Abundance of Oligoflexales bacteria is associated with algal symbiont density independent of thermal stress in Aiptasia anemones

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ABSTRACT

Many multicellular organisms, such as humans, plants, and invertebrates, depend on symbioses with microbes for metabolic cooperation and exchange. Reef-building corals, an ecologically important order of invertebrates, are particularly vulnerable to environmental stress in part because of their nutritive symbiosis with dinoflagellate algae, and yet also benefit from these and other microbial associations. While coral microbiomes remain difficult to study because of their complexity, the anemone Aiptasia is emerging as a simplified model. Research has demonstrated co-occurrences between microbiome composition and the abundance and type of algal symbionts in cnidarians. However, whether these patterns are the result of general stress-induced shifts or depletions of algal-associated bacteria remains unclear. Our study aimed to distinguish the effect of changes in symbiont density and thermal stress on the microbiome of symbiotic Aiptasia strain CC7 by comparing them with aposymbiotic anemones, depleted of their native symbiont, *Symbiodinium linucheae*. Our analysis indicated that overall, thermal stress had the greatest impact on disrupting the microbiome. We found that three bacterial classes made up most of the relative abundance (60-85 %) in all samples, but the rare microbiome fluctuated between symbiotic states and following thermal stress. We also observed that *S. linucheae* density correlated with abundance of Oligoflexales, suggesting these bacteria may be primary symbionts of the dinoflagellate algae. The findings of this study help expand knowledge on prospective multipartite symbioses in the cnidarian holobiont and how they respond to environmental disturbance**.**

INTRODUCTION

Biological organisms, from single cells to ecosystems, are influenced by symbiotic interactions (Boucher, 1985; University of Massachusetts Amherst Massachusetts Lynn Margulis, Margulis and Fester, 1991; Smith and Szathmary, 1997; Sachs *et al.*, 2004). Although symbioses are generally modeled and studied as two-way interactions, multipartite symbioses are also ubiquitous and have been well-documented in plants (Miransari, 2011; Antunes and Goss, 2015; Adeniji, Babalola and Loots, 2020; Afkhami *et al.*, 2020), humans (Wahida, Tang and Barr, 2021), and invertebrates (Worthen, Gode and Graf, 2006; Ffrench-Constant, Eleftherianos and Reynolds, 2007; Chaston and Goodrich-Blair, 2010; Stock, 2019). These multi-partner interactions can provide the host with essential metabolites, amino acids, and vitamins (Cleveland, Alan Verde and Lee, 2011; Adeniji, Babalola and Loots, 2020). Additionally, they can also confer defense (Zan *et al.*, 2019), facilitate host morphogenesis (Wichard, 2015) and aid resilience under environmental stress (Gupta *et al.*, 2021; Santoyo *et al.*, 2022).

Understanding the impact of multi-partner associations on the health and survival of marine invertebrates is crucial given they are among the most susceptible animals to the impacts of climate change (Mather, 2013; Lam *et al.*, 2020). The global decline of corals, which are cnidarian hosts that harbor intracellular populations of dinoflagellate algae in the family Symbiodiniaceae and other microbial associates (Pandolfi *et al.*, 2003) is already underway and is predicted to worsen with climate change (Hoegh-Guldberg *et al.*, 2007; Allemand and Osborn, 2019; Kleypas and Kleypas, 2019). Bleaching, which involves the expulsion of symbiotic algae in cnidarians resulting in the loss of color, can be induced by several factors, although elevated temperature, as noted by (Douglas, 2003)), is the most common cause. Yet there is also abundant variation in coral thermal tolerance evidenced by differences in bleaching among species, populations, and individuals (Dixon *et al.*, 2015; Thomas *et al.*, 2018; Drury, 2020). Collectively, these observations have led to the "Coral Probiotic Hypothesis" (Reshef *et al.*, 2006) which is based on the notion that symbiotic relationships with bacteria can increase coral resilience (Peixoto *et al.*, 2017).

The cnidarian-dinoflagellate symbiosis is better characterized in the literature than potential cnidarian-bacterial symbioses, and it remains unclear whether environmental or internal

host factors, like host genetics or microalgal symbiont type, modulate the composition of cnidarian microbiomes (Bourne, Morrow and Webster, 2016; van Oppen and Blackall, 2019; Barno *et al.*, 2021). One understudied theory postulates that well-known cnidarian-bacterial associates may actually be primary associates of Symbiodiniaceae, in both free-living and in-hospite states (Ritchie, 2012; Bernasconi *et al.*, 2019; Matthews *et al.*, 2020). Global datasets suggest that the identity of Symbiodiniaceae may contribute to structuring coral and anemone microbiomes (Bernasconi *et al.*, 2019). For instance, susceptibility to *Vibrio* pathogens was higher in *Acropora cytherea* corals hosting *Symbiodinium* than *Durusdinium* (formerly Clade A and D Symbiodinium, respectively) (Rouzé *et al.*, 2016). Additionally, the abundance of diazotrophs in *Montipora* corals also correlated with algal symbiont type (Olson *et al.*, 2009). Similarly, unique microbiomes were identified in symbiotic and aposymbiotic Aiptasia anemones (Herrera *et al.*, 2017) indicating presence of the symbiont influences the microbiome. However, insufficient evidence exists to verify this hypothesis and tracking bacteria in adult corals is nearly impossible, due to their high bacterial diversity (Blackall, Wilson and van Oppen, 2015), which poses a challenge for distinguishing obligatory and facultative bacterial symbionts. Furthermore, selectively eliminating holobiont members empirically is not feasible since reef-building corals cannot survive without their algal symbionts, who provide vital sugars and nutrients (Weis, 2008). The anemone Aiptasia (*Exaiptasia pallida*, *sensu stricto*) is a tractable model for studying the cnidarian microbiome as it harbors fewer bacterial OTUs, with diversity estimated to be around 1-2 orders of magnitude lower than their coral relatives, (Röthig *et al.*, 2016; Herrera *et al.*, 2017). Aiptasia are easy to maintain, engage in a nutritive symbiosis with Symbiodiniaceae similar to corals, and can reproduce asexually (Baumgarten *et al.*, 2015; Weis, 2019). Unlike their coral relatives, Aiptasia can be rendered aposymbiotic (free of their dinoflagellate algae) in laboratory conditions and lack a calcitic skeleton, which facilitates experimental manipulation (Lehnert *et al.*, 2014).

While associations between microbial community composition and the abundance and/or diversity of algal endosymbionts is consistent with the hypothesis that some microbes are primary associates of Symbiodiniaceae, these patterns cannot be distinguished from passive commensal relationships. Additionally, general stress may be responsible for changes in the host-photosymbiont relationship, leading to alterations in metabolite production and resulting in different selective pressures that favor distinct groups of commensals. For example,

Symbiodiniaceae taxa are known to differ in their metabolite production (Camp *et al.*, 2022). *Durusdinium* translocates less carbon to hosts than *Cladocopium (*Cantin *et al., 2009)* under thermal stress but *Cladocopium* translocates more carbon and nitrogen to hosts during non-stressful conditions (Pernice *et al.*, 2015), which could impact the consortium of microbiota in the holobiont. The host-photosymbiont relationship can also be affected by mild bleaching, which is another general stress response (Ortiz, Gomez-Cabrera and Hoegh-Guldberg, 2009). Here, we aimed to distinguish the effects of the stress response and that of symbiont density on microbial communities while controlling for host and symbiont genetic diversity. To achieve this, we used the emerging model organism Aiptasia clonal strain CC7, which harbors *Symbiodinium linucheae (*Starzak *et al.*, 2014*;* Baumgarten *et al.*, 2015*)* and conducted a comparison between the microbiomes of symbiotic anemones undergoing mild bleaching and aposymbiotic anemones, which were presumed to lack the microbes typically associated with *Symbiodinium*.

