remains unclear.

One host attribute that could contribute to such differences is the degree of genetic subdivision among populations. High coral population subdivision could facilitate local adaptation that may extend to the active selection of local symbionts and co-evolving partnerships to create the most beneficial microbiome, as seen in Symbiodiniaceae (LaJeunesse et al. 2003, Stat et al. 2008, Finney et al. 2010). Thus, if host genetic subdivision matters, then within-species compositional differences of microbiomes should increase with increasing population genetic structure of the host.

The co-occurring Caribbean gorgonians *Eunicea flexuosa* and *Gorgonia ventalina* offer an opportunity to address the extent to which host population genetic subdivision impacts the flexibility of the microbiome. Both soft corals are broadcast spawners that acquire their micro-algal endosymbionts via horizontal transmission (Andras et al. 2013, Prada et al. 2014). How these 2 hosts acquire their prokaryotic symbionts is not known, but many broadcast spawn-

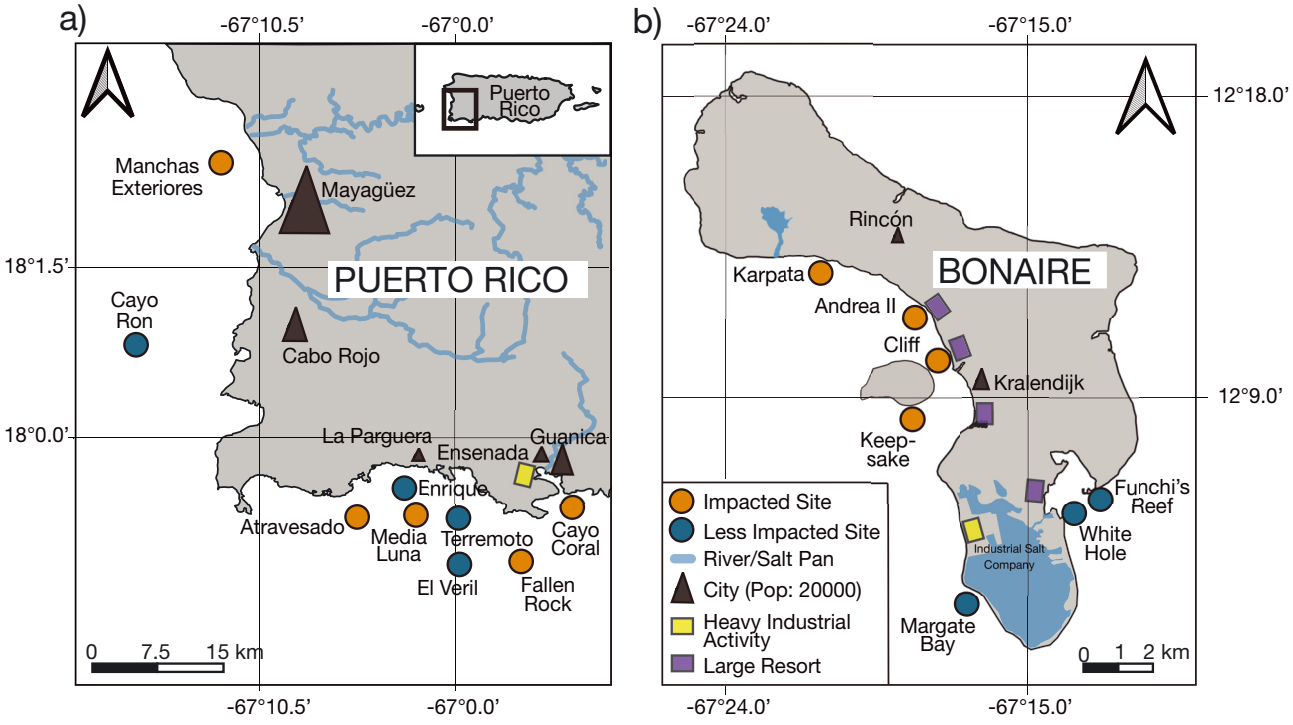


Fig. 1. Collection sites in (a) southwestern Puerto Rico and (b) Bonaire. These maps only indicate rivers (blue lines), salt pans (blue polygons), cities (black triangles with triangle size correlating positively with increasing city population size), areas of heavy industrial activity (yellow rectangles), and large resorts (purple rectangles) of particular interest for this study. Orange circles: impacted sites; blue circles: less impacted sites

ers acquire most, or all, of their symbionts horizontally during the larval phase or immediately following settlement (Apprill et al. 2009, Sharp et al. 2010, Lema et al. 2014a, Damjanovic et al. 2020). Despite these similarities, these 2 gorgonians show different levels of population genetic differentiation. *E. flexuosa* is genetically homogenous across its Caribbean range, although the nominal species includes 2 genetically and morphologically distinguishable depth-delimited lineages (Prada & Hellberg 2013). Each of these lineages harbors a unique strain of symbiotic micro-algae that does not shuffle, even when hosts are reciprocally transplanted (Prada et al. 2014). This strong and specific algal symbiosis and the high level of host connectivity within lineages suggests that *E. flexuosa* may be constrained in its ability to shift its microbial community in response to local environmental conditions. By contrast, *G. ventalina* displays high levels of genetic differentiation across its range (Andras et al. 2013) and its micro-algal endosymbionts change over distances as short as ~200 km (Kirk et al. 2005, 2009, Andras et al. 2011). This geographic differentiation in *G. ventalina*-associated micro-algal endosymbionts may suggest a relatively high potential for microbiome adaptability.

We compared compositional differences in the bacterial microbiomes of *G. ventalina* and *E. flexuosa* in parallel with both geographic and local environmental differences. Microbiome differentiation among populations was assessed by comparing microbiomes of corals collected from 2 islands located ~700 km apart: Puerto Rico and Bonaire. Local microbiome differentiation was compared in response to varying anthropogenic impact levels on each of these islands.

2. MATERIALS AND METHODS

2.1. Sample collection

We sampled from 9 sites in SW Puerto Rico (Fig. 1a) and 7 in Bonaire (Fig. 1b) (Table S1 in the Supplement at www.int-res.com/articles/suppl/m720p059_supp.pdf). To examine microbiome responses to local stress, we sampled from sites more and less impacted by anthropogenic activity within each island. Site selections were made based on proximity to human activity and literature on reef health (see Section 2.2). Samples were collected in Puerto Rico under permit O-VS-PVS15-SJ-00842-19052016 obtained via the Departamento de Recursos Naturales y Ambientales

and in Bonaire under an un-numbered permit provided by Stichting Nationale Parken (STINAPA) Bonaire and approved by the Directorate of Spatial Planning and Development.

Colonies of 2 Caribbean soft corals, *Eunicea flexuosa* 'shallow' lineage (Prada & Hellberg 2013) and *Gorgonia ventalina* were sampled via SCUBA diving (count details in Table S2). Colonies from the *E. flexuosa* shallow lineage were identified based on their distinctive branching morphology (Prada et al. 2008), and their lineage was later verified via genetic analysis (see Section 2.5). Sampled colonies were located >1 m apart and were free of lesions, encrusting organisms, and corallivores. Sampled corals were >1 m in height, equivalent to >20 yr old based on growth rates for *E. flexuosa* and other soft corals in the same family as *G. ventalina* (Cary 1914, Castanaro & Laskera 2003, Lasker et al. 2003). Coral tissue (~5 cm) was clipped from an axial branch tip from colonies located at <20 m depth for *E. flexuosa* and at <25 m depth for *G. ventalina* and placed into individual pre-labeled sealable bags. Corals produce a surface mucus layer that renews diurnally or hourly in some species and houses a more variable microbiome than coral tissue (Bourne & Munn 2005, Sweet et al. 2011, Weiler et al. 2018). In an effort to examine the impact of long-term exposure to anthropogenic stress on coral microbiomes, we focused only on the more stable tissue microbiome. Upon return to the surface, each branch was rinsed with filtered seawater to remove the mucus layer (Krupp 1985, Glasl et al. 2019, Marchioro et al. 2020). Following rinsing, branches were placed in individual sterile Whirl-Pak® bags, flash-frozen using a pre-charged (to −80°C) Bio-Bottle® Ultra-Freeze MAX, and moved to a −80°C freezer for storage.

Sediment (~50 g) and water (1 l) samples (3 each per site, 48 total; Table S2) were collected from all sites as environmental controls. Sediment was placed in a labeled bag at depth. At the surface, excess water was drained off and sediment was transferred to a sterile Whirl-Pak® bag. Water samples were collected using a bleach-rinsed, EtOH-sterilized 1.5 l Nalgene® bottle. Following the protocol set forth in Henson et al. (2016, 2018), upon returning to the surface, 1 l was filtered first through a 2 µm glass microfiber pre-filter to remove larger plankton and particulate matter, then through a 0.22 µm Sterivex filter to concentrate picoplankton. Each filter was placed in a sterile Whirl-Pak® bag. Approximately 20 ml of filtered water was saved in sterile centrifuge tubes for subsequent nutrient analyses. All environmental samples were flash-frozen at the surface using a pre-charged (to −80°C) Bio-Bottle® Ultra-Freeze MAX and were moved to a −80°C freezer for long-term storage.

2.2. Reef water nutrient testing and analyses

The filtered reef water samples and 2 µm pre-filters from each site were sent to the Wetland Biogeochemistry Analytical Services Lab at Louisiana State University (LSU) to be tested for nutrient parameters indicative of anthropogenic impact: nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}), and chlorophyll *a* (chl *a*) (Bell 1992, Bell et al. 2014, Slijkerman et al. 2014; Table S3). Nutrient samples were run with both internal and external calibration standards on an O.I. Analytical Flow Solutions IV Segmented Flow Auto Analyzer according to the following protocols: EPA 353.4 (NO_2^-, NO_3^-; Zhang & Peter 1997), EPA 350.1 (NH_4^+; US EPA 1993b), and EPA 365.5 (PO_4^{3-}; US EPA 1993a). Chl *a* values were obtained from the 2 µm pre-filters following the EPA 445.0 protocol (Arar & Collins 1997). Fluorescence was read on a Turner Designs TD-700 Fluorometer.

The resulting nutrient data were used to calculate dissolved inorganic nitrogen (DIN: $NH_4^+ + NO_x$) and the DIN:PO_4^{3-} ratio (Table S4). High N:P ratios can lead to nutrient-starved Symbiodiniaceae and lower tolerance to heat and light stress in corals (D'Angelo & Wiedenmann 2014). All nutrient data were log transformed in R, and the subsequent values were normalized using the R function 'scale'. The normalized values were used in principal component analysis (PCA) biplots (R package 'ggbiplot').

