Bacterial communities of *Cassiopea* in the Florida Keys share major bacterial taxa with coral microbiomes

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Abstract:

Interactions with microbial communities fundamentally shape metazoans' physiology, development, and health across marine ecosystems. This is especially true in zooxanthellate (symbiotic algae-containing) cnidarians. In photosymbiotic anthozoans (eg. shallow water anemones and corals), the key members of the associated microbiota are increasingly well studied, but there is limited data on photosymbiotic scyphozoans (true jellyfish). Using 16S rRNA barcoding, we sampled the internal and external mucus of the zooxanthellate Upside-Down Jellyfish, *Cassiopea xamachana* and *C. andromeda*, throughout eight sites covering the full length of the Florida Keys. We find these medusae to have low-diversity internal microbial communities distinct from the communities of their external surfaces and their environment. These internal communities are dominated by only three taxa: Endozoicomonadaceae, Mycoplasmataceae, and Vibrionaceae. External mucosal samples conform largely to the communities of surrounding sediment with the addition of Endozoicomonadaceae. The microbial taxa we identify associated with wild Florida Keys *Cassiopea* bear a strong resemblance to those

found within photosymbiotic anthozoans, increasing the known links in ecological position between these groups.

Key words: Cnidaria, microbiome, symbiosis, *Cassiopea*, Scyphozoa

1 INTRODUCTION

Cnidarian-photosymbiont interactions are central to ecosystem function in coral reefs and beyond. Algal photosymbionts, or zooxanthellae, are present in the well-known stony corals, octocorals, and anemones, but also play important roles in a variety of lesser-known jellyfishes, chief among them, jellyfish of the genus *Cassiopea* (Brown et al. 2017, Van De Water et al. 2018, Djeghri et al. 2019, Meyer et al. 2019). For symbiotic anthozoans, microbial communities play a key role in maintaining health within this photosymbiont partnership, with community members directly conferring disease resistance and heat tolerance on hosts (Baldassarre et al. 2022, Connelly et al. 2022, Ushijima et al. 2023). These communities are often specialized, with distinct corals demonstrating co-evolution with members of their bacterial communities (Pollock et al. 2018), but retain several prevalent clades, such as Endozoicomonadaceae, across many hosts (Huggett & Apprill 2019). While anthozoan communities are under active investigation, few analyses have documented the stability and nature of microbiome communities associated with zooxanthellate jellyfishes, a group with divergent life history traits from Anthozoa but with a similar obligate symbiosis with dinoflagellates of the clade Symbiodiniaceae.

The Upside-Down Jellyfish, *Cassiopea* spp., is a model organism for cnidarian-photosymbiont interactions (Ohdera et al. 2018, Puntin et al. 2022). These medusae are residents of tropical and subtropical waters globally and can survive in water that is frequently shallow (as little as 8 cm depth) and hot (up to 33˚C for *C. andromeda*) (Mammone et al. 2021, Béziat & Kunzmann 2022). *Cassiopea* obligatorily hosts algal symbionts, losing mass even when well fed while in aposymbiotic conditions (McGill & Pomory 2008). As in corals and other scyphozoans, mucus plays a central role for *Cassiopea*, presenting a sticky interface between the medusae and their benthic environment (Bakshani et al. 2018, Ames et al. 2020). In contrast to other symbiotic

cnidarians, such as stony corals, *Cassiopea* can tolerate intense temperature fluctuations. Understanding the *Cassiopea* natural microbial community could provide new potential insights into the function and diversity of cnidarian holobionts.

Past jellyfish (class Scyphozoa) microbial studies have focused on the microbiome of lab-reared specimens (Lee et al. 2018, Röthig et al. 2021) or limited sample sizes (Cleary et al. 2016, Hao et al. 2019, Peng et al. 2021). While these types of studies may be able to track state changes across life stages (Lee et al. 2018) and between condition types (symbiotic/aposymbiotic) (Röthig et al. 2021), there is little guarantee that the microbial communities of jellyfish kept under prolonged laboratory conditions translate to *in situ* conditions. Recent *in situ* studies have provided insights into microbial taxa associated with some scyphozoans ("true jellyfish") but have not included species of the genus *Cassiopea*. In the moon jellyfish, *Aurelia* spp., individuals from the Chinese coast were *Vibrionaceae*-dominated, while samples from the northwestern Atlantic were *Mycoplasma*-dominated (Daley et al., 2016; Peng et al., 2021). Between the Indonesian marine lakes Kakaban, Haji Buang, and Tanah Bamban, *Mastigias* microbial community composition varied significantly by lake, but consistently included *Oceanospirillales* (primarily *Endozoicomonas*-like) (Cleary et al. 2016). In samples across taxa, *Mycoplasma* is a common component of the identified gut and tissue microbiome of jellyfishes, though its function remains unknown (Weiland-Bräuer et al. 2015, Daley et al. 2016, Viver et al. 2017, Basso et al. 2019, Stabili et al. 2020, Peng et al. 2021). Pathogenesis is often a focus of scyphozoan microbiome discovery. *Tenacibaculum* has been identified in several species, e.g. *Cotyllorhiza tuberculata*, *Aurelia aurita*, and *Pelagia noctiluca*, and, as a fish pathogen, which can have important fisheries ramifications (Delannoy et al. 2011, Daley et al. 2016, Viver et al. 2017, Peng et al. 2021).

As most studied jellyfish populations occur in coastal waters only sporadically, the relative consistency of *Cassiopea* assemblages provides a system in which location effects can be analyzed at single time points. *Cassiopea* spp. presents an opportunity to act as a comparison group for the microbial communities associated with both other scyphozoans and symbiotic anthozoans. The ubiquity of *Cassiopea* assemblages within the Florida Keys allows for the examination of differences in the microbial community of one population (Florida Keys *Cassiopea*) at one time point across a broad geographic area.

The aim of this work is to identify the core components of the internal and external mucosal microbiomes of *Cassiopea* and compare the microbial communities of *Cassiopea* across sites of the Florida Keys.

2 MATERIALS & METHODS

2.1 Sampling

A total of 55 medusae were collected by wading in eight sites along the length of the Florida Keys in late August 2021 over eight days (see Table 1 for a list of sites). Species identification for each individual is reported in Muffett & Miglietta 2023 and summarized in Supplementary Table S1. At each site, coordinates, temperature, time of day, depth, salinity, and pH were recorded (Table 1). Salinity was measured with a refractometer, and temperature and pH were measured with an Apera A1209. A minimum of two and a maximum of 10 medusae were sampled per site. At each site and in between each medusa, all equipment was cleaned with absolute ethanol and 10% bleach solution. A 1 L water sample was collected from above (>10 cm above the benthos) each medusa aggregation and filtered (0.2 um pore size, Thermo Scientific Cat No.09-740-30G) with a manual sampler as described in the USGS manual water

sampling protocol (Laramie et al. 2015), replicate filters were placed in 15 mL of absolute ethanol or dimethyl sulfoxide ethylenediamine tetraacetic acid saturated salt storage solution (1 L pH 7.5: 93.06 g EDTA, 60 mL 20 % NaOH solution, 20 mL 25 % HCl, 40 mL DMSO, 800 mL water, NaCl to oversaturation; see ref. Pavlovska 2021) (commonly referred to as DESS), one filter in each buffer. At each *Cassiopea* medusa aggregation, duplicate scrapings of ~10 g of sediment collections (benthic sample) were placed in either 15 mL of absolute ethanol or DESS storage solution.