MATERIALS AND METHODS

2.1 Aiptasia rearing

Aiptasia anemones, clone strain CC7 (obtained from Dr. Cory Krediet, Eckerd College, FL, USA) were used in this study. Anemones were kept in 0.5 L polycarbonate tanks, filled with 0.2 µm filtered seawater (FSW) from Catalina Island (Catalina Water Co., Long Beach CA, USA) and maintained at 25°C on a light/dark (14:10 h) cycle under 12-20 µmol photons m -2 s -1. Animals were maintained in these common garden conditions at USC with weekly feeding (frozen brine shrimp, *Artemia salina*) and water changes for 24 months. A subset of Aiptasia animals were rendered aposymbiotic by menthol-induced bleaching (Matthews *et al.*, 2016) four months prior to experimental trials. Briefly, a 1.28M menthol solution was prepared (20% w/v in ethanol) and added to polycarbonate tanks containing FSW for a final concentration of 0.19 mmol l -1. Anemones were placed in the menthol solution for 8 h during the light period of the 14:10, light: dark cycle. The animals were then transferred overnight to tanks containing a final concentration of 0.10 M DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an algicide and photosynthesis inhibitor. This was repeated for four consecutive days. At the end of the fourth day, anemones were placed in black-out tanks with no light exposure and allowed to recover for

three days with one feeding. After the three-day recovery, the process was repeated once more and bleaching status was confirmed using a fluorescent microscope. An absence of red autofluorescence from dinoflagellate chloroplasts was noted and these aposymbiotic anemones were transferred to black-out tanks filled with 0.2 µm FSW and maintained in the dark for three months with the same feeding and water change regime as the symbiotic anemone stock.

2.2 Experimental design and sampling

Symbiotic and aposymbiotic anemone of approximately 2.5 cm length were selected for the 6-day thermal stress experiment. Aposymbiotic Aiptasia were added to the experimental design to act as a control for distinguishing baseline host stress responses from cnidarian-algal microbiome shifts. These aposymbiotic anemones were acclimated to the same light/dark (14:10 h) cycle as symbiotic anemones one week prior to the experiment start date. In addition, all anemones were starved for 2 weeks prior to sampling to avoid prey (shrimp, *Artemia sp.*) contamination.

Six 0.5 L tanks containing six symbiotic Aiptasia (n=36) and four 0.5 L tanks containing six aposymbiotic Aiptasia (n=24) were distributed evenly between experimental conditions (25 °C vs 32 °C, Fig. 1). The treatment temperature was set to 32 °C due to prior observation of slight bleaching and expulsion of *Symbiodinium* in Aiptasia at this temperature (Perez, Cook and Brooks, 2001)). To maintain and manipulate temperature, tanks used in the heat stress treatment were placed in 10 L bins containing two SL381 submersible water pumps, two 100W aquarium heaters, a HOBO temperature logger, and a digital, waterproof thermometer. Acclimatization of Aiptasia was reached by exposing them to a gradual temperature ramp over the course of four days. Temperature was increased from 25 $\pm$ 0.5 °C to 27 $\pm$0.5 °C the first day, to 30 $\pm$ 0.5 °C the second day, 32 $\pm$ 0.5 °C the third day and 33 $\pm$ 0.5 °C on the fourth day. Once Aiptasia were acclimated to the elevated temperature treatment, treatment was maintained at an average of 32 $\pm$ 0.5°C for 6 days with FSW changes every 2 days, in both control and heat-stress tanks. At the end of the 6-day exposure period, each anemone was rinsed three times with FSW in individual 60 x 15 mm petri dishes, placed in a 1.5 mL microcentrifuge tube and frozen at -80 °C, until processing.

2.3 DNA extractions and 16S rDNA sequencing

Individual anemone DNA was extracted by ethanol precipitation as detailed previously (https://openwetware.org/wiki/Ethanol_precipitation_of_nucleic_acids) with some modifications. Briefly, animals were placed in sterile, 2 mL polypropylene screw cap tubes (Merck KGaA, Germany) containing a thin layer of Zi/Si beads (100-500 mm diameter), 200 µL lysis buffer AP1 from a DNEasy Power Plant Kit (Qiagen, Germany), 2 µL RNAse (100 mg/mL, stock), and 2 µL Proteinase K (20 mg/mL, stock) and incubated at 55 °C for 10 min in a temperature-controlled water bath. Samples were then homogenized using the Omni bead beater (Omni International, USA) at 6.3 m/s, for 2 cycles of 30 seconds. The samples were then centrifuged for 5 min at 14,000 rpm and the proteinase K enzyme was heat-inactivated on a heat block (78-82 °C) for 3 min. The supernatant was transferred to a 1.5 mL microcentrifuge tube. Ethanol precipitation was performed as described (https://openwetware.org/wiki/Ethanol_precipitation_of_nucleic_acids) and the pellet was resuspended in 40 µL elution buffer (GenElute Bacterial Genomic DNA Kit, Merck KGaA, Germany). DNA was purified using the Zymo DNA Clean & Concentrator Kit (Zymo Research, USA) following the manufacturer's instructions.

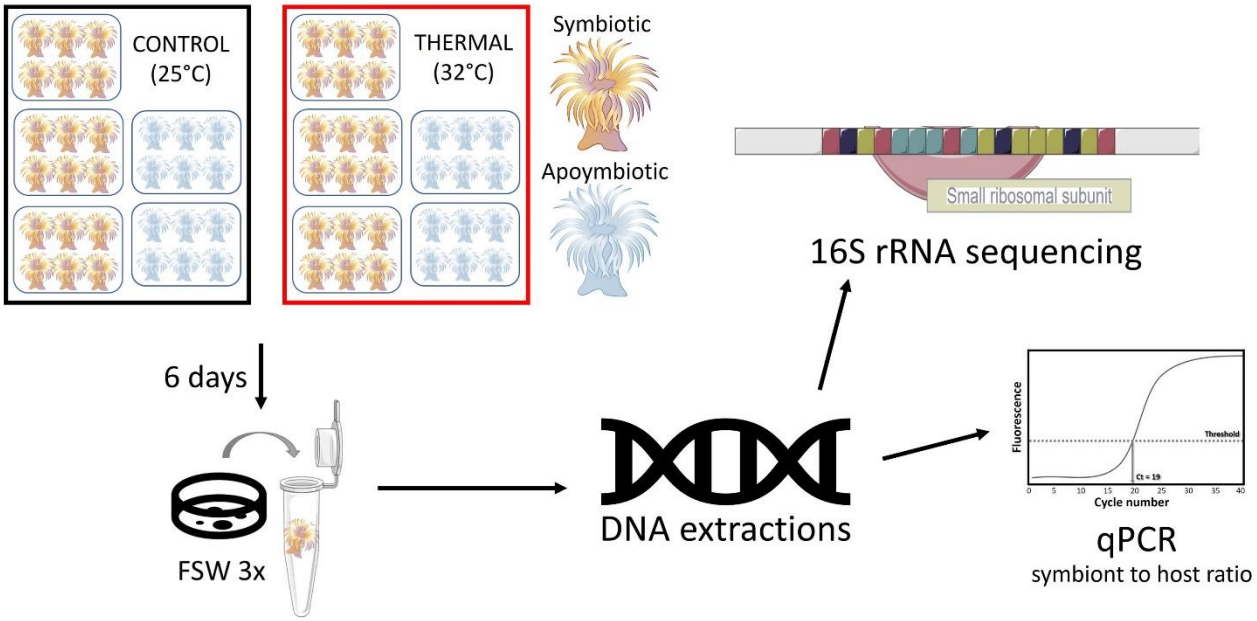

Figure 1. Study design for the mild thermal stress experiment. Each treatment condition received 5 tanks (3 tanks x 6 symbiotic anemones and 2 tanks x 6 aposymbiotic anemones; n= 30 per treatment). Following a 7-day ramping period, the peak temperature exposure continued for 6 days, and sampling was conducted on the last day. Each anemone was rinsed with filtered seawater (FSW) thrice, deposited into a microcentrifuge tube and stored at -80 °C until processing. DNA extractions were performed followed by 16S rRNA amplicon sequencing. Samples with remaining DNA were used in qPCR assays to determine symbiont (*Symbiodinium*) to host (Aiptasia) ratios.

Amplification of the V5/V6 region of the 16S rRNA gene was done using the 784F and 1061R primer set, which amplifies approximately 277 bp fragments with minor cross-amplification of host mitochondria and microalgal symbiont chloroplasts (Andersson *et al.*, 2008; Bayer *et al.*, 2013). Briefly, 50 ng of DNA were amplified in 25 ul reactions using 0.25 µL of 2,000 units/mL Q5 High-Fidelity DNA Polymerase (New England BioLabs Inc., Germany), 0.5 µL of 10 mM each dNTP, 0.25 µL of 10 µM forward and reverse primer each, 0.25 µL BSA 100X (New England BioLabs Inc., Germany), 5.0 µL 5X Q5 PCR Buffer (New England BioLabs Inc., Germany), 13.5 µL nuclease-free water, and 5 µL of 10 ng/µL DNA. PCR conditions were as previously described in Bayer et al. (2013) with the following modifications: 26 cycles of denaturation at 95°C for 60 sec and annealing at 55°C for 60 sec. 5 mL of the 16S rRNA amplified sample was added to a second round of 3-step PCR to incorporate sample-specific Illumina barcodes using an amplification profile of: 98°C 0:30, (98°C 0:10 | 59°C 0:30 | 72°C 0:30) x 4 cycles, 72°C 2:00. The barcoded samples were pooled in equimolar amounts (1 ng/µL) and sent for 250-bp paired-end sequencing on Illumina's MiSeq v2 PE 250 platform (USC Norris Comprehensive Cancer Center Molecular Genomics Core (NCCC), USA).