2.3. Designating the level of anthropogenic stress at local collection sites

The anthropogenic impact level at each collection site was designated using a 2-tier approach. The first tier was based on data from a literature search of reef quality including (1) percent live coral cover, (2) proximity to freshwater output, (3) proximity to or directly down-current from a city or resort, (4) industrial activity, and (5) nutrient loading/sedimentation (Goenaga & Cintrón 1979, Morelock et al. 2001, Larsen & Webb 2009, Stokes et al. 2010, Sommer et al. 2011, Slijkerman et al. 2014, de Bakker et al. 2016, Dutch Caribbean Nature Alliance 2017; Table S5). Each category was given an impact rating of low, moderate, or high (Table S5). Based on these cate-

gories, sites were given a preliminary designation of impacted or less impacted.

The second tier was made by coupling tier-one site parameter impact ratings with water quality parameters tested in this study (Tables S3–S6). Four such parameters were considered because they have clearly defined environmental thresholds for healthy reefs (Bell 1992, Bell et al. 2014, Slijkerman et al. 2014; Table S3) and/or best separated tier-one site types on a PCA plot (Fig. S1), as determined by % contributions from the R package 'factoextra' function 'get_pca_var' (Kassambara & Mundt 2020). Parameter impact level for DIN, PO_4^{3-}, and chl *a* were categorized as low (<0.6*x* threshold), moderate (0.6*x*–1*x* threshold), or high (>1*x* threshold) based on their established thresholds (Table S3). DIN:PO_4^{3-} ratios were lower than the ocean average (~22; Redfield 1934, Martiny et al. 2014; Table S4), but we were able to utilize them for inter-site comparisons within this study. The DIN:PO_4^{3-} ratio was given an impact rating of low: <2; moderate: 2–4; or high: >4 (Table S6). Finally, each parameter-level impact rating (low, moderate, or high) was assigned a point value of 0 pt, 1 pt, or 2 pts, respectively. Final site designations of impacted (>6 pts) and less impacted (<6 pts) were determined by summing points across all parameters (Table S6). This split sites on both islands into similar numbers of impacted and less impacted sites (Table S6, Fig. 1).

2.4. DNA extraction

All coral, sediment, and water (0.22 µm Sterivex filters) samples were prepared for DNA extraction in a biological safety cabinet (Baker) to minimize potential contamination. DNA was extracted using the Qiagen DNeasy PowerSoil® Kit. Coral tissue (~40–50 mg) was removed from the skeleton using a sterile razor blade. DNA extractions were completed following the modified PowerSoil protocol of Sunagawa et al. (2010) with an extended lysis step of ~24 h. To prepare the 0.22 µm Sterivex filters for DNA extraction, each plastic filter housing was cracked open using sterilized pliers, and a sterile razor blade was used to cut along the edge of the plastic filter holder to release the filter. Each filter was removed and placed in a PowerSoil bead-beating tube, and extractions were completed following the manufacturer's protocols. Sediment samples (~25 mg sediment per sample) were extracted following the manufacturer's protocols. We also extracted DNA from 6 kit control samples, one for each box of PowerSoil extraction kits, following the manufacturer's protocols. A Qubit2.0 Fluorometer (Life Technologies) was used to quantify DNA. All DNA samples were eluted in PowerSoil C6 elution buffer (10 mM Tris) and stored at −20°C.

2.5. PCR protocols, 16S rRNA amplicon library preparation, and sequencing

To confirm the shallow lineage designation of the sampled *E. flexuosa* colonies, the identity of a random subset (32 individuals) was verified (data not shown) by sequencing a small portion of the mt*MutS* gene (a mitochondrial MutS homolog originally coined mt*MSH* by France & Hoover 2001), a useful genetic marker for identifying soft coral species (Bilewitch & Degnan 2011). We targeted a 631 bp region of the mt*MSH* gene that can distinguish lineage-level differences in *E. flexuosa* using the primers (ND42599F: 5′-GCC ATT ATG GTT AAA CTA TTA C-3′, Mut-24584: 5′-TSG AGC AAA AGC CAC TCC-3′) and PCR protocols detailed in Prada & Hellberg (2013). Sanger-sequencing of the PCR product was completed on a 3130xl Genetic Analyzer (Applied Biosystems) at the LSU Genomics Facility.

For microbiome analysis, the extracted DNA was used to generate PCR amplicon libraries targeting the V4 region of the 16S rRNA gene present in prokaryotic taxa. Libraries were generated using a region-specific (515F–806R) primer set that includes a 12 bp barcoded sequence on the forward primer that supports pooling of up to 2167 samples in each lane (Caporaso et al. 2012, Apprill et al. 2015, Parada et al. 2016). Previous work on these 2 coral species found high levels of host contamination with the use of these primers but successfully increased prokaryotic read recovery by 67.0% in *E. flexuosa* and 56.7% in *G. ventalina* by adding custom peptide nucleic acid (PNA) clamps to each PCR reaction (Reigel et al. 2020). To mitigate host DNA contamination, we also added the custom PNA clamps to all PCR reactions following the protocol detailed in Reigel et al. (2020).

Barcoded amplicons were quantified with PicoGreen (Invitrogen) and an Infinite 200 PRO plate reader (Tecan). All volumes of each product were pooled to ensure that each was represented in equimolar amounts. Pooled amounts were cleaned using AMPure XP Beads (Beckman Coulter) and quantified a second time using a Qubit fluorometer (Invitrogen). Each pool was diluted to a concentration of 2 nM, denatured, and diluted to a final concentration of 6.75 pM with a 10% PhiX spike. Ampli-

con sequence data was generated using a 2 × 251 bp Illumina HiSeq2000. Library preparation and sequencing was completed at the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory.

2.6. Sequence processing

Amplicon sequence reads were imported into QIIME2 (Bolyen et al. 2019) and separately processed for grouping into both 97% operational taxonomic units (OTUs) and amplicon sequence variants (ASVs). ASVs can differ by as little as 1 bp across a nucleotide sequence and are better able to detect strain-level differences in prokaryotes, although many studies comparing the 2 analysis methods have shown that they result in similar biological signals (Chiarello et al. 2022). Analysis at the ASV level is computationally intensive, but OTU clustering at 97% identity reduces the size of the raw read data set and the computational requirements (Schloss et al. 2011, Chiarello et al. 2022). The per sample raw read counts for sediment and water were 5–9× greater than for coral (Table S7), making an ASV-level analysis for all samples computationally restrictive, but 97% OTU clustering was sufficient to detect differences in the bacterial communities of these disparate sample types (see Section 3.2). To examine finer scale compositional differences in the prokaryotic communities among coral samples, we used ASVs but excluded sediment and water samples.

For OTU clustering, the raw fastq-formatted files were joined using the 'vsearch join-reads' command. Quality control (QC) was performed on join-reads using the 'q-score-joined' command, with a min-quality threshold of 20 (Bokulich et al. 2013). QC-filtered reads were then dereplicated and clustered into 97% OTUs using the 'vsearch cluster-features-open-reference' command. Clustering and OTU taxonomic assignments were performed by referencing the Silva rRNA v.132 reference database (Quast et al. 2013).

For ASV clustering, fastq-formatted files were demultiplexed following the 'Atacama soil microbiome tutorial' pipeline provided at https://qiime2.org. The QIIME2 wrapper of the 'DADA2' package (Callahan et al. 2016) was used to denoise reads and complete QC steps. We removed low-quality (expected error rate of >2.0) and chimeric reads (method: 'consensus'), and truncated 10 bp from the 3' end of each reverse read because the quality score consistently dropped below 20 after 240 bp (as seen on the interactive QC plot produced during the demultiplexing step). Truncating low-quality bases prior to ASV analysis increases the overall quality of the read, resulting in higher read retention during QC and more accurate ASV inference (Werner et al. 2012, Mohsen et al. 2019). DADA2 was also used to infer ASVs, and the resulting ASVs were assigned taxonomy in QIIME2 using the Silva rRNA v.132 reference database (Quast et al. 2013). To focus the analyses on the bacterial symbionts, all resulting OTUs and ASVs that did not have at least phylum-level designations or were taxonomically identified as non-bacterial DNA (e.g. mitochondria, chloroplast, archaeal, or eukaryotic) were filtered from the data prior to downstream analyses. Resulting OTU and ASV tables and taxonomic assignments were exported from QIIME2 for further analyses in R.

2.7. Bacterial community analyses

Alpha and beta diversity for the bacterial microbiomes were calculated using the R package 'phyloseq' (McMurdie & Holmes 2013) and following a modified version of the R script from (Henson et al. 2018) for both the OTU data set (coral and environmental samples) and the ASV data set (coral samples only). Three different alpha diversity metrics (observed richness, Shannon's diversity index, inverse Simpson's diversity index) were calculated using the 'phyloseq' function 'estimate_richness'. Alpha diversity metric box plots were made using the R package 'ggplot2' (Wickham 2016). Statistical differences in alpha diversity metrics between sample types (OTU data set), between host species (ASV data set), and between islands (within each host species; ASV data set) were tested using independent non-parametric Kruskal-Wallis tests using the R package 'STAT' (Bolar 2022).

Before calculating beta diversity, all samples were normalized to account for among-sample differences in sequencing depth, which can impact diversity analyses (Weiss et al. 2017). The 'rarecurve' function in 'vegan' (Oksanen et al. 2018) was used (1000 iterations) to calculate rarefaction curves for both the OTU and ASV data sets. Results of the rarefaction curves are used to inform read abundance thresholds for subsequent rarefying of data sets. Samples were rarefied in QIIME2 (Weiss et al. 2017) by removing samples that did not meet the minimum read count and then resampling without replacement for the remaining samples. Rarefaction curves for the ASV data set (coral only; Fig. S2) indicated little gain in diversity for most coral samples beyond 15K reads

per sample and rarefying to 15K reads minimized the removal of coral samples due to low read counts. The OTU data set was also rarefied to 15K reads to standardize between the 2 methods. The rarefaction process for the OTU data removed 2 sediment and 73 coral samples, while for the ASV data set, 79 coral samples were removed. As such, all further references to microbiome results from work presented here are focused on bacterial taxa. The rarefied OTU and ASV data sets were used for all beta diversity analyses.