Each *Cassiopea* medusa was collected from shallow waters <1.5 m within a single assemblage at each site. Medusae were gently grasped by the oral arms, lifted from the water, swabbed along the apex of the bell, then placed bell-down onto a pre-prepared dissection plate (Figure 1). External bell swabs were done in replicate and placed in 3 mL DESS or absolute ethanol. Medusa oral arm base was bisected between oral arm groups, and replicate swabs were inserted and circled the digestive cavity. One internal swab was placed in DESS, the other in ethanol, as with external swabs. A section of bell tissue was then collected and placed in ethanol for mitochondrial species confirmation (see Muffett & Miglietta 2023). The size of each medusa was taken, and individual medusae were photographed. As sampling was done in a nonsterile environment, blanks for buffer with swab and buffer with filter were also taken.

2.2 Extraction and Sequencing

Samples in DESS and EtOH were maintained at room temperature for three to ten days and then frozen. DESS can be stored at room temperature for at least thirty days without significantly compromising community integrity (Pavlovska et al. 2021). DNA from all samples (see Supplementary Table S1) was then extracted using Zymobiomics DNA Kits (PN# D4300T). All samples were pre-tested through the ampliciation of the 16S rRNA gene (variable region V4)

amplifications. Only samples that produced visible bands for the V4 region were sent for 16S V3-V4 preparation and sequencing. While DESS preserved samples yielded good amplification results, EtOH-preserved samples largely failed to produce adequate amplification. As such, only a subset of EtOH-preserved samples were sent for sequencing. Sequencing was performed by the University of Texas Medical Branch Sequencing Lab. Library was prepared with Zymo Quick 16S V3-V4 kit (PN #D6400) in 20 uL reactions (10 uL Quick-16S qPCR premix, 4 uL Quick-16S V3-V4 primers (341f: (CCTACGGGDGGCWGCAG, CCTAYGGGGYGCWGCAG) and 806r: (GACTACNVGGGTMTCTAATCC)), 4 uL water and 2 uL sample). qPCR was run using Roche LightCycler 480 Instrument II (10min 95C, [30sec 95C, 30sec 55C, 3min 72C]x20). PCRs were enzymatically cleaned according to Zymo Quick-16S kit protocols, and barcodes were added in a secondary reaction at 20 uL (10 min 95˚C, [30 sec 95˚C, 30 sec 55˚C, 3 min 72˚C]x5). Plates included two replicate microbial community standards (Zymobiomics Cat. No. D6300) and two PCR negative controls. Samples were loaded at 10 pM, with 15% 10 pM PhiX sequencing control, and run on Illumina MiSeq with a 600 cycle v3 kit.

Three samples were sent to CD Genomics for full length 16S on PacBio Sequel using universal 16S primers (F: AGRGTTTGATYNTGGCTCAG; R:TASGGHTACCTTGTTASGACTT). Samples sent for full length 16S rRNA sequencing included gastrovascular cavity swab extractions from medusae Marathon Key GVC 30 and Key West GVC 96 and GVC 120. As the usable reads returned from this were low (597-12520 per sample), these full-length identities are not discussed in the main text. For further details, please refer to the appendix (A1).

2.3 Data analysis

Data generated was run through the mothur v1.47 (Schloss et al. 2009) pipeline with only limited deviations from the published standard mothur MiSeq protocols for ASV generation (full pipeline can be found in supplementary material S02) (Kozich et al. 2013). Sequences attributable to Chloroplast, Mitochondria, Eukaryota, and unattributable sequences were removed ("remove.seq"). All genera found in the negative controls (4 PCR controls, 5 sampling and extraction controls) at a rate greater than 1% were removed using "remove.lineage". Number of sequences removed at each of these filtering steps can be found in Supplementary Table S03. Based on the sequenced microbial community positive control, ASVs were oversplit, and the amplification was biased towards Gram-positive bacteria. Given this oversplitting, analysis for 16S rRNA gene V3-V4 data was lumped by classification at the family level.

The complete dataset (NCBI Bioproject Accession number PRJNA1020388) included 13.27 million sequences after merging ("make.contigs"), 7.17 million after screening for homopolymers and length ("screen.seqs": maxlength = 480, maxhomop = 8). Unique sequences were aligned to a 16S rRNA small subunit V3-V4 reference database generated from the Silva v. 132 full database ("pcr.seqs") and filtered again to produce 3.66 million unique sequences that were clustered into ASVs ("pre.cluster": diffs = 4). After chimera removal ("chimera.vsearch"), 2.27 million unique sequences were classified according to the Silva reference library v.132 ("classify.seqs"). ASVs were phylotyped at family level. After quality filtering, 947 families were present. Explorable phylotype datasets can be found in the supplementary materials (S4-S6).

2.4 Statistical analysis

Refraction curves, principal component analysis, non-metric multidimensional scaling (NMDS), diversity metrics, Dirichlet multinomial mixture and analysis of molecular variance

were computed in mothur (Schloss et al. 2009). NMDS plot was visualized using R 4.0.1 (R Core Team, 2021).

3 RESULTS

3.1 Data description

We analyzed the microbiome associated with 34 medusae from eight sites within the Florida Keys. For each medusa, we analyzed the microbial composition of their gastrovascular cavity and external mucus, as well as their benthic or water environment. All medusae were identified as *Cassiopea xamachana*, with the exception of four medusae identified as *C. andromeda* found in Key West, Cudjoe Key, and Key Largo (see Supplementary Table S1).

3.1.1 Distribution of sequences in full dataset

Almost all of the 5,431,716 final sequences were Bacteria as opposed to Archaea (98.93% to 1.07%). At the phylum level, 66.75% of sequences were Proteobacteria, 9.50% were Bacteroidetes, 8.58% were Tenericutes, and 2.45% were Cyanobacteria (Fig 2). No other phylum had greater than 2% relative abundance.

At the class level, Gammaproteobacteria comprised 48.90% of all sequences. Alphaproteobacteria, the next most abundant, comprised 12.07%. Bacteriodia (8.83%), Mollicutes (8.58%), Deltaproteobacteria (5.38%), Oxyphotobacteria (2.37%), and Verrucomicrobiae (1.27%) were the other classes with an relative abundance above 1%. The majority of Archaea within the dataset were from the classes Halobacteria (40.17%) and Woesearchaeia (33.94%).