2.4 16S rDNA sequencing and bioinformatic analysis

We successfully extracted DNA and sequenced the 16S rRNA gene of 59 individual anemones (initial n=60) but seven samples were discarded from the dataset due to low read yields (< 2,000). Paired-end reads were demultiplexed by the sequencing facility (USC NCCC Molecular Genomics Core) and quality checked with FastQC (Andrews, 2010). An amplicon sequencing variant (ASV) table was was generated with DADA2 (Callahan *et al.*, 2016) in R (R Core Team 2020) using the default filtering parameters (truncLen=c (240,160), maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE, compress=TRUE, multithread=FALSE). Taxonomy was assigned using the Ribosomal Database Project Classifier (Wang *et al.*, 2007) along with the SILVA SSU version 138.1 database, formatted for DADA2 (DOI/10.5281/zenodo.4587955). Chloroplast and mitochondria sequences were removed using the R package, Phyloseq (McMurdie and Holmes, 2013). The dataset yielded uneven library sizes, so samples were rarefied to an even depth of 26, 481 reads (Gloor *et al.*, 2017; Weiss *et al.*, 2017). Following rarefaction, the dataset consisted of 1, 377, 012 reads (Table A1). All statistical analyses and visualizations were conducted in R (R Core Team, 2020). Taxa plots, alpha diversity (Chao1 index), and beta-diversity visualizations

were conducted using the Phyloseq (McMurdie and Holmes, 2013), ggplot2 (Wilkinson, 2011), and Microbiome (Lahti and Shetty *et al*, 2019) packages. Differential tree matrices were generated and visualized using the Metacoder (Foster, Sharpton and Grünwald, 2017) package. Statistical output for differential heat trees can be found at Zenodo, doi.org/10.5281/zenodo.7693398.

Additional statistical analyses were conducted using the vegan (Dixon, 2003) package. A one-way ANOVA test was conducted on rarefied data, comparing alpha-diversity data (Chao1 scores) between the microbial assemblages of symbiotic and aposymbiotic anemone groups in different treatments (control vs thermal stress). Post-hoc pairwise comparisons were done using Tukey's HSD. Beta-diversity was visualized using a PCoA plot employing the weighted-Unifrac metric. The adonis2 function in vegan was used to conduct pairwise Permutational Multivariate Analysis of Variance (PERMANOVA) of microbial assemblage dissimilarities between treatment groups. Adonis2 was used due to even homogeneity of variances in all pairwise comparisons tested.

As the SILVA SSU v138.1 database only assigned some bacterial taxonomy to Order, we conducted a separate phylogenetic analysis of ASVs classified in the order Oligoflexales. A Bioconda (Grüning *et al.*, 2018) environment was used for this analysis. The filtered Phyloseq dataset was subsetted to include only Oligoflexales reads and resulting ASVs were then transferred to a fasta file and a standard NCBI nucleotide blast (Sayers *et al.*, 2023) was performed on the web interface, optimizing for highly similar sequences (megablast). The ASVs exhibited high quality (Table A2) matches to three uncultured bacterium clones, originating from one microbial survey (Randle *et al.*, 2020) conducted on Aiptasia strain CC7 (GenBank: MK571601.1/ MK571569.1) and Aiptasia strain H2 (GenBank: MK571216.1), as well as one uncultured proteobacterium clone (GenBank: FJ425635) found in the microbiome of scleractinian coral *Orbicella* (formerly *Montastrea*) *faveolata*. We queried our nine Oligoflexales ASVs and these previously published sequences with 16S rDNA sequences from 2 confirmed members of the Oligoflexales order (GenBank accession numbers AB540021.2 and OW948931.1) and ten members (7 families) of the Bdellovibrionota phylum. Sequence alignment was performed using the MUSCLE algorithm version 5.1 (Edgar, 2004) and a phylogenetic tree was constructed by maximum likelihood with ultrafast bootstrap (n= 1,000

replicates) in IQ-TREE version 2.2.0.3 (Kalyaanamoorthy *et al.*, 2017; Minh *et al.*, 2020). The resulting phylogenetic tree was visualized and annotated using the integrated web editor interface, Interactive Tree of Life (ITOL, https://itol.embl.de/).

2.5 Symbiont to host (S/H) cell ratio qPCR

To assess the effects of thermal stress on symbiont density in Aiptasia, we analyzed symbiont to host cell ratios (Mieog *et al.*, 2009) using nuclear ribosomal protein L10 as a reference gene for the host, Aiptasia (Poole, Kitchen and Weis, 2016) and the actin locus as a target in *Symbiodinium* (Palacio-Castro 2019). Nuclear ribosomal protein L10 primers were previously validated for Aiptasia specificity in qPCR assays (Poole, Kitchen and Weis, 2016) and used as a reference gene due to stable expression in Aiptasia (Kitchen and Weis, 2017). Previous qPCR assays targeting the actin locus gene in *Symbiodinium* sp. (clade A) showed amplification specificity with an estimated copy number for the actin locus at ~9 per cell (Palacio-Castro 2019). The primers used in this study for the host were 400nM nrp_L10-F (5'-ACGTTTCTGCCGTGGTGTCCC-3') and 400 nM nrp_L10-R (5'-CGGGCAGCTTCAAGGGCTTCA-3'). Primers used for *Symbiodinium* symbionts were 300 nM Aact_F (5'-ATGAAGTGCGACGTGGACAT-3') and 200nM Aact_R (5'-GGAGGACAGGATGGAGCCT-3'). All qPCR assays were performed on the Agilent AriaMx qPCR Machine (Agilent, USA). Each reaction totaled 20 µL volumes, using 10 µL Brilliant III Ultra-fast SYBR qPCR Master Mix (Agilent, USA), 6.1 µL Milli-Q water, 0.8 µL per primer (forward and reverse, final concentrations listed above), 0.3 µL 1:500 reference dye (SYBR) and 2 µL of template DNA (concentration range between 5-10 ng/ µL). The thermal profile was 50 °C 2:00, 95 °C 10:00 (95 °C 0:10 | 60 °C 1:00 | 72°C 0.20) x 40 cycles + melt curve profile of (95 °C 0:30 | 65 °C 0:30 | 95 °C 0:30) x 1 cycle. Due to low remaining DNA after 16S rRNA library preparation and sequencing, we used 15 aposymbiotic replicates and 21 symbiotic replicates (Table A3) for the qPCR assays. Each sample was assayed in duplicate, per target primer set. Cycle threshold (Ct) values were calculated by Agilent AriaMx qPCR machine when the first amplification cycle in a reaction exceeded the fluorescent baseline. All aposymbiotic anemones in the control (but not those in the thermally stressed samples) exhibited non-target amplification for the actin primer set, and upon analysis of the melt curve, we noticed a distinct peak between 83-84 °C for these samples, yet all other positive amplification reactions exhibited

a distinct peak between 85-87 °C. We surmised cross-amplification of an Aiptasia locus in the absence of a *Symbiodinium* target. Sanger sequencing of the different products revealed that actin samples with a melt product between 85-86 °C exhibited high quality matches to the actin gene locus in *Symbiodinium* spp. (GenBank: AB231899.1, NCBI nr BLAST, megablast, e-value= 8e-86, bit score= 329) whereas no matches were identified for the lower melt product samples, likely due to a high abundance of Ns in the sequences. Therefore, actin reactions exhibiting melt products < 84 °C were assigned a cycle number of 40 to account for this non-specific amplification (Table A4). Ct values were averaged between technical replicates and symbiont to host ratios were calculated using the formula ((2^ (Ct host)/(Ct sym))∗2) based on host/symbiont target ploidy (Aiptasia, host = 2, *Symbiodinium*, symbiont = 1) (Cunning and Baker, 2013*;* Palacio-Castro 2019).