Our sampling design includes 4 unique variables: sample type (coral, sediment, water), host species, geographic location, and anthropogenic impact on collection site. To determine the relative impact of each of these variables on microbiome composition (beta diversity), we completed 3 rounds of sequential non-parametric analysis of similarities (ANOSIM) tests using the rarefied 97% OTU and ASV data sets. ANOSIM tests, used to assess significant differences between groups of samples, were completed using the 'anosim' function (distance method: 'bray'; permutations: 10 000) in the R package 'vegan' (Oksanen et al. 2018). These tests provided us with a series of ANOSIM R-values, along with p-values denoting significance, for each variable. The R-values were utilized to parse the data into smaller subsets to examine differences between microbiomes that might be masked. For this analysis, ANOSIM R-values of >0.25 indicated different microbial communities, with higher values indicating more compositional differentiation, and R-values of <0.25 indicated similar microbial communities with subsequently lower differentiation. All ANOSIM tests completed in the sequential analysis returned significant p-values ($p < 0.001$).

Beta diversity differences and similarities between samples were visualized using non-metric multidimensional scaling (NMDS) plots made with the R package 'ggplot2' (Wickham 2016). An initial NMDS visualization of all samples (sediment, water, coral, and kit controls; OTU data set) showed that kit control samples clustered independently of all other samples, and they were removed from further analyses. Differences in beta diversity variance between species, between islands within species, and between site types from each island within a species were assessed with homogeneity of dispersion tests. Dispersion tests were run using the 'betadispersion' function from the R package 'micro4all' (distance method: 'Bray'; permutations: 999; Wentzien 2021).

The R package 'ampvis2' (K. S. Andersen et al. preprint, doi:10.1101/299537) was used with the coral-only ASV data set to further compare bacterial microbiome composition between host species, between islands within species, and between site types from each island within a species. The 'amp_ordinate' function, with 'Hellinger' transformations (Legendre & Gallagher 2001), was used to calculate and plot PCA biplots of host species microbiome composition (beta diversity) and ASVs that contribute the most to differences between species (based on abundance within a particular species). The 'amp_ordinate' function determines contributing ASVs by first filtering out very low-abundance (<0.1%) ASVs and transforming the data, and then calculating a distance matrix (Bray-Curtis). The function then performs an ordination and obtains axis scores for both the samples and each ASV. ASV axis scores are based on relative abundance within a host species microbiome such that each ASV is plotted closer to the host in which their abundance is higher. ASVs of relatively low abundance, or those with similar abundances in the microbiomes of both host species, are plotted at or near 0,0 coordinates. The 'tax_glom' function was used to collapse ASVs to the genus level. Genus-level box plots showing the relative abundance, in each host species, of the 15 most abundant genera in the ASV data set were calculated and plotted using the 'amp_box' function (specifications: sort_by: mean; adjust_zero: FALSE; normalize: FALSE).

'Core' bacterial ASVs for each coral host and each island were identified in 2 ways. First, the 'amp_core' function in 'ampvis2' was used to create a scatterplot of ASVs that were abundant (≥1% relative abundance per sample) versus present (>0% relative abundance per sample) in a defined group of samples (e.g. *G. ventalina* from Puerto Rico). Second, the 'amp_venn' function was used to identify the 70 and 90% 'core' ASVs, as those occurring in at least 70% of samples and 90% of the samples, at any abundance, in a defined group. 'Core' ASV parameters were aligned with those used by 36 publications that identified 'core' microbial taxa in marine invertebrate associated-microbiomes, most at the 97% out level or higher, as having a relative abundance from >0 to >0.5% and occurring in >30–100% of samples (reviewed in Neu et al. 2021). Average read abundance per sample of each identified 70% 'core' ASV was calculated as: (total reads per group) / (rarefied reads per sample). The resulting values for each 70% 'core' ASV were combined to obtain the read abundance per sample for the 'core' ASVs within each group.

Differential abundance between impacted and unimpacted sites was examined for the top 10 most

abundant ASVs within the rarefied data set for each host from each island. This method resulted in 4 groups of 10 ASVs (1, *G. ventalina* from Bonaire; 2, *G. ventalina* from Puerto Rico; 3, *E. flexuosa* from Bonaire; and 4, *E. flexuosa* from Puerto Rico) that were independently tested for differential abundance between the site types. Abundance data for each ASV was first checked for normality and homogeneity of variance, but none met both assumptions; therefore, significance was calculated using a series of non-parametric *t*-tests (Kruskal-Wallis) performed in JMP Pro® v.14. (SAS Institute).

2.8. *Endozoicomonas* haplotype map construction

The 16S rRNA (V4 region) gene sequences representing the 30 most abundant *Endozoicomonas* ASVs across all samples in the rarefied coral data set were aligned using MUSCLE (Edgar 2004) in AliView (Larsson 2014). The resulting nexus-formatted alignment was input to the program 'PopART' for haplotype construction and visualization (settings: median-joining network, ε = 0; Bandelt et al. 1999, Leigh & Bryant 2015).

2.9. Literature search: host genetics and microbiome differentiation

We conducted a literature search to compare the geographic genetic population differentiation of coral hosts, including both hard and soft corals, with the differentiation of their microbiomes. To this aim, Google Scholar was searched (July 2022) to identify all publications on coral microbial communities, with no publication date limitations, using a list of search terms including 'coral microbiome', 'coral microbial communities', 'coral microbiota', 'soft coral microbiomes', 'soft coral microbial communities', 'octocoral microbiomes', 'octocoral microbial communities', 'hard coral microbiomes', 'hard coral microbial communities', 'scleractinian microbiomes', 'scleractinian microbial communities', and 'coral reef microbiomes'. Only coral microbiome research comparing ≥3 locations (minimally a distinct site or reef within a broader geographic area) and with sample sizes of >3 corals per location or species were included. Studies that used stress treatments were not included, as these factors are known to impact microbiome composition.

For each microbiome study that met the defined parameters, Google Scholar was again searched for any publications reporting geographic genetic differentiation of the host corals. Search terms were unique for each host species, but general formats included 'species name + genetics', 'species name + geographic differentiation', 'species name genetic differentiation', 'species name + genetic subdivision', 'species name + population subdivision', and 'species name + population genetics'. We made efforts to find multiple references with the same results for both host genetics and microbiome differentiation. Even with these relatively open parameters (no publication date limitations, few locations, and low sample sizes), little work has been done that fit these criteria.

We also included 2 exceptional studies, both of which examined just 2 locations. Goldsmith et al.'s (2018) work on *Primnoa resedaeformi* included 2 locations but was the only study we found that compared host genetics and microbiome composition from the same samples. Chapron et al. (2020) compared microbiomes of *Lophelia pertusa*, also from just 2 locations, but their work was corroborated by another source (Kellogg et al. 2017) and included a sizable number of samples (23 coral colonies).

The microbiomes of each coral were designated as either 'more' or 'less' differentiated based on the findings and conclusions in each publication. Due to the various types of analyses used to determine microbiome compositional and host genetic differences across the publications, a broader approach was taken to determine the designations. If the results indicated significant compositional differences between locations, the host was labeled as having a 'more differentiated' microbiome, while hosts that did not show significant compositional differences between locations were considered 'less differentiated'. Similarly, a host was labeled as having 'more genetic subdivision' if the results in the publication indicated significant genetic differences between locations, while a host with 'less genetic subdivision' did not show evidence of genetic differences or was considered genetically homogenous across locations.

3. RESULTS

3.1. Sequencing results

Sequencing returned a total of 177 939 712 reads across all samples. QIIME has unique QC processes for 97% OTU (all samples) and ASV (corals only) analyses, but both removed 2 *Gorgonia ventalina* samples because none of their associated reads passed QC filters. After QC for the OTU analysis

(corals + environmental samples), 117 082 482 reads remained. Sediments had the highest average read counts across all sample types (Table S7). Following QC filtering for the ASV analysis, coral samples retained a total of 33 712 508 reads. Samples from *G. ventalina* averaged ~71K reads per sample, while *Eunicea flexuosa* returned ~117K reads per sample (Table S7).

After rarefying to 15K reads per sample and removing samples that did not meet this threshold, the OTU analysis retained 156 *E. flexuosa* and 123 *G. ventalina* samples, while the ASV analysis retained 152 and 121 samples, respectively (Table S7). The rarefied OTU reads were grouped into 62 347 OTUs at 97% similarity and the rarefied ASV data set of corals grouped reads into 14 782 ASVs.

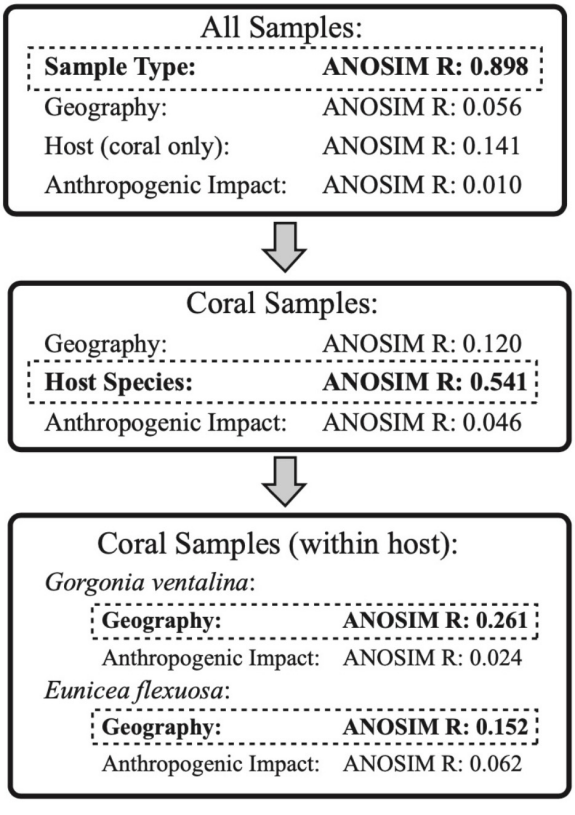


Fig. 2. Sequential ANOSIM R results for the analysis of beta diversity. The initial round was completed using 97% operational taxonomic unit data. The 2 subsequent rounds used amplicon sequence variant data from corals only. Results in bold and outlined indicate the highest ANOSIM R-value for that round and denote the factor that accounts for the greatest amount of variation in prokaryotic community composition between groups of samples. All ANOSIM test results were significant ($p < 0.001$)