3.1.2 Distribution of sequences within gastrovascular cavity (GVC) samples

Within gastrovascular cavity samples, 70.40% of the 2,485,856 total sequences were Proteobacteria, 17.89% Tenericutes, 4.02% Bacteriodetes, 1.75% Cyanobacteria, and 1.19% Chlamydiae. 73.20% of all sequences from gastrovascular cavities belonged to just three orders, Oceanospirillales (42.01%), Mycoplasmatales (17.80%), and Vibrionales (13.38%). The Oceanospirillales sequences identified within the GVC were monotypic at the family level, with 98.07% identified to the family Endozoicomonadaceae. This was true of the Mycoplasmatales as well, 99.96% were identified as sequences from the family Mycoplasmataceae.

3.1.3 Distribution of sequences within external mucus samples

Of the 2,012,513 total sequences from external mucosal swabs, 65.57% were Proteobacteria, 12.62% Bacteriodea, 3.17% Cyanobacteria, and 2.54% Planctomycetes.

At the order level, 19.34% of sequences were Oceanospirillales, 6.65% unclassified Gammaproteobacteria, 6.33% Rhodobacterales, 4.89% Desulfobacterales, 4.78% Chromatiales, 4.30% Flavobacteriales, with fourteen additional orders comprising between 1 and 2.5% of abundance each.

84.40% of the external swab Oceanospirillales sequences were placed within the family Endozoicomonadaceae. The families Rhodobacteraceae (6.33%), Chromatiaceae (4.29%), Flavobacteriaceae (3.22%), and Desulfobacteraceae (3.35%) also had abundances greater than 2%.

3.1.4 Benthic and water samples

Benthic samples were primarily dominated by Proteobacteria (51.28%) and Bacteroidetes (19.77%). At the order level, Desulfobacterales (9.67%), unclassified Gammaproteobacteria

(7.50%), Rhodobacterales (7.16%), Flavobacteriales (5.96%), and Chromatiales (4.16%) were most common.

Proteobacteria (68.26%) and Bacteroidetes (14.91%) were the primary phyla of water samples. Primary orders within water samples were Oceanospirillales (15.30%), Rhodobacterales (14.96%), SAR11 (11.92%), Flavobacteriales (10.76%), and Chromatiales (7.41%). Only 1.35% of water sample Oceanospirillales sequences were assigned to Endozoicomonadaceae.

3.2 Diversity

Alpha diversity was lower in the gastrovascular cavity samples than in external mucus, benthic, or water samples. The Shannon and Inverse Simpson of gastrovascular cavity communities was low (H = 1.73+/-1.81, 1/D = 4.13+/-8.97) when compared to external mucus (H = 3.83+/-1.37, 1/D = 23.46 +/-21.96) and environmental samples (Water: H = 3.19+/-1.24, 1/D = 13.98489+/-15.12037; Benthic: H = 4.21+/-0.33, 1/D = 30.07+/-9.51) (Figure 3) (ANOVA, df = 3, p-val = <2e-16). Family richness was significantly lower within medusae, 155 (+/-158) families were observed in gastrovascular samples, and 270 (+/- 195) were observed in external samples (ANOVA, df = 3, p-val = 6.69e-09).

3.3 Community identity

External and internal communities were distinct from each other (AMOVA, Fs = 20.146, df = 1, p-value: <0.001). GVC communities were distinct from benthic and water comparison groups as well (AMOVA, GVC-Benthic Fs = 15.28, df = 1, p-value: <0.001; GVC-Water Fs = 11.87, df = 1, p-value: <0.001) (Figure 4). External mucus communities were distinct from water (AMOVA, Fs = 8.257, df = 1, p-value: <0.001) but not statistically significant from benthic

samples (AMOVA, Fs = 3.214, df = 1, p-value: 0.01). Clustering difference was most distinct between GVC and environmental samples.

Within gut microbial communities, Dirichlet multinomial mixture analysis defined two community profiles, either low Endozoicomonadaceae with higher Vibrionaceae and Rhodobactereaceae (Community Type 1) or high Endozoicomonadaceae with low Vibrionaceae and high Mycoplasmataceae (Community Type 2), with Endozoicomonadaceae being the largest determining component. These profile types were not evenly distributed across sites (to see Dirichlet partitions refer to S7, to see likelihoods of Dirichlet models refer to S8, for final model parameters see S9, for comparative value of community members see S10, for Dirichlet types by site refer to S12, to see 16S rRNA gene histograms grouped by site, refer to supplementary figure S11).

Community Type 1 sites were Lower Matacumbe Key, Bahia Honda Key, and Key Largo. Sites that included a mix of community type 1 and 2 individuals were Tavernier (C2:C1 = 2:1) and Marathon Key (C2:C1 = 5:2). The Lower Keys sites, Key West (C2:C1 = 3:1), Cudjoe Key (C2:C1 = 6:0) and Big Pine Key (C2:C1 = 4:0), were primarily community profile 2. Comparative star plots of community components separated by site can be seen in Figure 5.

3.3 Core microbiome

Core microbiome for the purposes of this study was evaluated based on 100% occurrence of a family and a relative abundance of > 0.2% of reads in all 34 gastrovascular cavity samples. This is a conservative component definition compared to other core microbiome work (Neu 2021). The most abundant clade, Endozoicomonadaceae, was present in 30 of the 34 samples at this level, as was Vibrionaceae. For Mycoplasmataceae, 26/34 samples met a 0.2% threshold.

There were no medusae where all three of these families were absent from the gastrovascular cavity, and three medusae in which two of the three were absent.

3.5 Host medusa species identity

The mitochondrial haplotype of the non-native *Cassiopea*, *Cassiopea andromeda,* a cryptic sister species to *C. xamachana*, was found in four of the 34 medusae sampled. The four *C. andromeda* mitotype individuals included were from the Key Largo (1), Cudjoe Key (2), and Key West (1) sites. The gastrovascular communities of these individuals were not different from *C. xamachana* individuals (AMOVA, Fs = 0.722, df = 1, pval = 0.775).

4 DISCUSSION

By analyzing the microbiome of 34 *Cassiopea* medusae from eight locations within the Florida Keys, we demonstrate clear differences in the microbial communities present on their external and internal surfaces. Gastrovascular cavity samples have lower diversity than the sediment, external surfaces, and the water column surrounding medusae. This is consistent with results for multiple other scyphozoans— including *Cotylorhiza tuberculata* (Viver et al. 2017). We also record variation in community composition between sites, a feature consistent with results from other species, such as *Aurelia, Mastigias,* and *Tipedalia* (Weiland-Bräuer et al. 2015, Cleary et al. 2016).