2.6 S/H ratio statistical analyses

A dataset containing only symbiotic Aiptasia with S/H ratios, their alpha-diversity scores (Shannon, Chao, Observed, Fisher and Simpson) and select bacterial abundance counts (Oligoflexales and Staphylococcus) was used to generate a Pearson's correlation matrix using the corrplot package in R (Wei and Simko, 2021). The lm command was used for regression analysis of Oligoflexales abundance counts on S/H ratio in symbiotic anemones. We implemented a linear mixed effects model with a fixed effect of treatment and a random effect of tank to test whether S/H ratios were reduced in symbiotic anemones response to heat treatment using the nlme (Pinheiro and Bates, 2023; Bates and Pinheiro, 1998), and lme4 (Bates *et al.*, 2015) packages.

RESULTS

3.1 Microbial assemblages in Aiptasia

After ASV calling with DADA2, the sequence table yielded 4, 996, 356 reads and after chloroplast and mitochondria removal, 4, 984, 116 reads and 4, 471 ASVs remained (Table A1). Rarefaction and filtering of taxa occurring at least 3 times in more than 4 samples (the minimum number of replicates per tank) yielded a final dataset of 1, 338, 082 reads and 774 ASVs. The dataset was dominated by Alphaproteobacteria (66 %), Gammaproteobacteria (19%), and

309 Bdellovibrionota (4%) (Fig. A1). All other taxonomic classes were present in abundances < 3%,
310 except for Oligoflexia, which occurred at 4% relative abundance in symbiotic anemones under
311 control conditions (Fig. A1). The predominant genera in all samples were *Cognatishimia*, an
312 unnamed bacterium from the family Rhodobacteraceae and *Alcanivorax* (Fig. A2).

313 3.2 Community dynamics differ by treatment and symbiotic state

314 Alpha diversity was assessed by estimating species richness using the Chao1 index. Lower
315 within-sample diversity was observed in symbiotic anemones exposed to heat, but within-sample
316 diversity was consistent between other treatment groups (Fig. 2a). Although alpha diversity
317 differed between groups on average (ANOVA, p=0.009, Table A5), significant differences were
318 detected for only one pairwise comparison: heat-stressed symbiotic anemones and control
319 symbiotic anemones (Tukey multiple comparison of means, p=0.016, Table A5). A marginal
320 pairwise difference was detected between symbiotic and aposymbiotic anemones under heat
321 stress (Tukey multiple comparison of means, p=0.05, Table A5).

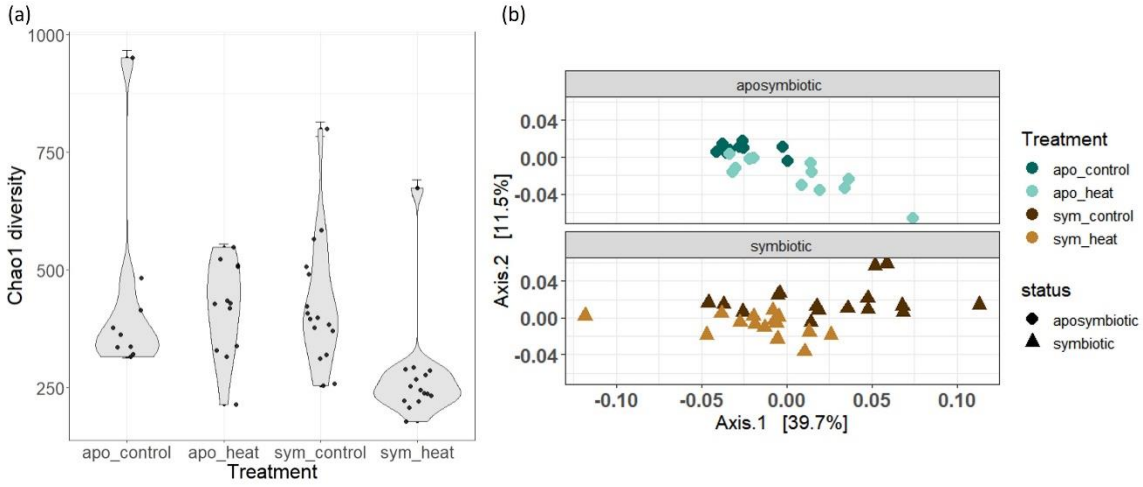

Figure 2. Alpha diversity and beta diversity differences of microbial assemblages in Aiptasia. (a) Alpha diversity index, Chao1, by treatment group. Standard error for Chao1 is represented by the error bars. (b) Beta differences by weighted Unifrac, principal coordinates of analysis (PCoA) on aposymbiotic samples (control, dark blue circles vs heat stressed, light blue circles) and symbiotic (control, dark brown triangles vs heat stressed, light brown triangles).

322

323 A principal coordinate analysis (PCoA, weighted Unifrac) also revealed differences in
324 beta diversity between symbiotic and aposymbiotic pairs in response to treatment (Fig. 2b).
325 Namely, beta diversity in heat-stressed symbiotic anemones converged whereas beta diversity in
326 control symbiotic anemones did not (PERMANOVA, p= 0.001, Table A6), indicating microbial
327 community composition in symbiotic anemones became more similar to each other after heat

328 treatment. The opposite pattern was observed in aposymbiotic anemones: convergence was
329 observed in the control group and divergence in the heat treatment (Fig. 2b, PERMANOVA,
330 p=0.001, Table A6). Differences in beta diversity were also observed between symbiotic and
331 aposymbiotic animals under control conditions (ANOSIM R= 0.28, p = 0.003, Table A6).

332 We further explored taxa responsible for beta diversity disparities by generating a
333 differential heat tree and visualizing statistically dissimilar taxa between pairwise comparisons.
334 We detected differential abundance of several taxa (Wilcoxon Rank Sum tests, FDR-adjusted p-
335 values < 0.05. Statistical output: Zenodo, doi.org/10.5281/zenodo.7693398) but most noticeably
336 Firmicutes, Oligoflexales, Oceanospirillales, Planctomycetes and Alteromonadales (Fig. 3).
337 Firmicute abundances were higher in aposymbiotic and symbiotic controls (Fig 3a, 3d), and
338 Oligoflexales abundances were highest in symbiotic anemones under control conditions (Fig.
339 3b). Bacteria from the family *Methylophilaceae* exhibited a similar pattern as Oligoflexales but
340 overall abundance was low, and the difference between aposymbiotic and symbiotic controls was
341 only ~ 100 raw counts. In contrast, Oligoflexales abundances in the aposymbiotic and symbiotic