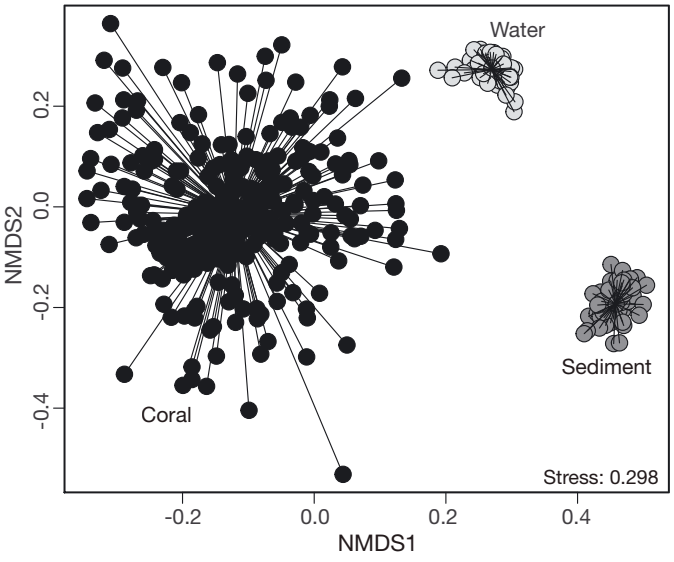


Fig. 3. Non-metric multidimensional scaling (NMDS) of beta diversity (Bray-Curtis) for coral (black), sediment (dark gray), and water (light gray) samples. Black lines connect points of the same type to the centroid for each group

3.2. Relative impact of sample variables on microbiome composition

To assess the relative impact of sample type, host species, geography, and anthropogenic impact on the sampled microbiomes (bacteria only), we completed 3 rounds of sequential ANOSIM (Fig. 2). The first round used 97% OTU data from all samples. Sample type (i.e. coral vs. sediment vs. water) had the highest impact on microbiome composition (ANOSIM R: 0.898, $p < 0.001$). Sediment and water communities were tightly clustered regardless of collection location, while coral communities were more variable (Fig. 3). Coral microbiomes were also less diverse (Chao1) than either sediment or water microbial communities (Kruskal-Wallis $\chi^2_2 = 203.13$, $p < 0.0001$; Fig. S3)

Because sample types had such distinct bacterial microbiomes, for the 2 subsequent ANOSIM tests, we used ASV data from the coral samples only to compare the impact of host species, geography, and anthropogenic impacts on microbiome composition. Within coral samples, host species identity had the highest impact on microbiome composition (ANOSIM R: 0.541, $p < 0.001$). Therefore, for the final round of ANOSIM, coral host species were individually assessed for the impacts of geography and anthropogenic impact level on microbiome composition. Geography had the greatest impact on composition for both species (Fig. 2), with localized anthro-

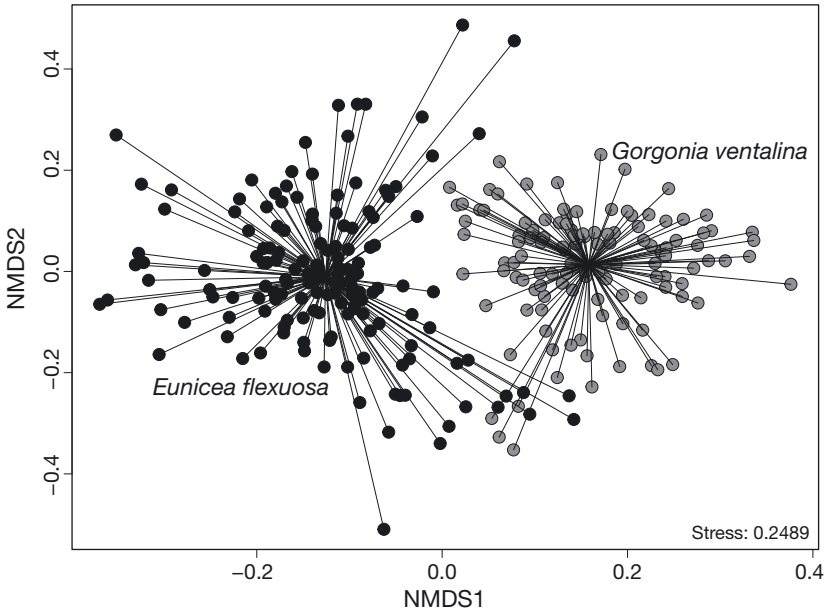


Fig. 4. Non-metric multidimensional scaling (NMDS) plot of microbial community beta diversity (Bray-Curtis) for samples from *Eunicea flexuosa* (black) and *Gorgonia ventalina* (gray). Black lines connect points from the same species to the centroid of each group

pogenic impact only becoming an important determinant of microbiome structure within species on each island.

3.3. Coral microbial community differentiation: host species identity

The identity of the coral host species played the primary role in structuring microbiome composition (Figs. 2 & 4). Each host species had distinct microbial communities with little overlap (ANOSIM R: 0.541, $p < 0.0001$; Figs. 2 & 4), but did not differ in their compositional variance (betadispersion: $F = 2.85$, $p = 0.089$). Microbial diversity was higher in *G. ventalina* samples than in *E. flexuosa* for all 3 metrics (observed richness: $\chi^2_1 = 5.211$, $p = 0.02$, Shannon's diversity: $\chi^2_1 = 24.072$, $p < 0.0001$, inverse Simpson's diversity: $\chi^2_1 = 25.254$, $p < 0.0001$; Fig. S4).

To determine which ASVs contributed the most to differences in host microbiomes, a PCA biplot of host species microbial composition was overlaid with ASV species scores based on abundance (Fig. 5a). Of the 14 782 ASVs in the data set, 7565 had an abundance of >0.1% across both corals, and 6, all *Endozoicomonas* ASVs, had higher abundances in one of the 2 hosts and contributed significantly to the overall compositional differences. These 6 *Endozoicomonas* ASVs were split between hosts: 3 were in high abundance only in *E. flexuosa* and 3 others, only in *G. ventalina* (Fig. 5a). *Mycoplasma*, occurring almost exclusively in *E. flexuosa* samples (Fig. 5b), also appears as a host differentiator: 3 distinct *Mycoplasma* ASVs were found in high abundance only in *E. flexuosa* (Fig. 5a). Two additional abundant ASVs, an *Escherichia-Shigella* and *Endozoicomonas*-10, were distributed relatively equally between host species (Fig. 5a). *Escherichia* and *Shigella* are distinct genera but their 16S rRNA sequences are nearly indistinguishable (Gwak & Rho 2020); therefore, we chose to retain the collective genus name, *Escherichia-Shigella*, from the Silva rRNA v.132 reference database used for taxonomic classification of ASVs in this study and by other researchers (Gwak & Rho 2020, Baltazar-Díaz et al. 2022, Castillo-Lopez et al. 2023).

3.4. Coral microbial community differentiation: geography

Once host species were separated, microbial communities were assessed for island-specific compositions and bacterial taxa that were differentially abundant between locations. The microbiomes of *G. ventalina* were distinct between the islands (ANOSIM R: 0.261, $p < 0.001$; Figs. 2, 3, & 6a), and Bonaire colonies were slightly more variable (average distance to the median: Bonaire: 0.600; Puerto Rico: 0.596) but this was not significant (betadispersion: $F = 0.045$, $p = 0.826$). Of the 70% 'core' microbiome, 2 of the most common ASVs were shared among all *G. ventalina* samples (Figs. 6b & S5c) and accounted for 17.5% of the reads per sample (Fig. 6b). One of these ASVs, *Endozoicomonas*-2, also contributed to differences between host species microbiomes (Fig. 5b). In addition to their shared 'core' ASVs, *G. ventalina* from both islands had independent 'core' ASVs (Fig. S5c). Bonaire had 3 'core' ASVs (2 unique *Endozoicomonas* and *Pirellulaceae*-1) that together accounted for 9.3% of the reads from its *G. ventalina* samples (Fig. 6b). Puerto Rico also had a 'core' ASV (*Gammaproteobacteria*-1) that accounted for 7.7% of reads (Figs. 6b & S5c). In the 90% core microbiome, only one ASV is shared among all *G. ventalina* samples, but both islands

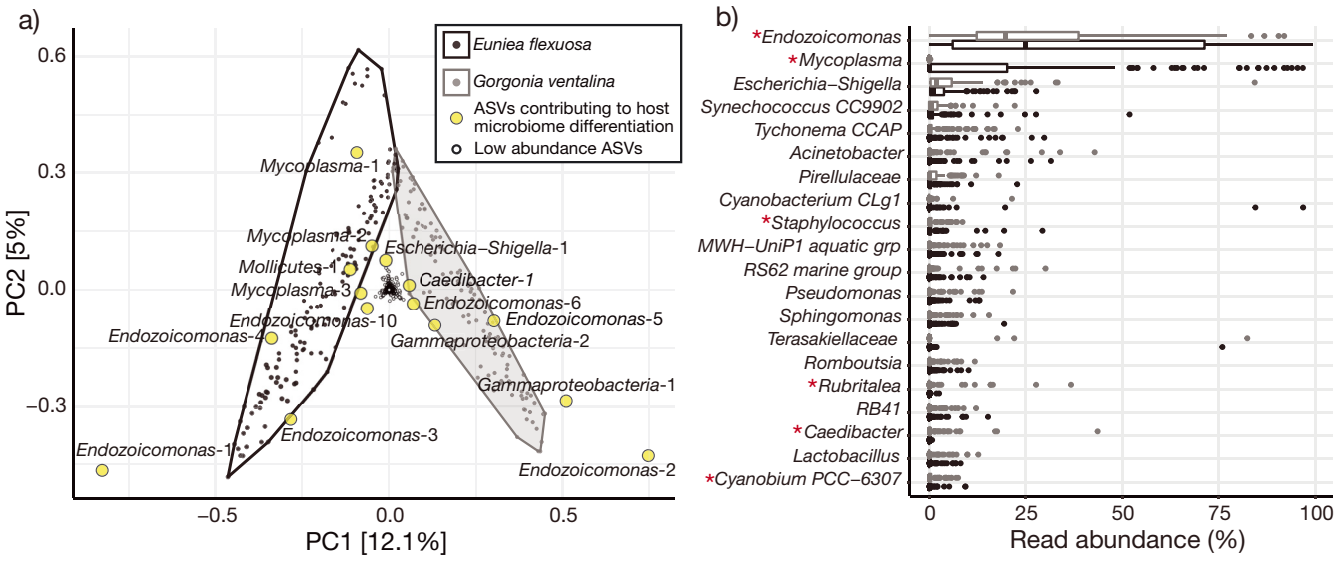


Fig. 5. Bacterial taxa that contribute most to differences between coral host species (gray: *Gorgonia ventalina*; black: *Eunicea flexuosa*). (a) Principal component analysis (PCA) biplot indicating amplicon sequence variants (ASVs) (n = 7565; abundance across all samples > 0.1 %) that contribute most to microbiome compositional differences between *G. ventalina* and *E. flexuosa* samples. Relative proximity of an ASV to microbiome samples from a specific coral host indicates its abundance within that host (i.e. closer: higher abundance). The 15 ASVs (of the 7565 ASVs on the plot) that contribute the most to differences between the host species' microbiomes are labeled by genus. Low abundance ASVs are located near (0, 0) and have little impact on the observed differences between host microbiomes. ASV abundance data have been transformed using the 'Hellinger' transformations (Legendre & Gallagher 2001). (b) Box plots of average relative read abundance for the 20 most abundant bacterial genera across both corals. Asterisks denote genera with significantly different abundances between host species. The lower and upper hinges represent the first and third quartiles. The lower and upper whiskers extend to either the largest or smallest value that is no more than 1.5× the interquartile range (i.e. distance between the first and their quartiles). Outliers are plotted as individual points. ASVs without a genus-level designation are identified to their lowest taxonomic rank