4.1 Diversity and consistency

Across all sites, the gastrovascular cavities of *Cassiopea* medusae showed no core microbiome. The external microbial community of *Cassiopea* from the Florida Keys adheres fairly closely to the epibenthic microbial community at each site, with the addition of Endozoicomonadaceae for most individuals. Endozoicomonadaceae may be a primary mucosal

component and extend to the exterior of the body through mucus exchange, as it is less prevalent on the external surface than internal for most individuals. Notably, the abundant Mycoplasmataceae and Vibrionaceae from the gastric cavity of *Cassiopea* were not found in any external mucus samples. Oceanospirillales (order of *Endozoicomonas*) was the only bacterial order in which external mucus samples were enriched relative to the benthos. The *Cassiopea* bell microbiome Shannon Diversity Index values found in this study are higher than those reported in the low-diversity mucosa, oral arms and umbrella of a previously studied pelagic rhizostome, *Rhizostoma pulmo*, a likely result of the constant contact of *Cassiopea* with the epibenthos (Basso et al. 2019). The lack of overlap between diverse external communities and low diversity internal communities in *Cassiopea* is notable and consistent with the significant differences in body compartment microbiome of other scyphozoans and anthozoans (Weiland-Bräuer et al. 2015, Hao et al. 2019, Bonacolta et al. 2021). While the lack of diversity in scyphozoan microbiomes may seem distinct from many described stony coral microbial communities, these stony coral microbial profiles are often produced using whole body sampling. Lower diversity gastrovascular cavities with distinct community composition may also be a feature of stony corals (Agostini et al. 2011, van Oppen & Blackall 2019).

Microbial communities across sites within this study were not significantly different from each other, as gastrovascular cavity components remained dominated by three taxa throughout the Keys. This suggests that while lacking a formal "core" microbiome, *Cassiopea* gastric cavities may naturally rely on some combination of several microbial key taxa. The lack of obligate bacterial taxa in the *Cassiopea* gastrovascular cavity is also consistent with other microbial hosts, notably the anemone *Exaiptasia diaphana*, another successful model cnidarian (Brown et al. 2017). While *Cassiopea* require their algal symbiont, bacterial partners may be

transient, and bacteria may have far less significance to host health for *Cassiopea* than for specialized obligate hosts (e.g., leafhoppers) (Riviere et al. 2013, Sharp et al. 2017, Hammer et al. 2019, Wu et al. 2023). Without a better understanding of specific metabolic function of different bacterial associates, microbiome importance cannot be determined. We identified Endozoicomonadaceae, Mycoplasmataceae, and Vibrionaceae as the three most common bacterial families found within the gastrovascular cavity of *Cassiopea*. These are all commonly associated with coral microbial communities (Neave et al. 2016, Pollock et al. 2018, Huggett & Apprill 2019b). Below, we provide information on these common bacterial components of cnidarian holobionts.

4.2 Endozoicomonadaceae

The large proportion of Endozoicomonadaceae in internal and external samples fits within a broader framework of both Endozoicomonadaceae and known cnidarian-associated microbial communities; *Endozoicomonas* strains are present in a variety of invertebrates, including many corals, sponges and sea anemones, but also scyphozoans and cubozoans (Neave et al. 2016, Tinta et al. 2019, Huggett & Apprill 2019, Maher et al. 2020, Pogoreutz et al. 2022). Some hosts house multiple distinct *Endozoicomonas* genotypes, which indicates that different *Endozoicomonas* clades may provide different metabolic services to a host (e.g., Vitamin B12 metabolism) (Neave et al. 2017). Endozoicomonadaceae is not restricted to symbiotic taxa but has been more commonly identified in healthy scleractinian corals than bleached and diseased ones (McDevitt-Irwin et al. 2017). For these taxa, it may play a role in a variety of fundamental *Symbiodinium*–host interactions, including nutrient transport and symbiont cell breakdown (Neave et al. 2017). Notably, Endozoicomonadaceae alone is not an arbiter of microbiome health, its disappearance and reappearance without apparent host health declines has been identified in

octocorals (Van De Water et al. 2018, Pogoreutz et al. 2022). In coral, members of *Endozoicomonas* are found in close proximity to *Symbiodinium*, it is unknown whether this feature is shared with *Cassiopea* (Neave et al. 2017).

Endozoicomonas is abundant in the microbial community of the rhizostome scyphozoan *Mastigias* (Cleary et al. 2016). *Mastigias*, like *Cassiopea*, host zooxanthellae from the clade *Symbiodiniaceae*, however not all symbiotic rhizostomes appear to harbor the microbe, and not all taxa with the microbe are symbiotic (Cleary et al. 2016, Viver et al. 2017). *Endozoicomonas* may be common across medusae, but the difficulty of culturing some strains may have excluded them from the results of past culture-based analyses (Neave et al. 2017, Pogoreutz et al. 2022). Greater clarity is needed on the function of *Endozoicomonas* for these medusae and other taxa.

4.3 Mycoplasmataceae

Mycoplasmataceae, as with Endozoicomonadaceae, is present in studied coral and scyphozoan microbiomes (Daley et al. 2016, Viver et al. 2017, Huggett & Apprill 2019, Peng et al. 2021). While intracellular parasitism is common for members of Mycoplasmataceae, those associated with cnidarians may be located extracellularly and exist as opportunistic commensals (Neulinger et al. 2009, Van De Water et al. 2018). *Mycoplasma* sp. is noted as a potential endosymbiont in lab-raised *Aurelia* polyps, while adults from many jellyfish populations are known to have them (Weiland-Bräuer et al. 2015, Stabili et al. 2020, Peng et al. 2021). Whether this group represents an endosymbiont, a result of prey capture, or a parasite, its exclusivity to the gastrovascular tract in *Cassiopea* sampled within the Keys is notable, as other studies have not demonstrated gastrovascular cavity localization (Weiland-Bräuer et al. 2015, Stabili et al. 2020).

4.4 Vibrionaceae

Vibrionaceae is both a core part of coral microbiomes (Sweet & Bulling 2017, Huggett & Apprill 2019) and known to facilitate metamorphosis within *Cassiopea* (Hofmann et al. 1978, Neumann 1979), however, it is a highly diverse family. While *Vibrio* spp. are found in gut communities of presumed healthy scyphozoans (Daley et al. 2016, Peng et al. 2021), they can also be associated with senescence and disease (Kramar et al., 2019; Tinta et al., 2012). For *Cassiopea* specifically, an increase in *Vibrio* may be associated with an aposymbiotic or stressed state (Carabantes et al. 2022). Given the variation in Vibrionaceae species and strains, family-wide statements on the beneficial or harmful nature of Vibrionaceae within *Cassiopea* gastrovascular cavities should not be made.