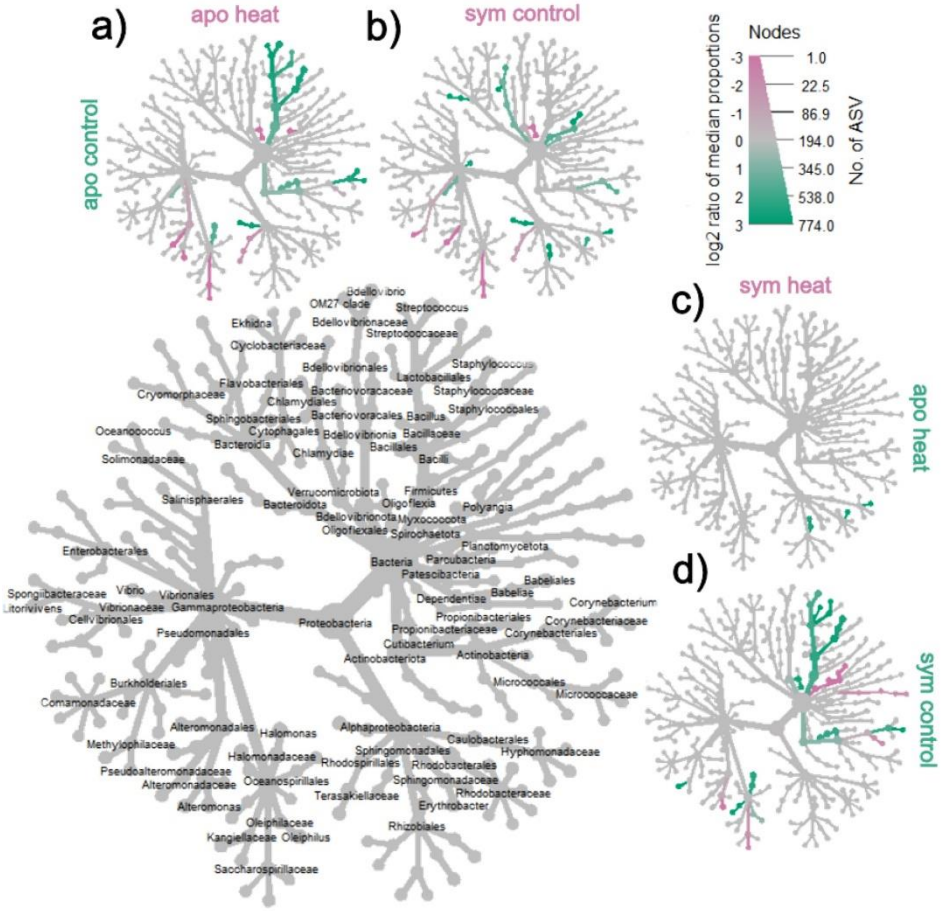

Figure 3. Differential heat trees illustrating pairwise comparisons between groups of interest. Amplicon data was used to visualize microbial taxonomic composition in Aiptasia, using the R package, Metacoder (Foster et al. 2017). The bigger tree with taxon labels on the lower left serves as a key for the smaller pairwise-comparison trees surrounding it a) aposymbiotic control vs aposymbiotic heat, b) aposymbiotic control vs symbiotic control, c) symbiotic heat-stressed vs aposymbiotic heat-stressed and d) symbiotic heat-stressed vs symbiotic control. Taxon color (diverging scheme from pink to green) is represented by log-2 ratio of median proportions of reads observed by treatment group. Significantly differentially abundant taxa, determined by Wilcoxon rank sum tests followed by FDR correction, colored in pink are more prominent in the groups shown in the columns and those colored in green are more prominent in the groups shown on the rows, e.g., Oligoflexales are significantly more abundant in symbiotic control (green) anemones than symbiotic heat-stressed but Myxococcota are enriched in symbiotic heat-stressed (pink). Size of tree nodes corresponds to ASV richness, as denoted in the color and size key in the upper right. Statistical output of differential abundance analysis is archived at Zenodo, doi.org/10.5281/zenodo.7693398

controls differed by 1-3 orders of magnitude (Fig. 4b). Alteromonodales and Oceanospirillales abundance both decreased in heat-stressed anemones, regardless of symbiotic state (Fig. 3c).

3.3 Oligoflexales order are are associated with symbiotic state and are lost under thermal stress

Oligoflexales abundances were elevated in symbiotic anemones under control conditions but marginally elevated under heat treatment in aposymbiotic anemones relative to aposymbiotic controls (Fig. 3a, 3b). Total abundance counts clearly displayed this categorical difference as well (Fig. 4b). In addition to these categorical differences in Oligoflexales abundance by anemone symbiotic state and treatment (Fig. A1, 3, 4b), we also observed quantitative differences in abundance as a function of algal symbiont density. Symbiont to host (S/H) cell ratios of symbiotic anemones decreased under thermal stress indicating mild bleaching ($p=0.047$, Fig. A3, Table A7). Total S/H ratios for aposymbiotic anemones averaged around 0 but values slightly increased in anemones exposed to thermal stress (Fig. A3, Table A4).

A correlation matrix was built to examine pairwise relationships between the S/H ratio of symbiotic Aiptasia, alpha diversity, and Oligoflexales abundance counts. A moderate correlation between S/H ratio and the abundance of Oligoflexales bacteria was detected (Pearson's Correlation, $p=0.013$, Fig. A4, Table A8). Regression analysis verified this positive correlation, revealing that 53% of the observed variation in Oligoflexales abundance across samples was explained by differences in S/H ratio ($p < 0.001$, Fig. 4a).

3.4 Oligoflexales may diversify under thermal stress

A phylogenetic tree plotting Oligoflexales ASV abundance showed symbiotic anemones in control conditions initially hosted 9 distinct ASVs but lost 1 after thermal stress (Fig. A5). In contrast, aposymbiotic anemones only harbored 2 ASVs under control conditions, whereas 8 Oligoflexales ASVs were detected in aposymbiotic anemones which experienced thermal stress (Fig. A5). We conducted a more detailed phylogenetic analysis to further investigate relationships among these distinct ASVs and other Oligoflexales variants identified in prior studies. Oligoflexales ASVs from this study grouped closely with three uncultured bacterium clones originating from an unrelated study on Aiptasia clonal strains CC7 and H2 (Randle *et al.*, 2020) and one uncultured proteobacterium clone (GenBank: FJ425635) found in the microbiome

of the scleractinian coral *Orbicella* (formerly *Montastrea*) *faveolata* (Fig. A6). Other Oligoflexales representatives were more distantly related (Fig. A6).

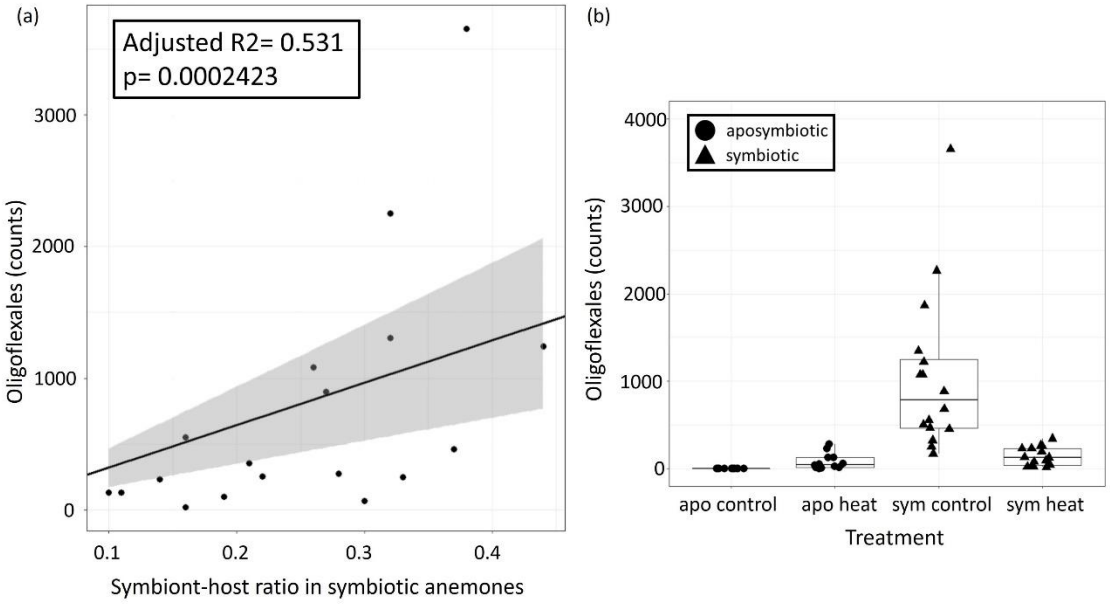

Figure 4. Oligoflexales in the microbiome of Aiptasia. (a) Regression line through the origin and linear model results testing the relationship between the dependent variable, Oligoflexales bacterial counts (y axis) and explanatory variable, symbiont to host ratio (S/H) (x axis). (b) Total counts of Oligoflexales in rarefied data, by treatment group (black circles= aposymbiotic anemones, black triangles= symbiotic anemones).

DISCUSSION

Overall, understanding the ecological dynamics of algal-microbe interactions in cnidarians may be important for developing strategies to mitigate the impacts of climate change (Matthews *et al.*, 2020). (Frommlet *et al.*, 2015) found that a diverse community of bacteria facilitated the formation of symbiolites (spheroid, aragonite structures) in ex-hospite Symbiodiniaceae cultures. This discovery highlighted a unique approach that could potentially aid in coral calcification within reefs. Similarly, many eukaryotic algae are auxotrophs for the prokaryote-produced B vitamins, like B12, and must obtain B12 from symbionts or dietary sources for proper metabolic function and growth (Croft, Warren and Smith, 2006; Helliwell *et al.*, 2011; Grossman, 2016). Furthermore, microbiome and algal symbiont co-occurrences may be a result of metabolic cooperation, as dinoflagellates may rely on necessary metabolites produced by bacteria and vice-versa (Cruz-López and Maske 2016; Grossman 2016; Kurihara et al. 2013). Additional possible functional roles of bacteria associated with Symbiodiniaceae may also span DMSP production, enhancing iron bioavailability and sulfur cycling (Lawson et al.

2018).

Here, we investigated the ecology of microbial communities, associated with Aiptasia, and how they are affected by presence of the algal symbiont and elevated temperatures. Despite treatment, Alphaproteobacteria and Gammaproteobacteria remained the predominant microbial taxa. Differences in beta diversity were observed between symbiotic and aposymbiotic animals under both control and thermal stress conditions but greater community similarity was observed among microbial populations in both symbiotic and aposymbiotic anemones exposed to heat stress. Additionally, elevated temperature decreased species richness in symbiotic anemones.We also show that Oligoflexales bacteria are part of the rare microbiome in symbiotic anemones but significantly decreased in abundance following thermal stress. The abundance of Oligoflexales was positively correlated with higher S/H cell ratio indicating symbiont density, rather than heat stress per se, impacted their abundance in Aiptasia.