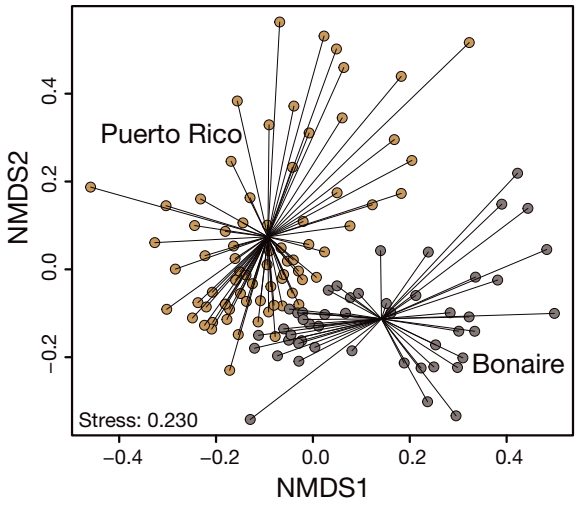
have 2 independent 'core' ASVs (Fig. S5d). Overall, *G. ventalina* from Bonaire were more diverse (average richness [observed ± SE]: 118.8 ± 15.2) than those from Puerto Rico (72.4 ± 6.4; Kruskal-Wallis $\chi^2_1 = 16.74$, $p < 0.001$; data not shown).

Despite the ~700 km distance between Puerto Rico and Bonaire, and in contrast to *G. ventalina*, *E. flexuosa* microbiomes showed little difference between the islands (ANOSIM R: 0.152, $p < 0.001$; Figs. 2, 3 & 6c), but compositional variance was higher across Bonaire samples (average distance to the median: Bonaire: 0.648; Puerto Rico: 0.596; betadispersion: $F = 9.575$, $p = 0.002$). The 3 ASVs in the *E. flexuosa* 70 % 'core' accounted for 27.6 % of all reads per sample (Figs. 6d & S6c). Two of these ASVs, *Endozoicomonas*-1 and *Endozoicomonas*-3, were large contributors to differences between host species microbiomes based on their relative abundances (Fig. 5b). Only the 3 *E. flexuosa* 'core' ASVs were both abundant and prevalent in the Bonaire samples (Fig. S6a). Puerto Rico samples had an additional 'core' ASV, *Endozoicomonas*-4 that was present in only 30 % of the *E. flexuosa* samples from Bonaire (Figs. 6c & S6b,c). The 90 % 'core' microbiome for *E. flexuosa* only contained a single ASV, shared among *E. flexuosa* from both islands, and no island-specific 'core' ASVs (Fig. S6d). Alpha diversity (richness [observed]) within *E. flexuosa* did not differ between the 2 islands (average richness [observed ± SE]: Bonaire: 68.9 ± 5.2; Puerto Rico: 63.8 ± 4.3; Kruskal-Wallis $\chi^2_1 = 1.804$, $p = 0.179$; data not shown).

3.5. Coral microbial community differentiation: localized anthropogenic impact

As with geography, differences between sites with different levels of local anthropogenic impact were greater for *G. ventalina* than for *E. flexuosa* (Fig. 7). The *G. ventalina* microbiome was highly differentiated by impact level in Bonaire (ANOSIM R: 0.512, $p < 0.0001$; Fig. 7a) and also Puerto Rico, although less so (ANOSIM R: 0.081, $p = 0.0002$; Fig. 7b). Similarly, Bonairian *G. ventalina* samples from impacted sites had higher compositional variance than those from less impacted sites (average distance to median: 0.611 and 0.538, respectively; betadispersion: $F = 6.332$, $p = 0.013$), while Puerto Rican *G. ventalina*

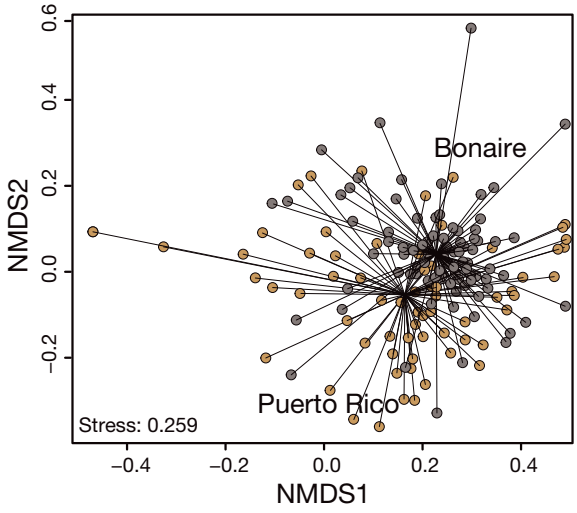
a) *Gorgonia ventalina*



b)

	Unique ASVs (70% Core)	Taxonomic Identity of Core ASVs	Read abundance per sample (%)
Gorgonia ventalina	2	*Endozoicomonas*-2	17.5
		Escherichia-Shigella-1	
Bonaire	3	*Endozoicomonas*-3	9.3
		Endozoicomonas-5	
		Pirellulaceae-1	
Puerto Rico	1	*Gammaproteobacteria*-1	7.7

c) *Eunicea flexuosa*



d)

	Unique ASVs (70% Core)	Taxonomic Identity of Core ASVs	Read abundance per sample (%)
Eunicea flexuosa	3	*Endozoicomonas*-1	27.6
		Endozoicomonas-3	
		Escherichia-Shigella-1	
Bonaire	0	----------	----
Puerto Rico	1	*Endozoicomonas*-4	6

Fig. 6. Geographic variation in microbial community composition and 70% core for (a,b) *Gorgonia ventalina* and (c,d) *Eunicea flexuosa*. (a,c) Non-metric multidimensional scaling (NMDS) plots of beta diversity (Bray-Curtis) show microbial composition for samples from Bonaire (dark gray) and Puerto Rico (tan). (b,d) The taxonomic identity and average read abundance (%) for ASVs that comprise the 70% core of both host species within each island

samples had similar variance regardless of impact level (average distance to the median: impacted sites: 0.567; less impacted: 0.618; betadispersion: F = 4.078, p = 0.06).

Bonairian *E. flexuosa* microbiomes from impacted and less impacted sites were distinct (ANOSIM R: 0.211, p < 0.0001; Fig. 7c), but far less so than *G. ventalina* samples from the same island (Fig. 7a). Although significant, there was little explanatory variation between site types in *E. flexuosa* from Puerto Rico (ANOSIM R: 0.001, p = 0.004; Fig. 7d). Furthermore, *E. flexuosa* samples from impacted sites on Bonaire had higher compositional variance relative to those from less impacted sites (average distance to the median: 0.640 and 0.606, respectively), but not significantly so (betadispersion: F = 2.391, p = 0.127). In Puerto Rico, there was no difference in compositional variance between site types (average distance to the median: impacted sites: 0.587; less impacted: 0.598; betadispersion: F = 0.101, p = 0.736).

To determine which ASVs were key in differentiating the 2 site types, we examined the 10 most abundant ASVs of both host species from each island independently. In *G. ventalina* from Bonaire, the top 10 ASVs accounted for 37% of all reads per sample, whereas in Puerto Rico, they accounted for 43%. Within the top 10 ASVs for *G. ventalina* from each