4.5 Caveats: seasonality and size

Seasonal changes are a feature of some cnidarian-associated microbial communities (Sharp et al. 2017, Stabili et al. 2020). In an outdoor laboratory setting, this is true of *Cassiopea* as well (Carabantes et al. 2022). As these samples were taken during the hottest time of the year in the Florida Keys (August), this community may not be representative of other timepoints. Winter communities may harbor different taxa or taxa in different proportions (Sharp et al. 2017). As the internal microenvironment of *Cassiopea* alters over the course of a day with regard to light and dark cycles, it is reasonable to expect that it may become more or less hospitable to some microbiota as temperature and light cycles adjust throughout the year (Arossa et al. 2021).

As *Cassiopea* medusae grow at rates commensurate with their feeding, there is no guarantee that the size of medusae in this study is representative of their age. Developmental changes are known to facilitate changes to microbial communities within other scyphozoans, but

size of adults has not been tested (Weiland-Bräuer et al. 2015, 2020, Lee et al. 2018). As medusa size and site were not independent (smaller medusae and larger medusae were not usually seen in mixed assemblages), we cannot determine whether size class had an impact.

4.6 Laboratory cultures

Cassiopea are primarily used for in-lab studies on symbiosis (McGill & Pomory 2008, Klein et al. 2016, Mammone et al. 2021). Some of these use wild-caught individuals, and others use lab-bred cultures, many of which have been held for years. The lab-raised *Cassiopea* used in Rothig et al. 2021 appear to have a completely different microbiome than the wild individuals caught here (Röthig et al. 2021). This potential discrepancy should be considered when using wild-caught or captive-bred individuals, as captive-bred symbiosis and tolerance levels may differ from medusae in the wild. When held in outdoor ponds, the community seems to match wild-type more fully (Carabantes et al. 2022). These discrepancies in microbiome are found in other model cnidarians, such as *Exaiptasia* (Brown et al. 2017). The information on wild *Cassiopea* microbial communities from the Florida Keys presented here may allow researchers working with lab-raised individuals to better contextualize the taxa that they find in cultured medusae.

5 CONCLUSION

The microbiome of *Cassiopea* within the Florida Keys demonstrates strong body compartment discrimination and includes a central group of families overrepresented in the gut and external mucus. All of these families, Endozoicomonadaceae, Vibrionaceae, and Mycoplasmataceae, are found in other medusae and anthozoans. Despite this overrepresentation of some microbial taxa, *Cassiopea* lacks a conservatively defined core microbiome. The

consistency of *Cassiopea* microbial communities across the Florida Keys demonstrates that specific taxa are strongly associated with *Cassiopea* and that this can extend across many sites with diverse physical conditions. This work provides a greater understanding of the wild microbiome of the common lab organism *Cassiopea*.

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Table 1: List of collection sites with latitude, longitude, salinity, pH, surface temperature, average diameter of collected medusae from the site, and date of collection.

Site Name	Latitude	Longitude	Salinity (ppt)	pH	Temp (C)	Average depth (m)	Average col. medusa diam (cm)	Date (AM/PM)
Key West	24.561526	-81.7881727	36	8.2	30.4	0.56	6.62	15.8.2021 PM
Cudjoe Key	24.6775378	-81.4991819	36	8.4	32.6	0.36	5.91	17.8.2021 AM
Big Pine Key	24.6978731	-81.3572574	35	7.7	29.1	0.15	4.43	15.8.2021 PM
Bahia Honda Key TP1	24.6825793	-81.2293673	37	8.3	31.8	0.86	12.41	13.8.2021 PM
Bahia Honda Key TP2	24.6825793	-81.2293673	36	8	29.4	0.51	10.28	15.8.2021 AM
Marathon Key	24.69396	-81.0980507	31	8.1	35.2	0.08	6.53	11.8.2021 PM
Lower Matacumbe Key	24.8582157	-80.7267927	35	8.1	32.9	0.89	11.40	16.8.2021 PM
Tavernier	25.0233783	-80.4940316	31	8.1	33.3	0.35	12.26	16.8.2021 AM
Key Largo	25.0872104	-80.4415797	35	7.8	32.0	0.56	4.30	18.8.2021 AM

Figure 1: Sampling strategy for medusae collection onsite.