Exploring the cnidarian-algal-bacteria tripartite symbiosis, Oligoflexales as primary associates of Symbioidiniaceae

We examined the role of symbiotic algae in recruiting microbial taxa that may be specific to the microbiomes of symbiotic Aiptasia and identified two taxa (Oligoflexales and *Methylophilaceae*) that were significantly abundant in symbiotic Aiptasia only and decreased in relative abundance with symbiont loss both independent of and as a result of thermal stress (Fig. 3b,3d; 4). We focused on exploring Oligoflexales because these taxa were present in higher relative abundances and had been previously reported as associates of Aiptasia (Randle *et al.*, 2020; Maire, Blackall and van Oppen, 2021). Whereas we could not find a consistent record of *Methylophilaceae* in symbiotic Aiptasia. Despite numerous surveys conducted on the Aiptasia microbiome (Röthig *et al.*, 2016; Herrera *et al.*, 2017; Ahmed *et al.*, 2019; Hartman, van Oppen and Blackall, 2020; Costa *et al.*, 2021), Oligoflexales remained undetected until recent research reported their presence in Aiptasia strain CC7 (Randle *et al.*, 2020) and in the microbiome of Aiptasia acontia in strains AIMS1, AIMS2, AIMS3, and AIMS4 (Maire, Blackall and van Oppen, 2021). Two factors likely account for these results: (1) the utilization of identical sequencing primer sets across all three studies, which document the existence of Oligoflexales,

418 including our own, and (2) the classification of taxonomy based on the most recent release of the
419 SILVA database (v138, issued in 2019). We chose to use the 784F/1061R primer set because it
420 captures global bacterial diversity while exhibiting low amplification of chloroplast and
421 mitochondrial host DNA (Bayer *et al.*, 2013; Andersson *et al.*, 2008; Bayer *et al.*, 2013) (Bayer
422 *et al.*, 2013). Furthermore, as Oligoflexales were recently recognized as a novel order under the
423 Bdellovibrionota phylum (Nakai *et al.*, 2014; Waite *et al.*, 2020)), older databases may designate
424 them as "unclassified/uncultured bacterial clones". Based on our research and the prior studies,
425 we conclude that Oligoflexales are a consistent component of the symbiotic microbiome in
426 Aiptasia.
427
428 Bacteria belonging to the Oligoflexales order are Gram-negative, oligotrophic
429 spirochaetes, and only one species, *Oligoflexus tunisiensis*, has been described and isolated
430 (Nakai *et al.*, 2014, 2016). We conducted a phylogenetic analysis of nine Oligoflexales ASV
431 sequences against *O. tunisiensis* and other members of the parent phylum, Bdellovibrionota
432 (Waite *et al.*, 2020). Our ASVs formed a sister clade to *O. tunisiensis* and *Pseudobacteriovorax*
433 *antillogorgiicola*, an isolate from gorgonian corals in the family Pseudobacteriovoracacea
434 (McCauley, Haltli and Kerr, 2015) (Fig. A6). This suggests a close relationship between them.
435 Currently, Pseudobacteriovoracacea are classified as Bdellovibrionales, but a proposal to
436 reclassify them as Oligoflexales was submitted (Hahn *et al.*, 2017), which is consistent with our
437 findings grouping them with *O. tunisiensis*.
438
439 Although the ecological role of Oligoflexales in symbiotic Aiptasia remains a mystery, it
440 has previously been suggested to comprise a set of taxa that aid thermotolerance in high salinities
441 (Randle *et al.*, 2020). The genome sequence of *O. tunisiensis* also provides clues on possible
442 metabolic capabilities in Oligoflexales. Nakai et al. (2016) observed an incomplete
443 denitrification pathway in *O. tunisiensis*, which resulted in the conversion of nitrate/nitrite
444 (NO_3/NO_2) to nitrous oxide (N_2O). Heterotrophic bacteria are known to recycle fixed nitrogen
445 from the environment through denitrification, and those with a complete pathway can reduce
446 fixed nitrogen to dinitrogen (N_2) gas (Knowles, 1982). Nitrogen recycling by the host, Aiptasia,
447 regulates algal symbiotic density (Cui *et al.*, 2019) but bacterially regulated nitrogen may play a
448 role in maintenance of the cnidarian-algal symbiosis as well, since nitrogen cycling is a hallmark

of reciprocal bacterial association in cnidarian holobionts (Knowlton and Rohwer, 2003; Peixoto *et al.*, 2017). It is unclear if the Oligoflexiales in the present study possess a similar denitrification pathway, but the genomic evidence from *O. tunisiensis* suggests that further research is needed to investigate this possibility.

We observed a higher abundance of Oligoflexales ASVs in symbiotic Aiptasia under control conditions, compared to symbiotic animals in the heat-stress treatment. In contrast, aposymbiotic Aiptasia in the control showed only 1 ASV but experienced an increase in diversity and abundance of ASVs in thermally stressed, aposymbiotic anemones (Fig. A5). While superficially this observation initially appears to contradict the notion that Oligoflexiales are associates of Symbiodiniaceae, we believe this pattern can be explained by the dynamics of symbiont maintenance and proliferation in Aiptasia (Jinkerson *et al.*, 2022) and is actually fully consistent with the primary algal symbiont hypothesis.

Aposymbiotic Aiptasia anemones can retain remnant populations of algal symbionts, even in the absence of light. *S. linucheae* do not need to photosynthesize to be maintained in Aiptasia in dark conditions (Jinkerson *et al.*, 2022). Jinkerson et al (2022) also found that *S. linuchae* did not proliferate *in-hospite* in the dark, but algal cell density significantly increased after subsequent transition to light conditions. We theorize that a small, non-detectable (by PCR) population of algal symbionts remained in the aposymbiotic group, despite continuous darkness for three months. This *S. linucheae* population likely proliferated during the combined light and heat-stress periods as confirmed by the slight increase in S/H ratios in this population (Fig. A3, Table A4), in contrast to the aposymbiotic controls only exposed to light. A recent study showed significantly higher proliferation of *S. linucheae* in Aiptasia at 32 °C, after 28 days compared to ambient temperatures (25 °C), suggesting cell division rates initially increased in response to elevated temperature but then declined after 12 weeks of sustained thermal stress (Herrera *et al.*, 2021). The slight increase in algal abundance in the aposymbiotic population subjected to thermal stress was concomitant with an increase and diversification of Oligoflexales (Fig 4b, Fig A5). Whereas the opposite pattern (Oligoflexales loss) was observed in symbiotic Aiptasia exposed to heat stress. S/H ratios were lower (Table A4, Fig. A3) in symbiotic anemones exposed to thermal stress, and significant differences were observed between treatments (Table

A7), indicating symbiont loss. Thermal stress can promote bleaching in symbiotic cnidarians leading to dysbiosis (Weis, 2008; Wooldridge, 2009)) but the mechanisms are not thoroughly established. One theory suggests dysbiosis may occur as a cascade effect that begins with a decrease in the photosynthetic efficiency of the symbiont, followed by a reduction in ammonium assimilation by the host, leading to an increase in the available ammonium pool. This, in turn, stimulates algal growth, and eventually, the host becomes unable to keep up with the resulting internal metabolic shifts, leading to the expulsion of the symbionts (Cui *et al.*, 2019; Rädecker *et al.*, 2021). We hypothesize that heat-stressed, aposymbiotic Aiptasia did not experience the same metabolic constraints and were able to adequately support growth of algal populations, leading to slightly higher symbiont abundance and increased abundance/diversity of Oligoflexales bacteria (Fig. 4b, Fig. A5).