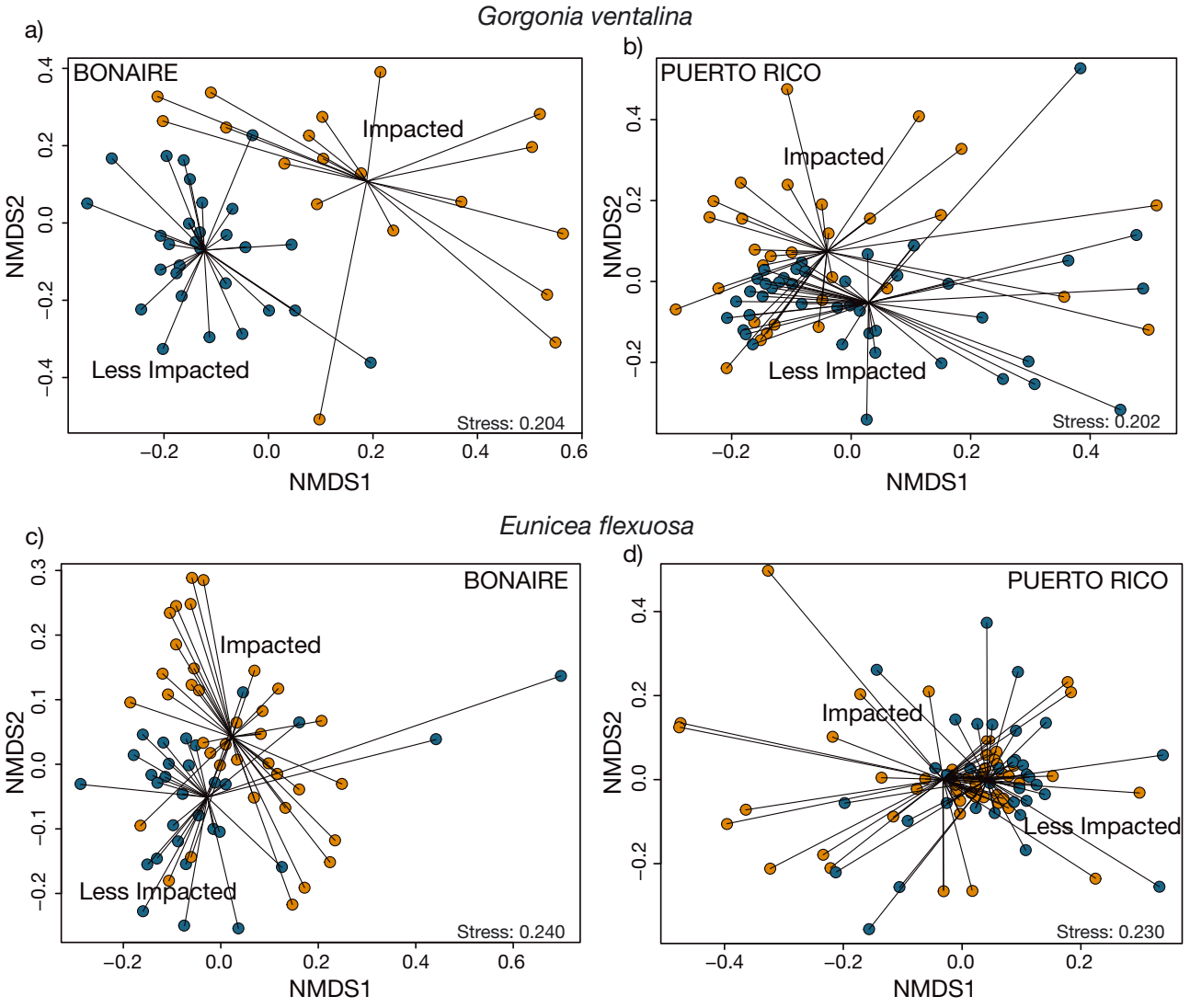


Fig. 7. Non-metric multidimensional scaling (NMDS) plot comparisons of coral microbiome compositions (Bray-Curtis) from anthropogenically impacted (orange) and less impacted (blue) sites for *Gorgonia ventalina* from (a) Bonaire and (b) Puerto Rico, and *Eunicea flexuosa* from (c) Bonaire and (d) Puerto Rico

island, 6 were differentially abundant between impacted and less impacted sites in Bonaire (Fig. 8a) and 3 in Puerto Rico (Fig. 8b). Of these ASVs, 2 were the same: an *Endozoicomonas*-2 and a *Tychonema*-1. *Endozoicomonas*-2 was more abundant and present in >90% of the sample colonies (Table S8, at less impacted sites in Bonaire; Kruskal-Wallis χ^2_1 = 21.9505, $p < 0.0001$; Fig. 8a) while it was more abundant at impacted sites in Puerto Rico (Kruskal-Wallis χ^2_1 = 3.850, p = 0.049; Fig. 8b), despite its almost ubiquitous presence in colonies at both site types (Table S8). The *Tychonema*-1 ASV was more abundant at less impacted sites on both islands (Bonaire: Kruskal-Wallis χ^2_1 = 4.399, p = 0.036; Puerto Rico: Kruskal-Wallis χ^2_1 = 5.343, p = 0.021; Fig. 8a,b).

Other ASVs that differed in abundance between *G. ventalina* from impacted and less impacted sites in Bonaire included *Gammaproteobacteria*-1, a *Caedibacter*-1, and *Oligoflexaceae*-1 (Kruskal-Wallis, all $p < 0.05$; Fig. 8a). Both the *Gammaproteobacteria*-1 and the *Oligoflexaceae*-1 were not found in any *G. ventalina* samples from impacted sites on Bonaire (Table S8). A single ASV of *Burkholderiaceae*-1, a bacterial family that includes many pathogenic species, was more abundant in *G. ventalina* from impacted sites (Kruskal-Wallis χ^2_1 = 7.579, p = 0.006; Fig. 8a). In Puerto Rico, the third differentially abundant ASV between *G. ventalina* samples was *Escherichia-Shigella*-1 — a genus with many known pathogens — which was more abundant at impacted

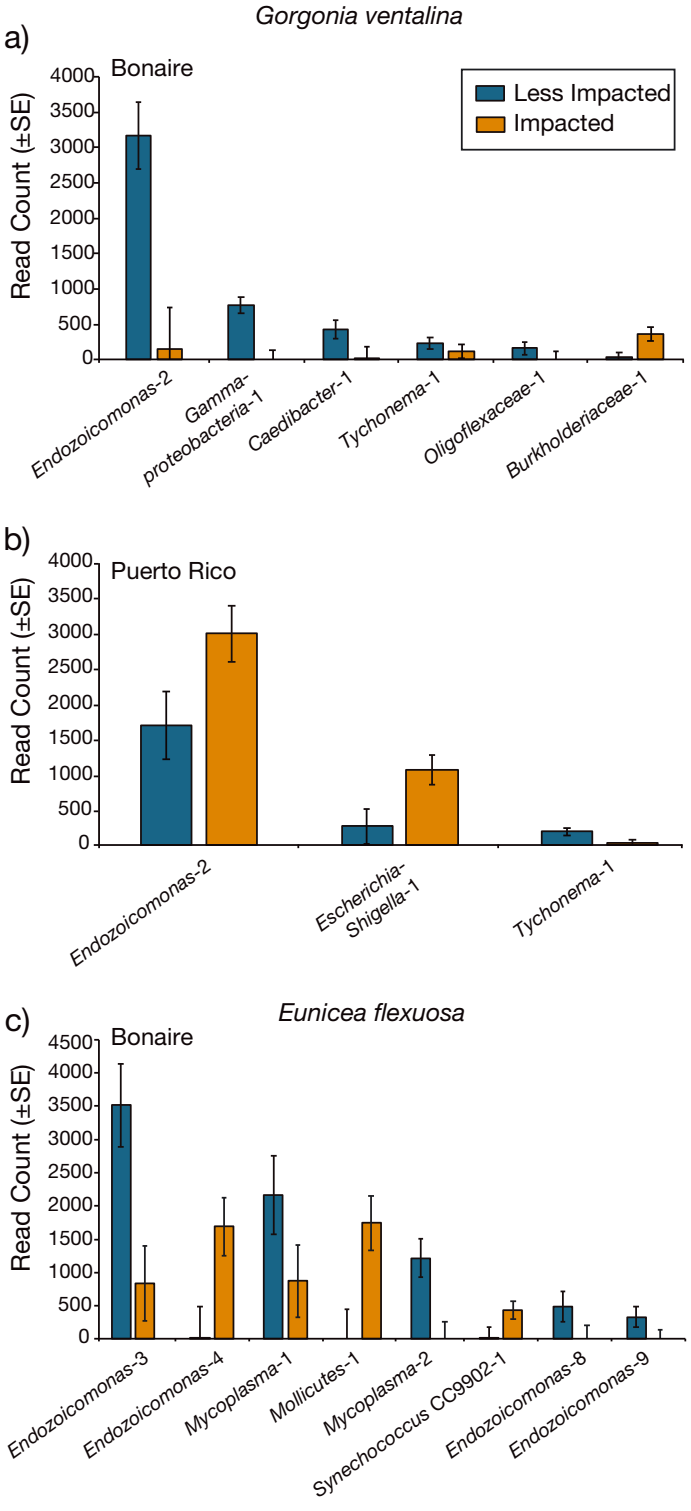


Fig. 8. Read abundance per sample (±SE) for differentially abundant ($p < 0.05$) amplicon sequence variants (within 10 most abundant) between impacted and less impacted sites for *Gorgonia ventalina* from (a) Bonaire and (b) Puerto Rico, and (c) *Eunicea flexuosa* from Bonaire

sites (Kruskal-Wallis $\chi^2_1 = 6.796$, $p = 0.009$; Fig. 8b) but present in a relatively equal percentage of colonies from both site types (impacted: 95.3%; less impacted: 96.7%; Table S8).

Eight of the 10 most abundant ASVs from Bonairian *E. flexuosa* (which accounted for 52% of all reads per sample) were differentially abundant between impacted and less impacted (Fig. 8c). Of these 8 ASVs, 4 were *Endozoicomonas*: 3 that were more abundant at less impacted sites ($p < 0.05$) and a fourth that was more abundant at impacted sites (Kruskal-Wallis $\chi^2_1 = 15.295$, $p < 0.001$; Fig. 8c). None of these 4 matched those that were differentially abundant between the site types for *G. ventalina* samples. Also included in the 8 differentially abundant ASVs were 2 *Mycoplasma*, both more abundant at less impacted sites (Kruskal-Wallis $\chi^2_1 = 9.025$, $p = 0.003$, and $\chi^2_1 = 22.443$, $p < 0.001$; Fig. 8c). *Mycoplasma*-2 was only present in colonies from less impacted sites (Table S8). Two additional ASVs, a *Mollicutes* and a *Synechococcus*, were more abundant in samples from impacted sites (Kruskal-Wallis $\chi^2_1 = 16.899$, $p < 0.001$, and $\chi^2_1 = 6.080$, $p = 0.014$, respectively; Fig. 8c). None of the 10 most abundant ASVs in Puerto Rican *E. flexuosa* were differentially abundant between site types.

3.6. Distribution and relationships of *Endozoicomonas*

Endozoicomonas was by far the most abundant bacterial genera across both host species. Of the 14 783 unique ASVs present in the rarefied data set, 727 were identified as *Endozoicomonas*, accounting for over one-quarter (27.7%) of all reads. In *E. flexuosa* samples, this percentage was even higher (35.2%). The 70% 'core' for both host species contained 2 *Endozoicomonas* ASVs, one of them shared (see Figs. 6b & 7b).