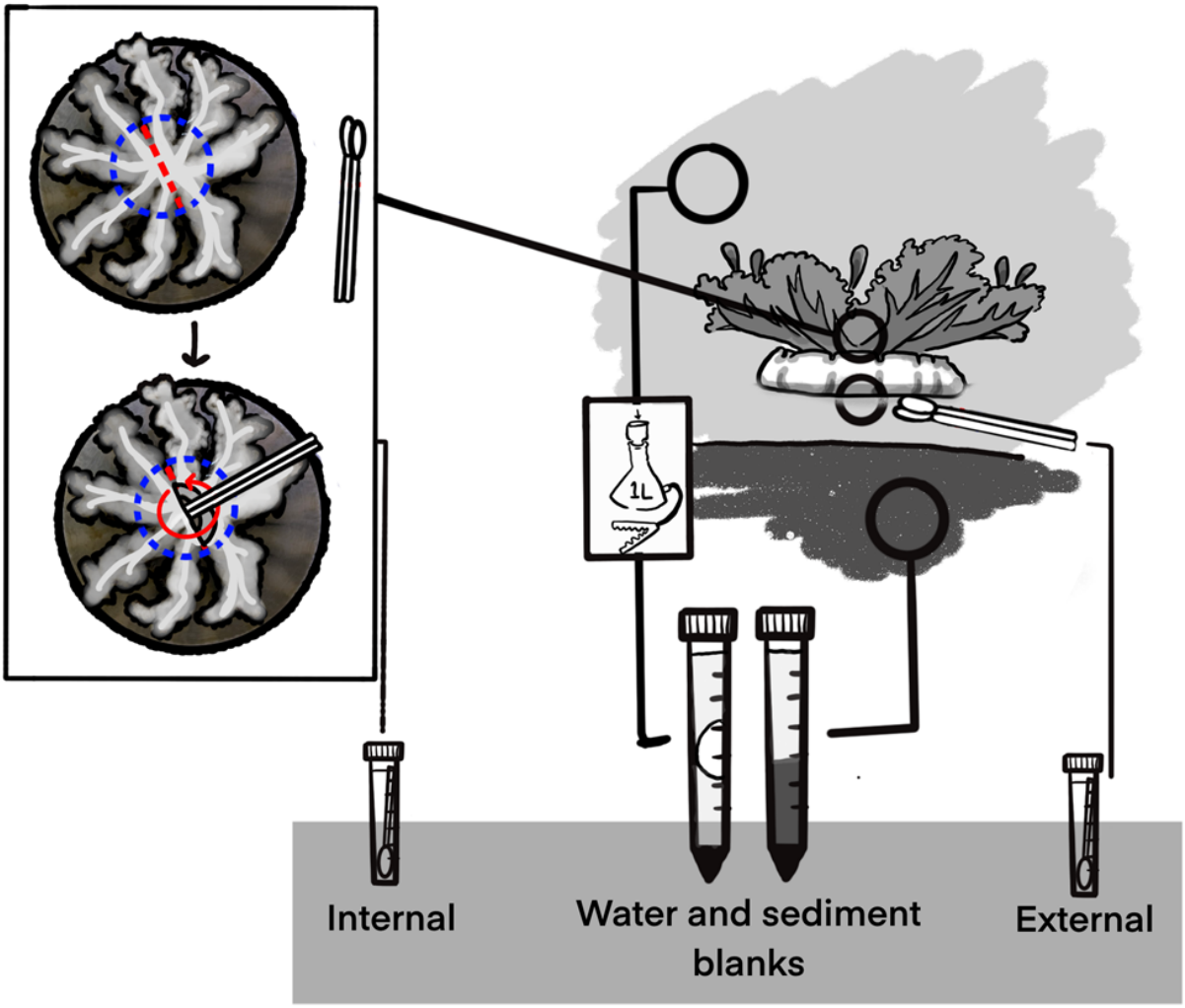


Figure 2. (left) phylum level and **(right)** class level distribution of sequences in samples aggregated by sample type**.**

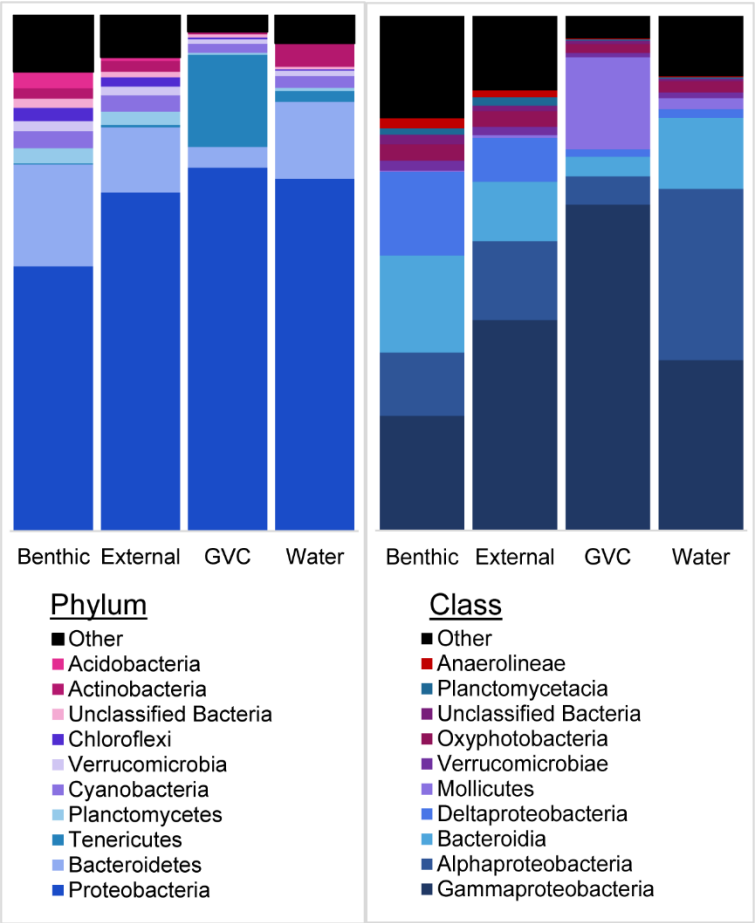


Figure 3. Order level diversity of samples by body compartment, including the top 20 most common orders by name.

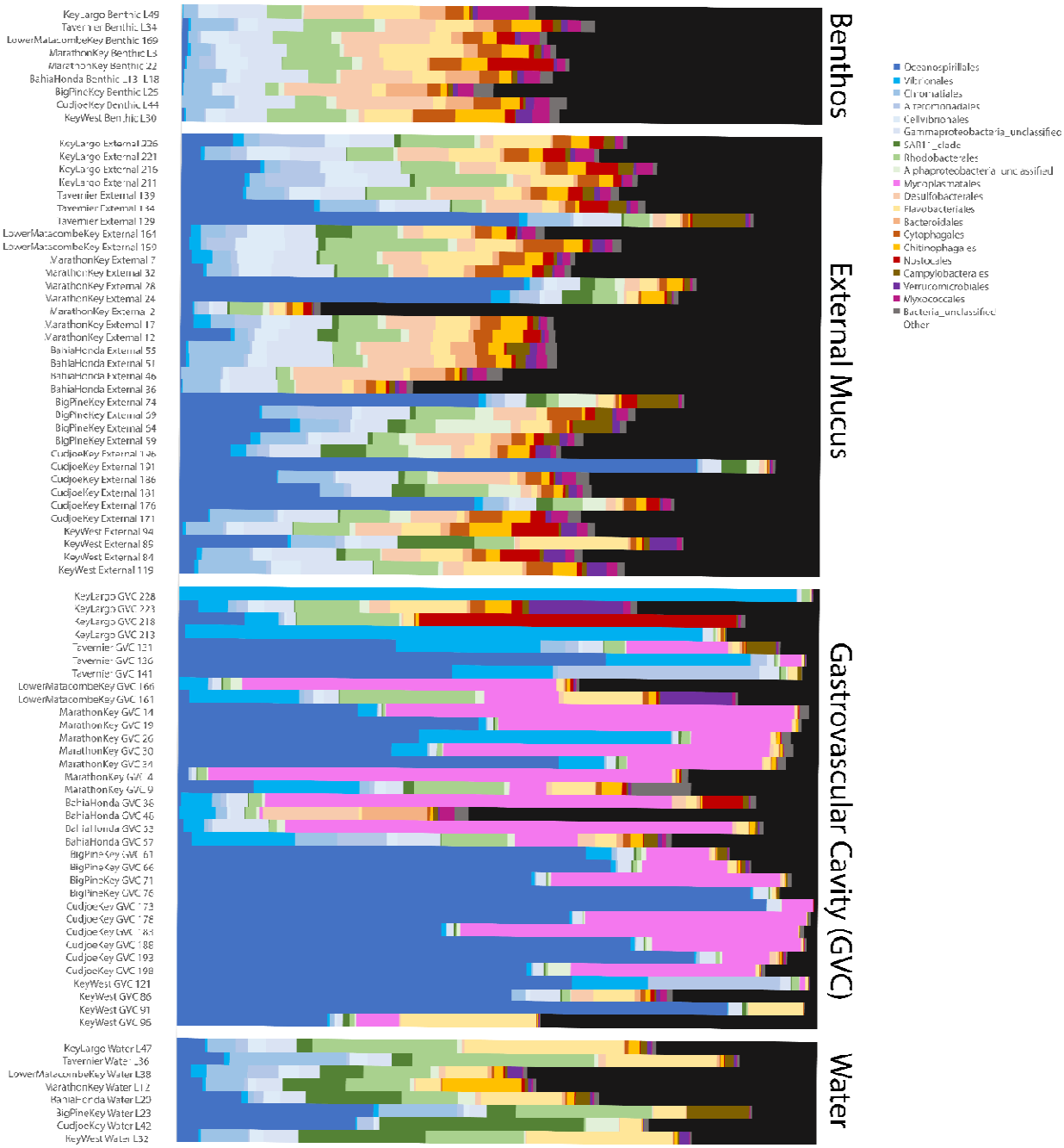


Figure 4. Two-dimension NMDS plot segregated by sample type. Stress = 0.255711, R-squared for configuration: 0.750874