Lastly, it is a possibility that Oligoflexales observed in aposymbiotic anemones under heat conditions were environmentally acquired. We used 0.2 µm FSW to maintain our cultures but previous research has demonstrated that *O. tunisiensis* is part of the 0.2 µm filtrate culturable fraction (Nakai *et al.*, 2014). Although *O. tunisiensis* can reach up to 10 µm in length, and are between 0.4 - 0.8 µm wide, they can compress through 0.2 µm filter pores. We do not have morphological data for the Oligoflexales in this study, so we cannot eliminate the possibility of environmental contamination. However, all animals were clonal propagates and were maintained using 0.2 µm FSW, originating from the same 20 L tank, yet displayed distinct microbiome profiles according to treatment (Fig 3). Furthermore, the disparity of Oligoflexales abundances between treatment groups (Fig. 4b) indicates symbiotic-state specificity in control conditions. Environmentally acquired or not, Oligoflexales may play an important role in the holobiont as endosymbiotic partners that only associate with cnidarians when microalgal symbionts are present, as rare members of the symbiotic microbiome. It is possible that Oligoflexales, like rare members of the coral core microbiome, are widespread in anemone tissue, particularly in close proximity to algal symbionts (D Ainsworth *et al.*, 2015) and future work should aim to fully characterize their abundance and distribution.

Microbiome assemblages of aposymbiotic and symbiotic Aiptasia

Microbial assemblages in Aiptasia from wild and cultured populations (including clonal strain CC7) in ambient conditions showed consistent similarity at the phylum level but not at lower taxonomic levels (Brown *et al.*, 2017). In all our samples, regardless of treatment, *Alcanivorax* sp. from the Oceanospirillales order, *Cognatishimia* sp., and an unclassified bacterium from the Rhodobacteraceae family from the Rhodobacterales order accounted for around 60-85% of the total relative abundance (Fig. A2). This observation, that 2-3 taxa are numerically dominant in Aiptasia, contradicts other microbial surveys on Aiptasia that identified a wider range of taxonomic groups within the same relative abundance range (Röthig *et al.*, 2016; Randle *et al.*, 2020; Curtis *et al.*, 2023). The importance of functional redundancy in shaping microbial communities in Aiptasia is emphasized by the conflicting results obtained from various studies. Functional redundancy refers to a diverse range of bacteria with similar capabilities, able to perform similar functions in the same niche (Louca *et al.*, 2018). Functional redundancy is an advantageous strategy for ecosystem stability and may play a role in the resilience of hosts, like corals, facing environmental disturbances (Voolstra and Ziegler, 2020).

Prior work conducted by (Röthig *et al.*, 2016; Ahmed *et al.*, 2019; Randle *et al.*, 2020) showed that aposymbiotic and symbiotic Aiptasia (CC7), hosted distinct microbiomes. Here we expanded upon this finding by introducing a stressor to assess the response of the aposymbiotic microbiome and the symbiotic microbiome under thermal stress (Fig. A1). Although alpha-diversity did not differ between symbiotic status (Fig. 2a), beta-diversity was dissimilar (Fig. 2b, Table A6), which prompted us to explore which taxa were responsible for these observations. We observed a significant difference in the relative abundance of Oligoflexales, Saccharospirillaceae, Pseudoalteromonadaceae, and Methylophilaceae families between symbiotic and aposymbiotic animals, with these taxa being more prevalent in the former (Fig. 3b). Our findings align with other studies that have demonstrated differences in relative abundance of microbiome composition between aposymbiotic and symbiotic states in strain CC7 (Röthig *et al.*, 2016; Sydnor, 2020; Curtis *et al.*, 2023). Nevertheless, the four differentially abundant taxa we identified were not previously reported, suggesting genotype by environment and symbiotic state all influence microbial assemblages in Aiptasia.

Microbiome fluctuations caused by heat stress changed the rare microbiome

Here, we aimed to identify microbial taxa that are linked with symbiotic states in a defined clonal strain of Aiptasia and tracked microbial fluctuations of these taxa following mild bleaching. The dominant taxa in all Aiptasia were found to be unaffected by both temperature and symbiotic state. However, the rare microbiome of symbiotic Aiptasia was significantly affected by temperature stress. In contrast to the other treatment groups, symbiotic Aiptasia exhibited a reduction in alpha-diversity and beta-diversity convergence after six days of exposure to heat stress (Fig. 2). A decrease in alpha-diversity was also observed in another study exposing symbiotic Aiptasia CC7 to short-term heat stress (Sydnor, 2020), but it appears to be a temporary phenomenon since a long-term study by (Ahmed *et al.*, 2019)) on Aiptasia CC7, surveying microbial communities under continuous heat stress (32°C for two years) demonstrated an increase in both alpha-diversity and number of bacterial taxa relative to paired controls. Here, symbiotic Aiptasia experienced microbiome restructuring when subjected to heat stress (Fig. 3d) and the resulting assemblage was most similar to that observed in heat-stressed aposymbiotic anemones (Fig. 3c). This suggests that short-term heat stress in Aiptasia may be the main driver that led to a convergence of microbial communities in both aposymbiotic and symbiotic animals.

However, possible symbiont gains in the aposymbiotic and symbiont loss in the symbiotic anemones exposed to thermal stress (Fig. A3) may also be contributing to the convergence of microbial communities. Symbiotic anemones exhibited increased relative abundance of several bacteria taxa, most noticeably Oligoflexales, Saccharospirillaceae, and Pseudoalteromonadaceae compared to aposymbiotic anemones under control temperatures (Fig. 3b). These same taxa increase in relative abundance in heat-stressed aposymbiotic anemones compared to control aposymbiotic anemones (Fig. 3a) until their relative abundance is indistinguishable from those observed in the symbiotic anemones in response to thermal stress (Fig. 3c). Whereas their abundances decrease in response to heat stress in symbiotic anemones (Fig. 3d). The most parsimonious explanation for these apparently contradictory changes in response to heat stress is that it is not thermal stress, but algal symbiont density which influences patterns of convergence. Additional time-course studies examining repopulation of algal symbiont communities would provide additional support for this hypothesis.

It is unclear whether changes in the relative abundance or composition of the microbial community affected the physiology or fitness of aposymbiotic and symbiotic Aiptasia hosts as we did not conduct any host-specific assays, but we can confirm that no Aiptasia died during the course of this experiment. While we have identified a strong association between the abundance of Oligoflexales and algal endosymbionts independent of thermal stress, whether these bacteria are mutualists or commensals remains unresolved. Additional work on patterns of localization, metabolic exchange, and spatial and temporal fidelity are needed. But given the tractability of the Aiptasia model, this represents a promising future study system for investigating multi-partner symbioses. Understanding how the absence of a member in a multipartite symbiosis impacts the resilience of other organisms in the holobiont can uncover valuable insights into how symbiotic organisms respond to environmental challenges.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

EGA and CDK conceived and designed the field experiment and obtained funding. EGA and MJF performed anemone maintenance, aposymbiotic generation and sampling for the experiment. EGA completed DNA extractions, library preparation, qPCR and all bioinformatic and statistical analyses and wrote the first draft of the manuscript. All authors contributed to

600 revisions.

601 DATA ACCESSIBILITY

602 Scripts for data analysis and statistical output for differential heat trees generated using the
603 Metacoder R package are archived at Zenodo, doi.org/10.5281/zenodo.7693398. It can also be
604 viewed at https://github.com/symbiotic-em/aiptasia_thermal_stress. Demultiplexed sequences
605 are available at the National Center for Biotechnology Information (NCBI) Sequence Read
606 Archives (SRA) under accession code: PRJNA929535.

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610 REFERENCES

611 Adeniji, A.A., Babalola, O.O. and Loots, D.T. (2020) 'Metabolomic applications for understanding complex
612 tripartite plant-microbes' interactions: Strategies and perspectives', *Biotechnology reports (Amsterdam,*
613 *Netherlands)*, 25, p. e00425. Available at: https://doi.org/10.1016/j.btre.2020.e00425.

614 Afkhami, M.E. *et al.* (2020) 'Tripartite mutualisms as models for understanding plant-microbial interactions',
615 *Current opinion in plant biology*, 56, pp. 28–36. Available at: https://doi.org/10.1016/j.pbi.2020.02.003.

616 Ahmed, H.I. *et al.* (2019) 'Long-Term Temperature Stress in the Coral Model Aiptasia Supports the "Anna
617 Karenina Principle" for Bacterial Microbiomes', *Frontiers in Microbiology*. Available at:
618 https://doi.org/10.3389/fmicb.2019.00975.

619 Allemand, D. and Osborn, D. (2019) 'Ocean acidification impacts on coral reefs: From sciences to solutions',
620 *Regional Studies in Marine Science*, p. 100558. Available at: https://doi.org/10.1016/j.rsma.2019.100558.

621 Andersson, A.F. *et al.* (2008) 'Comparative analysis of human gut microbiota by barcoded pyrosequencing', *PloS*
622 *one*, 3(7), p. e2836. Available at: https://doi.org/10.1371/journal.pone.0002836.