Of the 30 most abundant *Endozoicomonas* ASVs (Fig. 9), most were species-specific (14 for *E. flexuosa* and 6 for *G. ventalina*). Many of these species-specific ASVs are most closely related to other ASVs tied to the same host (e.g. *Endo.*-1 in Fig. 9), defining host-specific clades. The most numerically abundant ASVs overall tended to be prevalent among colonies as well: *Endozoicomonas*-1 was detected in over 80% of *E. flexuosa* colonies samples, while *Endozoicomonas*-2 was found in almost 90% of *G. ven-*

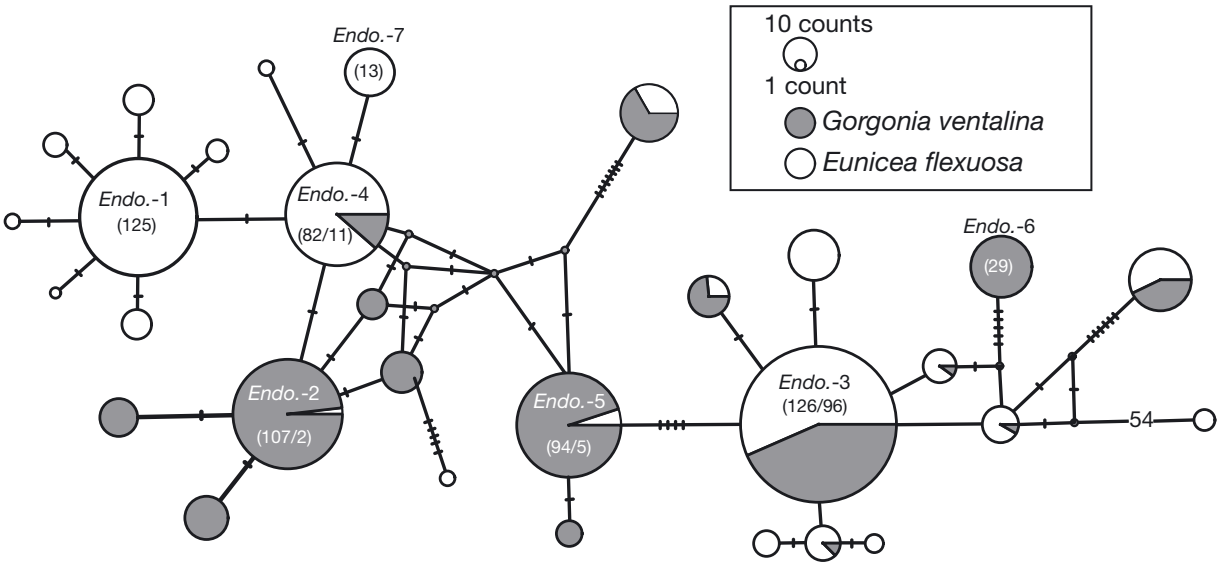


Fig. 9. Haplotype network of the 30 most abundant *Endozoicomonas* amplicon sequence variants (ASVs) in *Gorgonia ventalina* (n = 121) and *Eunicea flexuosa* (n = 152). Size of the open circles is proportional to the number of coral colonies sharing that ASV, with exact colony prevalence noted parenthetically for the 7 most common ASVs. Black dots on the connecting branches: inferred mutational steps between ASVs. Named ASVs correspond to those mentioned in previous figures

talina colonies. Some ASVs may be geographically constrained: of the 30 most common *Endozoicomonas* ASVs, 8 were private to Puerto Rico and 3 were limited to Bonaire (data not shown). Of these 11 endemic ASVs, 10 were taxon-specific. *Endozoicomonas*-3, the most common *Endozoicomonas* ASV, was shared relatively equally between the 2 species and was present in over 80% of all corals sampled (Fig. 9).

3.7. Comparison of coral genetics and microbiome differentiation

To determine if, like algal symbionts, prokaryotic microbiomes match coral host genetics, a literature search was conducted to identify healthy coral hosts with available genetic and microbiome comparisons between sites within their ranges and did not include any confounding stress treatments. The search uncovered 8 hosts that matched our criteria. In all of them, genetic differentiation was mirrored by their microbial composition (Table 1). Three corals (*Stylophora pistillata*, *Porites astreoides*, and *Orbicella faveolata*) had a low degree of host genetic subdivision and more geographically homogenous microbiomes. By contrast, 5 corals (*Pocillopora damicornis*, *Lophelia pertusa*, *Seriotopora hystrix*, *Primnoa resedaeformi*, and *Acropora cervicornis*) had high levels of both host genetic and microbiome differentiation between sites. Only a single study (on *P. resedaeformi*) compared both host genetics and microbiome composition from the same samples (Goldsmith et al. 2018; Table 1).

4. DISCUSSION

4.1. Host microbiomes are species-specific

Host species was the strongest differentiator of coral microbiome composition in our data. Many other coral species also exhibit distinct microbiome compositions, often mirroring host phylogenies, that are maintained through space and time (Rohwer et al. 2002, Carlos et al. 2013, La Rivière et al. 2015, Chu & Vollmer 2016). Differences between *Gorgonia ventalina* and *Eunicea flexuosa* microbiomes could largely be attributed to 2 bacterial genera: *Endozoicomonas* and *Mycoplasma*. *Mycoplasma* was present almost exclusively in *E. flexuosa* samples. By contrast, both species had a high abundance of *Endozoicomonas*, but this genus comprises many species-specific strains. We uncovered over 30 host-specific *Endozoicomonas* ASVs (Fig. 9); none were shared in the respective cores of either species. The closest relatives to common species-specific ASVs were often additional species-specific ASVs (see Fig. 9), consistent with long-term fidelity to one host. *Endozoicomonas* are thought to benefit their hosts by

Table 1. Examples, including citation information, of coral host species with more or less genetic subdivision and their associated micro biome differentiation (more or less). Relevant studies are cited along with sampling location information

	Host genetics			Microbiome composition		
Coral species	Host genetic subdivision across space	Locations compared	Source	Micro-biome diff-erentiation	Locations compared	Source
Stylophora pistillata	Less	8 sites across the Western Pacific (Japan, Malaysia, Australia)	Takabayashi et al. (2003)	Less	2 sites in Taiwan	Hong et al. (2009)
		3 sites across the Great Barrier Reef and 1 site in the Coral Sea	Klueter & Andreakis (2013)		3 sites in Taiwan (most dominant bacteria showed no differentiation by site)	Yang et al. (2017)
Porites astreoides	Less	25 sites across the Caribbean	Riquet et al. (2022)	Less	3 sites in Panama and 2 sites in Bermuda	Hong et al. (2009)
Orbicella faveolata	Less	4 sites across the Caribbean	Severance & Karl (2006)	Less	4 sites across the Caribbean	Morrow et al. (2012)
		>15 sites in the Florida Reef Tract	Baums et al. (2010)			
Pocillopora damicornis	More	3 sites in the Great Barrier Reef	Ayre et al. (1997)	More	4 sites on the Great Barrier Reef	Bourne & Munn (2005), Hong et al. (2009)
		9 sites across the eastern tropical Pacific	Combosch & Vollmer (2011)		5 sites in the Red Sea	Osman et al. (2020)
		>20 sites across the entire Pacific range of *P. damicornis*	Forsman et al. (2013)			
Lophelia pertusa	More	4 sites in the Gulf of Mexico and 7 in the N. Atlantic Ocean	Morrison et al. (2011)	More	1 site in the Atlantic and 4 sites in the Gulf of Mexico	Kellogg et al. (2017)
		5 sites in NE Skagerrak	Dahl et al. (2012)		2 sites in the Mediterra-nean Sea	Chapron et al. (2020)
		10 sites along the northern European coast	Le Goff-Vitry et al. (2004)			
Seriatopora hystrix	More	12 sites in the Great Barrier Reef	Ayre & Dufty (1994)	More	3 sites on the Great Barrier Reef	Pantos et al. (2015)
		22 sites in the Great Barrier Reef	Kininmonth et al. (2010)		5 sites in the Red Sea	Osman et al. (2020)
Primnoa resedaeformi	More	2 sites in the North Atlantic	Goldsmith et al. (2018)	More	2 sites in the North Atlantic	Goldsmith et al. (2018)
Acropora cervicornis	More	>200 sites (wild and nursery stock) from Florida, Dominican Republic, and Cayman Islands	Drury et al. (2017)	More	Nursery stock originating from sites across the Florida Keys	Williams et al. (2022)

promoting glucose production and preventing mitochondrial damage caused by oxidative stress (Ding et al. 2016). Their prevalence and species-specificity in our data adds support to the growing body of evidence suggesting that members of this genus co-evolve with their coral hosts (Bayer et al. 2013, La Rivière et al. 2013, Neave et al. 2017, Pogoreutz et al. 2018, Pollock et al. 2018).

Additional support for the specificity of host–symbiont relationships in both *E. flexuosa* and *G. ventalina* comes from the lack of shared 'core' ASVs. Just one 70% 'core' ASV, *Escherichia-Shigella*-1, was found in both host species (~2.6% of reads in the rarefied data set). Other studies have indicated the presence of multiple 'core' bacterial taxa shared across coral species (Ainsworth et al. 2015, Kellogg et al. 2017, van de Water et al. 2017), although all of these results were based on OTUs instead of ASVs. Kellogg (2019) examined the 'core' bacteria across 7 coral species and found more shared taxa at the OTU level than at the ASV level. OTU analyses can inflate the number of 'core' taxa by condensing 16S rRNA (V4 region) amplicon bacterial sequences that differ by as much as 7 bp of ~250 bp (for 97% OTUs) into a

single taxonomic unit, thereby obscuring ecological patterns (Callahan et al. 2017). Thus, the fine-scale resolution of ASVs may uncover cryptic symbiont diversity (Callahan et al. 2017, García-García et al. 2019).

4.2. Genetically homogeneous hosts have more homogeneous microbiomes

Despite their similar algal symbiont acquisition strategies (Andras et al. 2013, Prada et al. 2014), we anticipated that the bacterial microbiomes of sympatric *E. flexuosa* and *G. ventalina* hosts would exhibit different levels of differentiation, as seen in their algal symbiont communities. The microbiome of *E. flexuosa* was relatively homogenous across ~700 km and only showed differentiation between stress levels in Bonaire. Further, *E. flexuosa* had fewer island-specific 'core' ASVs (none in Bonaire and one in Puerto Rico) than *G. ventalina* (3 in Bonaire and one in Puerto Rico; see Fig. 6). The 3 'core' ASVs across all *E. flexuosa* colonies accounted for more than one-quarter of the read abundance (27.5% per sample) in the species, suggesting that not only are these 'core' taxa ubiquitous across the host's range but they are relatively abundant in the host microbiome. By contrast, the *G. ventalina* microbiome showed high compositional differentiation, with 82.5% of their prokaryotic symbionts showing variable presence and absence across all colonies from both islands. The 2 *G. ventalina* species-wide 'core' ASVs, including the one shared with *E. flexuosa*, only accounted for 17.5% of the read abundance for the species. Furthermore, *G. ventalina* microbiomes displayed differentiation at the local level in response to anthropogenic impacts in both Puerto Rico and, to a higher degree, Bonaire.