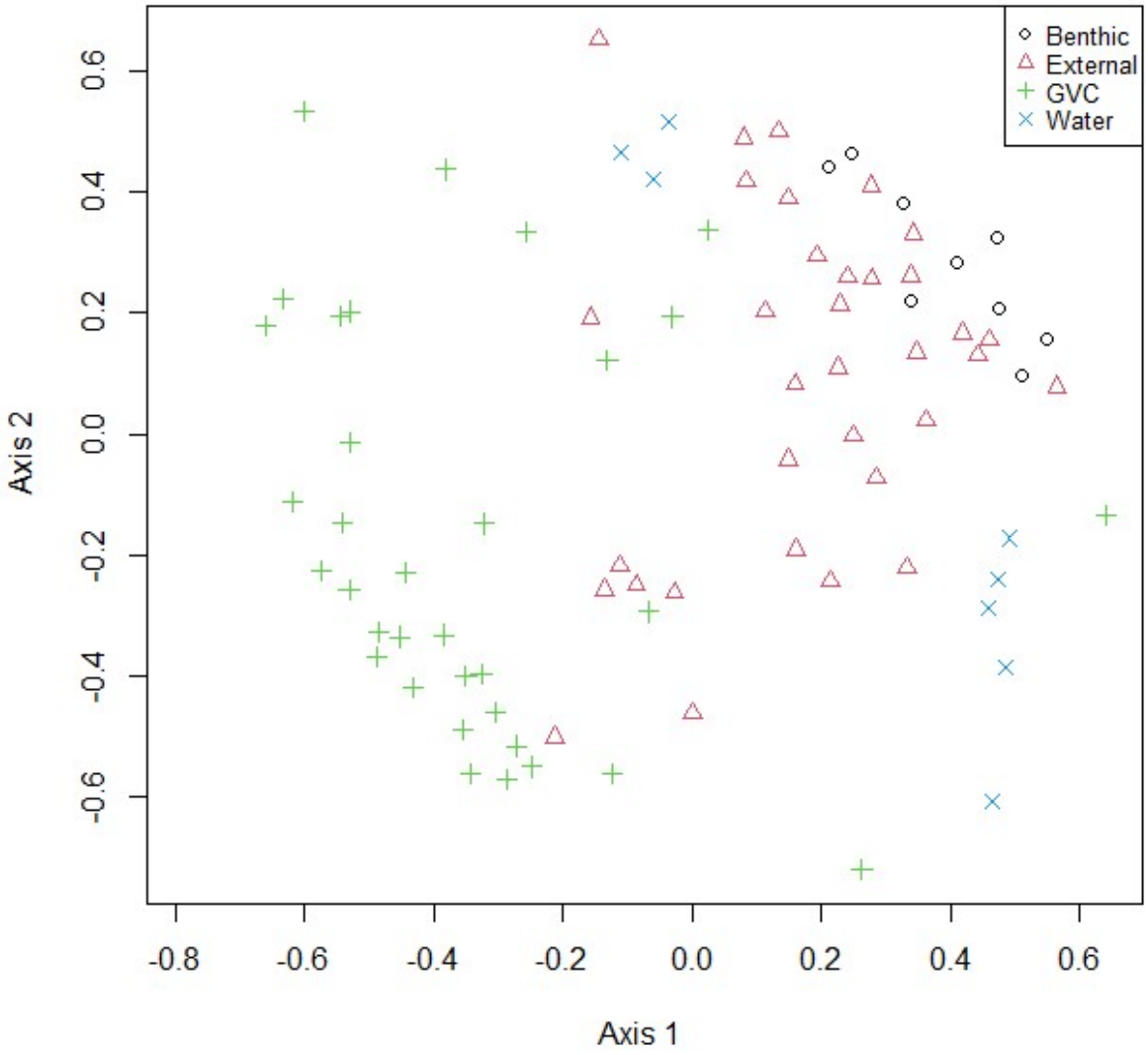
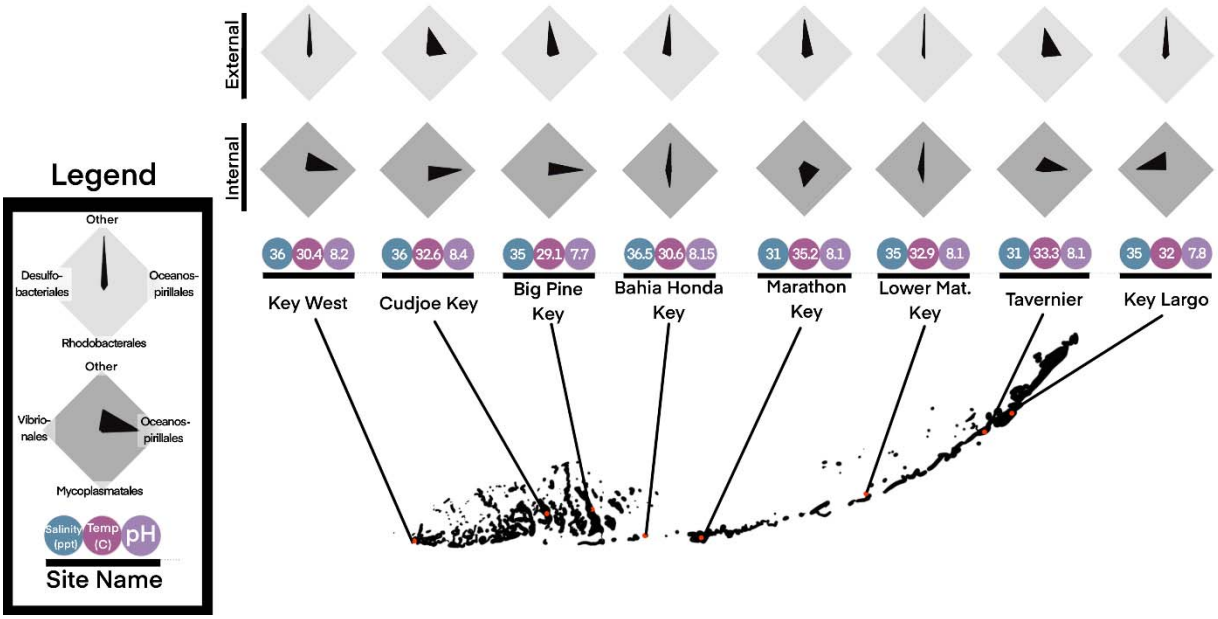


Figure 5. Star plots of community composition for internal and external mucus swabs at each site separated by order (three most prominent orders and "other"). Each spoke of the star plot ranges from 0% (center) to 100% (edge of shaded diamond). External group spokes (going clockwise): Other, Oceanospirillales, Rhodobacterales, and Desulfobacterales. Internal group: Other, Oceanospirillales, Mycoplasmatales, and Vibrionales. Each site's measured environmental conditions (salinity, water surface temperature, and pH) are presented below star plots. Sites are mapped onto their location within the Florida Keys.