Variation in microbiome composition within a coral species has been explained by factors including habitat differences, season, location, host physiology, and stress levels (Hong et al. 2009, Morrow et al. 2012, Haydon et al. 2022). We excluded several of these possibilities by sampling 2 broadcast-spawning corals from the same sites and in the same season. In addition, both hosts acquire their algal symbionts horizontally (Andras et al. 2013, Prada et al. 2014). While their mode of prokaryotic symbiont acquisition is unknown, limited evidence shows that these microbes are also acquired from the environment in broadcast-spawning corals (Apprill et al. 2009, Sharp et al. 2010, Lema et al. 2014a, Damjanovic et al. 2020). Having ruled out the above-mentioned potential sources of microbiome variation among coral hosts, the different levels of host population genetic differentiation remain as a strong candidate factor.

E. flexuosa is a depth specialist, with 2 depth-delineated lineages that, when reciprocally transplanted, incur high mortality (Prada & Hellberg 2013). Each of these lineages has a tight symbiosis with their own lineage-specific symbiotic micro-algae that is maintained across their range (Prada & Hellberg 2014). Glasl et al. (2017) found that corals with strict depth distributions had more stable microbiomes than those of depth-generalists. We sampled only the shallow ecotype of *E. flexuosa*, a depth specialist that is panmictic across the Caribbean (Prada & Hellberg 2013). By contrast, depth-restricted lineages are not known in *G. ventalina*, but populations can be genetically differentiated over a scale of <2 km (Andras et al. 2013).

Levels of micro-algal endosymbiont differentiation commonly echo those of their coral host (LaJeunesse et al. 2003, Stat et al. 2008, Finney et al. 2010, Prada & Hellberg 2014) but little research has directly examined whether this also holds true for prokaryotic symbionts. A literature search (Table 1) found that coral hosts lacking genetic subdivision (Takabayashi et al. 2003, Klueter & Andreakis 2013) housed more homogenous microbiomes (Hong et al. 2009, Morrow et al. 2012, Yang et al. 2017) than genetically subdivided hosts and did not display large, localized changes in microbiome composition. By contrast, hosts with higher levels of genetic subdivision (Ayre & Dufty 1994, Ayre et al. 1997, Kininmonth et al. 2010, Combosch & Vollmer 2011, Forsman et al. 2013) have more variable microbiomes that show location-specific signatures (Bourne & Munn 2005, Pantos et al. 2015, Osman et al. 2020). Clearly, this association needs to be tested on more host species; however, microbiome differentiation, or the lack thereof, in *E. flexuosa* and *G. ventalina* aligns cleanly with host population genetic differentiation.

Mechanistically, how could higher genetic subdivision of the coral host lead to greater differentiation of its microbes? One way could be if microbial communities were transmitted vertically via larvae, as has been shown in some brooding corals (Sharp et al. 2012). In this case, microbial communities would, much like vertically transmitted symbiotic micro-algae, reflect the subdivision of their host (LaJeunesse et al. 2003, 2004a, Stat et al. 2008, Finney et al. 2010). Even maternal inheritance of a relatively small proportion of the microbial community could have a strong impact on cross-generation community similarity, given that some microbiome members shape whether other

symbionts can join from the environment (Teplitski & Ritchie 2009, Welsh et al. 2017, Zhou et al. 2020, Monti et al. 2022). Another, non-exclusive, way would be via host genome-encoded microbial sorting mechanisms, such as NON-type receptors and C-type lectins, 2 families that have expanded in symbiotic anthozoans (Emery et al. 2021). Population differentiation of hosts at such sorting loci could cause hosts to choose differentiated microbiomes, even from a homogeneous environmental pool of microbes.

Differences in host population genetic subdivision could have implications for the divergence of coral-produced chemical cues used for symbiont attraction. Coral symbionts exhibit chemotaxis to coral-exuded chemicals, which helps maintain tight coral–microbe symbioses (Tout et al. 2015, Bourne et al. 2016). If chemical exudates vary across the range of subdivided corals, this could drive the acquisition of location-specific symbionts. Tout et al. (2015) found that the level of chemotaxis exhibited by coral symbionts depended on their associated hosts. One host species, for example, had symbionts that exhibited strong chemotaxis towards its exudates, while the symbionts of a different host species did not show as strong of an attraction to their host's exudates. Host subdivision may contribute to this variation in symbiont chemotaxis. The microbes in waters directly adjacent to colonies of less subdivided hosts display a lower potential for chemotaxis (Silveira et al. 2017, Walsh et al. 2017, Weber et al. 2019) than those of highly subdivided corals (Tout et al. 2015, Weber et al. 2019). Thus, it seems that low levels of host differentiation may tie corals strictly to a common set of symbionts shared by most, or all, members of the same host species, regardless of environmental factors, while highly differentiated hosts are more plastic in their symbiont acquisition, perhaps attributed to the diversity or specificity of their chemical exudates.

4.3. Host identity impacts microbiome response to anthropogenic stress

Consistent with its own greater geographical differentiation, the *G. ventalina* microbiome composition was highly flexible in response to varying levels of anthropogenic impact within islands. By comparison, the *E. flexuosa* microbiome only showed differentiation, and less strongly than *G. ventalina*, in composition between impacted and less impacted sites in Bonaire. For both coral hosts, microbiome distinctions between locally impacted and less impacted sites were stronger in Bonaire than in Puerto Rico.

Puerto Rican reefs are in poor condition due to anthropogenic activity (Morelock et al. 2001, Cusey et al. 2002), certainly more so than Bonairian reefs (Steneck et al. 2013, Slijkerman et al. 2014, Dutch Caribbean Nature Alliance 2017), as reflected by our water quality data. This finding may exemplify the 'Anna Karenina principle' ('All happy families are alike; each unhappy family is unhappy in its own way' – Tolstoy) for microbiomes (Zaneveld et al. 2017), in which dysbiotic individuals have a more variable microbiome than healthy individuals, or on a larger scale, corals collectively located on lower quality reefs would have more microbiome variability. Here, corals living on less impacted reefs in Bonaire (where water quality is higher) are healthier, so when exposed to stressors, such as at the impacted sites, their microbiomes increase in variance (i.e. become more dysbiotic). This has resulted in more distinct compositions between impacted and less impacted sites in Bonaire In Puerto Rico, where water and reef quality are worse, corals, even at 'better' sites, already have dysbiotic microbiomes, so variances for both site types are similar.

Coral communities from impacted sites were enriched for bacterial taxonomic groups known to be pathogenic, including *Burkholderiaceae*, *Mollicutes*, and *Escherichia-Shigella* (Fig. 8). Most of these potential pathogenic taxa were also present, albeit in low abundance, in less impacted corals. Stressed corals commonly experience increases in potential pathogenic bacteria (McDevitt-Irwin et al. 2019). Interestingly, *Escherichia-Shigella* was found in both coral hosts from all locations but was only differentially abundant between impacted and less impacted *G. ventalina* from Puerto Rico. *Escherichia-Shigella* has been identified as a common member of the microbiomes of 6 Caribbean gorgonians but did not always increase when the corals were experimentally stressed (McCauley et al. 2020). Unlike other studies examining coral microbiomes under stress, we did not see high abundances of *Vibrio* spp. at any location (McDevitt-Irwin et al. 2019). However, *Vibrio*, a genus containing many potential pathogens, is commonly associated with diseased corals and with high temperatures (Bally & Garrabou 2007, Vega Thurber et al. 2009, Sweet et al. 2012), stressors not known to be present in our study.

Of the 10 most abundant ASVs in *E. flexuosa* microbiomes, 8 were differentially abundant between impacted and less impacted sites in Bonaire, including 2 *Mycoplasma* ASVs. Many *Mycoplasma* species are considered pathogenic, although some are commonly associated with healthy corals, often at

high abundance (Neulinger et al. 2009, Gray et al. 2011, Holm & Heidelberg 2016, Woo et al. 2017). Coral-associated *Mycoplasma* occur near the distal ends of spirocysts, allowing them to uptake leaked nutrients from coral-captured prey while being protected by the coral surface mucus layer, but they appear to be neither harmful nor helpful (Neulinger et al. 2009). Both *Mycoplasma* ASVs were more abundant in *E. flexuosa* from less impacted sites, which supports their role as beneficial, or at least commensal, symbionts.

The abundance of *Endozoicomonas* variants is often negatively correlated with anthropogenic impact (Meyer et al. 2014, Morrow et al. 2015, McDevitt-Irwin et al. 2017), so the increase in abundance of some *Endozoicomonas* ASVs at presumably stressed sites was unexpected. While *Endozoicomonas* is generally considered a beneficial symbiont, some of its traits imply pathogenic capabilities (Ding et al. 2016). Thus, some *Endozoicomonas* phylotypes may not be beneficial or, when a coral is stressed, a normally beneficial symbiont may become an opportunistic pathogen. An increase in pathogenic bacteria under stress could also cause a decrease in beneficial bacteria like *Endozoicomonas* (Meyer et al. 2014). More likely, *Endozoicomonas* ASVs are differentially 'useful' to a host under stress and the abundances of these symbionts alter accordingly. Gene content of *Endozoicomonas* strains associated with different coral hosts suggested different functional capacities (Neave et al. 2017), consistent with the idea that the various *Endozoicomonas* ASVs we found could be providing unique abilities to their hosts. Diverse microbial communities often have taxa and/or ecotypes that perform a similar function but have a unique response to perturbations or adaptations to different niches, providing stability to the microbial community (McCann 2000, Konopka et al. 2015). Both *E. flexuosa* and *G. ventalina* microbiomes house >24 bacterial isolates, including *Endozoicomonas*, that inhibited the growth of known coral pathogens, including several *Vibrio* species, even under thermal stress (Monti et al. 2022). It is possible that several of the *Endozoicomonas* ASVs found in the corals from more impacted reefs may be useful in deterring pathogenic infections.

5. CONCLUSIONS

Our study sought to explore the spectrum of microbial community variation in 2 co-habiting coral hosts with different levels of geographic genetic differentiation. Our comparison of inter-species microbiome differences confirmed what others have seen: host species have largely unique microbiomes. Only when hosts were separated did evidence of geographic and local stress-related differences become visible. Intra-species microbiome variation, however, was highly dependent on host identity and aligns with the level of geographic genetic differentiation displayed by our hosts, which appears to be common in other coral species as well. Continued research is needed to determine if this holds true for all corals.

Data availability. The data sets presented in this study can be found at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA870491. All 16S rRNA gene fastq files are located under BioProject No. PRJNA870491, except 4 each of sediment and water samples (SD1, SD10, SD19, SD28 and S1, S10, S19, S28, respectively) from Puerto Rico that are accessioned under BioProject No. PRJNA643267 found at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA643267.

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