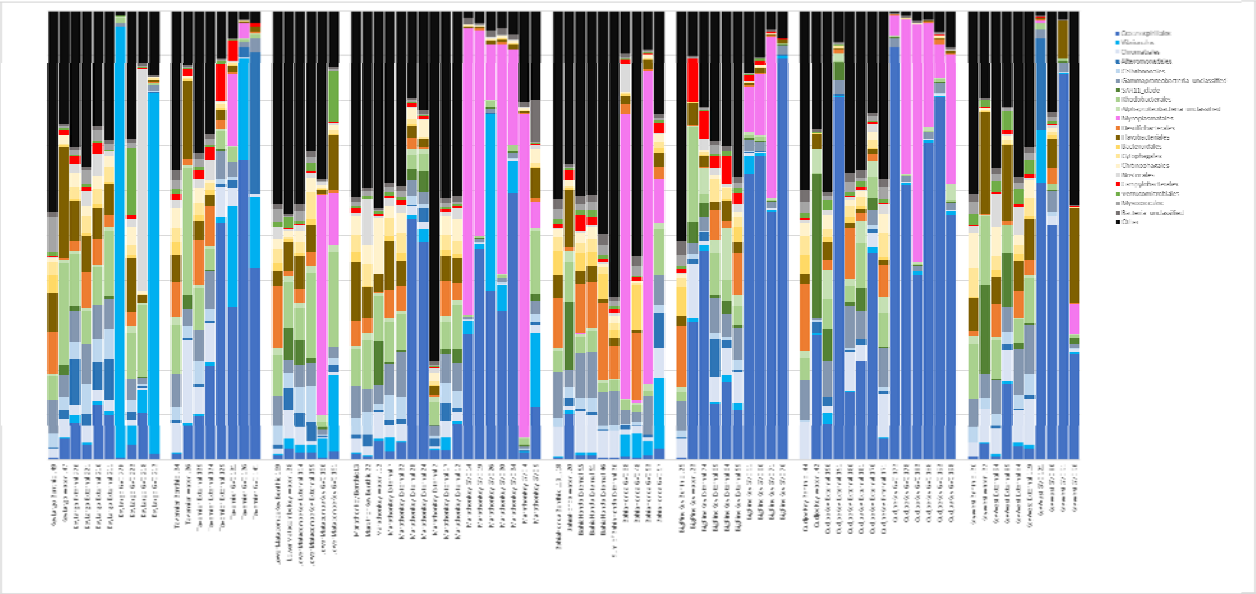
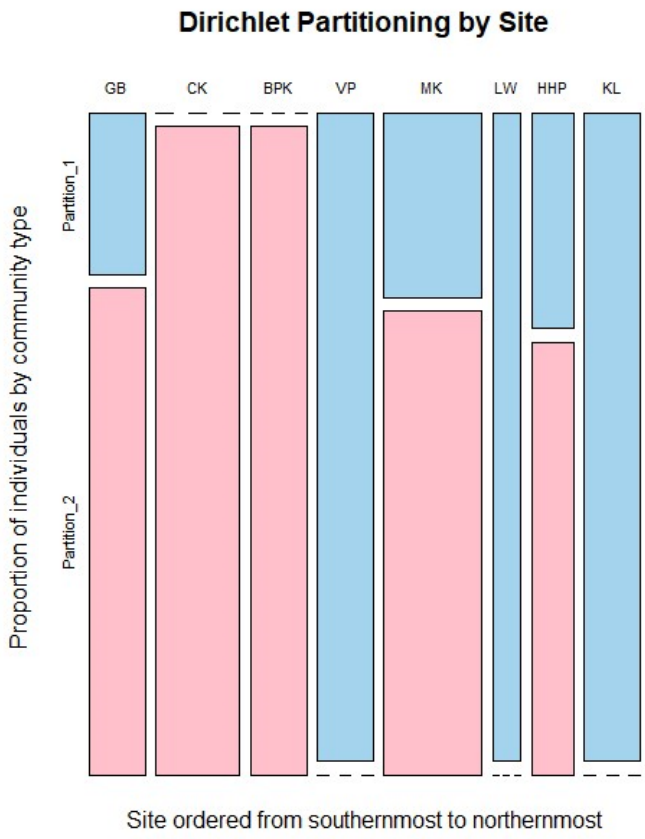


A1 FULL LENGTH 16S IDENTITIES

Full length 16S rRNA gene sequences (BioProject: PRJNA1020446) were run on mothur v1.48.0 with deviation from the MiSeq SOP on trim length. ASV cutoff was set to five differences, and ASV representative sequences were compared against the NCBI GenBank (Sept 24, 2023) database using BLAST to find nearest matches by e-value.

In order to identify core microbial groups to a lower taxonomic level, three internal medusa gastrovascular swabs (medusae Kmuffet_96, Kmuffet_120 and Kmuffet_30) from Marathon Key (MK) and Key West (GB) were sequence for full length 16S rRNA gene (see S1 for location of samples). As the primers were not chloroplast exclusionary, the Key West sites produced few useable sequences (Kmuffet_96: 597 and Kmuffet_120: 1097). The Marathon Key medusa (Kmuffet_30), likely bleached at time of collection, returned 12,520 non-chloroplast sequences. The Mycoplasma-like full length 16S rRNA gene sequence (Genbank Acc. No. OR592270) from Marathon Key and Key West has no closely related sequences currently identified (greatest identity 88.43% to sequence HQ393440). The *Endozoicomonas* strain from both Marathon Key and Key West (Genbank Acc. No. OR592271) has 99.93% identity to *Endozoicomonas atrinae* strain WP70(T) collected from the intestine of South Korean *Atrina pectinata* (Accession NR_134024)(Hyun et al. 2014) and 99.80% identity (<5 nucleotide differences) with isolates collected from *Gorgonia ventalina* (GenBank Acc. No. GU118345) in Bocas del Toro, Panama (Sunagawa et al. 2010). *Endozoicomanas* and *Mycoplasma*-like isolates were the only two groups to have ASVs with over 100 sequences.

S11: Order level bar plots grouped by location



S12: Mosaic plot of Dirichlet partition identity (community type) by site