623 Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available at:
624 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

625 Anon, 2020. Anaconda Software Distribution, Anaconda Inc. Available at: https://docs.anaconda.com/.

626 Antunes, P.M. and Goss, M.J. (2015) 'Communication in the Tripartite Symbiosis Formed by Arbuscular
627 Mycorrhizal Fungi, Rhizobia and Legume Plants: A Review', *Roots and Soil Management: Interactions between*
628 *Roots and the Soil*, pp. 199–222. Available at: https://doi.org/10.2134/agronmonogr48.c11.

629 Barno, A.R. *et al.* (2021) 'Host under epigenetic control: A novel perspective on the interaction between
630 microorganisms and corals', *BioEssays: news and reviews in molecular, cellular and developmental biology*, 43(10),
631 p. e2100068. Available at: https://doi.org/10.1002/bies.202100068.

632 Bates, D. *et al.* (2015) 'Fitting Linear Mixed-Effects Models Using**lme4**', *Journal of Statistical Software*. Available

633 at: https://doi.org/10.18637/jss.v067.i01.

634 Bates, D.M. and Pinheiro, J.C. (1998) 'LINEAR AND NONLINEAR MIXED-EFFECTS MODELS', *Conference*
635 *on Applied Statistics in Agriculture* [Preprint]. Available at: https://doi.org/10.4148/2475-7772.1273.

636 Baumgarten, S. *et al.* (2015) 'The genome of Aiptasia, a sea anemone model for coral symbiosis', *Proceedings of*
637 *the National Academy of Sciences of the United States of America*, 112(38), pp. 11893–11898. Available at:
638 https://doi.org/10.1073/pnas.1513318112.

639 Bayer, T. *et al.* (2013) 'The microbiome of the Red Sea coral Stylophora pistillata is dominated by tissue-associated
640 Endozoicomonas bacteria', *Applied and environmental microbiology*, 79(15), pp. 4759–4762. Available at:
641 https://doi.org/10.1128/AEM.00695-13.

642 Bernasconi, R. *et al.* (2019) 'Global Networks of Symbiodinium-Bacteria Within the Coral Holobiont', *Microbial*
643 *ecology*, 77(3), pp. 794–807. Available at: https://doi.org/10.1007/s00248-018-1255-4.

644 Blackall, L.L., Wilson, B. and van Oppen, M.J.H. (2015) 'Coral-the world's most diverse symbiotic ecosystem',
645 *Molecular ecology*, 24(21), pp. 5330–5347. Available at: https://doi.org/10.1111/mec.13400.

646 Boucher, D.H. (1985) *The Biology of Mutualism: Ecology and Evolution*. Oxford University Press on Demand.
647 Available at: https://books.google.com/books/about/The_Biology_of_Mutualism.html?hl=&id=dWuQS_3F2P0C.

648 Bourne, D.G., Morrow, K.M. and Webster, N.S. (2016) 'Insights into the Coral Microbiome: Underpinning the
649 Health and Resilience of Reef Ecosystems', *Annual review of microbiology*, 70, pp. 317–340. Available at:
650 https://doi.org/10.1146/annurev-micro-102215-095440.

651 Brown, T. *et al.* (2017) 'Worldwide exploration of the microbiome harbored by the cnidarian model, Exaiptasia
652 pallida (Agassiz in Verrill, 1864) indicates a lack of bacterial association specificity at a lower taxonomic rank',
653 *PeerJ*, 5, p. e3235. Available at: https://doi.org/10.7717/peerj.3235.

654 Callahan, B.J. *et al.* (2016) 'DADA2: High-resolution sample inference from Illumina amplicon data', *Nature*
655 *methods*, 13(7), pp. 581–583. Available at: https://doi.org/10.1038/nmeth.3869.

656 Camp, E.F. *et al.* (2022) 'Proteome metabolome and transcriptome data for three Symbiodiniaceae under ambient
657 and heat stress conditions', *Scientific data*, 9(1), p. 153. Available at: https://doi.org/10.1038/s41597-022-01258-w.

658 Cantin, N.E. *et al.* (2009) 'Juvenile corals can acquire more carbon from high-performance algal symbionts', *Coral*
659 *reefs*, 28(2), pp. 405–414. Available at: https://doi.org/10.1007/s00338-009-0478-8.

660 Chaston, J. and Goodrich-Blair, H. (2010) 'Common trends in mutualism revealed by model associations between
661 invertebrates and bacteria', *FEMS microbiology reviews*, 34(1), pp. 41–58. Available at:
662 https://doi.org/10.1111/j.1574-6976.2009.00193.x.

663 Cleveland, A., Alan Verde, E. and Lee, R.W. (2011) 'Nutritional exchange in a tropical tripartite symbiosis: direct
664 evidence for the transfer of nutrients from anemonefish to host anemone and zooxanthellae', *Marine Biology*, pp.
665 589–602. Available at: https://doi.org/10.1007/s00227-010-1583-5.

666 Costa, R.M. *et al.* (2021) 'Surface Topography, Bacterial Carrying Capacity, and the Prospect of Microbiome
667 Manipulation in the Sea Anemone Coral Model Aiptasia', *Frontiers in microbiology*, 12, p. 637834. Available at:
668 https://doi.org/10.3389/fmicb.2021.637834.

669 Croft, M.T., Warren, M.J. and Smith, A.G. (2006) 'Algae need their vitamins', *Eukaryotic cell*, 5(8), pp. 1175–1183.
670 Available at: https://doi.org/10.1128/EC.00097-06.

671 Cui, G. *et al.* (2019) 'Host-dependent nitrogen recycling as a mechanism of symbiont control in Aiptasia', *PLoS*
672 *genetics*, 15(6), p. e1008189. Available at: https://doi.org/10.1371/journal.pgen.1008189.

673 Cunning, R. and Baker, A.C. (2013) 'Excess algal symbionts increase the susceptibility of reef corals to bleaching',
674 *Nature Climate Change*, pp. 259–262. Available at: https://doi.org/10.1038/nclimate1711.

675 Curtis, E. *et al.* (2023) 'Bacterial microbiome variation across symbiotic states and clonal lines in a cnidarian
676 model', *Frontiers in Marine Science*. Available at: https://doi.org/10.3389/fmars.2023.1113043.

677 D Ainsworth, T. *et al.* (2015) 'The coral core microbiome identifies rare bacterial taxa as ubiquitous
678 endosymbionts', *The ISME journal*, 9(10), pp. 2261–2274. Available at: https://doi.org/10.1038/ismej.2015.39.

679 Dixon, G.B. *et al.* (2015) 'CORAL REEFS. Genomic determinants of coral heat tolerance across latitudes', *Science*,
680 348(6242), pp. 1460–1462. Available at: https://doi.org/10.1126/science.1261224.

681 Dixon, P. (2003) 'VEGAN, a package of R functions for community ecology', *Journal of Vegetation Science*, pp.
682 927–930. Available at: https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.

683 Douglas, A.E. (2003) 'Coral bleaching--how and why?', *Marine pollution bulletin*, 46(4), pp. 385–392. Available at:
684 https://doi.org/10.1016/S0025-326X(03)00037-7.

685 Drury, C. (2020) 'Resilience in reef-building corals: The ecological and evolutionary importance of the host
686 response to thermal stress', *Molecular ecology*, 29(3), pp. 448–465. Available at:
687 https://doi.org/10.1111/mec.15337.

688 Edgar, R.C. (2004) 'MUSCLE: a multiple sequence alignment method with reduced time and space complexity',
689 *BMC bioinformatics*, 5, p. 113. Available at: https://doi.org/10.1186/1471-2105-5-113.

690 Ffrench-Constant, R.H., Eleftherianos, I. and Reynolds, S.E. (2007) 'A nematode symbiont sheds light on
691 invertebrate immunity', *Trends in parasitology*, 23(11), pp. 514–517. Available at:
692 https://doi.org/10.1016/j.pt.2007.08.021.

693 Foster, Z.S.L., Sharpton, T.J. and Grünwald, N.J. (2017) 'Metacoder: An R package for visualization and
694 manipulation of community taxonomic diversity data', *PLOS Computational Biology*, p. e1005404. Available at:
695 https://doi.org/10.1371/journal.pcbi.1005404.

696 Frommlet, J.C. *et al.* (2015) 'Coral symbiotic algae calcify ex hospite in partnership with bacteria', *Proceedings of*
697 *the National Academy of Sciences of the United States of America*, 112(19), pp. 6158–6163. Available at:
698 https://doi.org/10.1073/pnas.1420991112.

699 Gloor, G.B. *et al.* (2017) 'Microbiome Datasets Are Compositional: And This Is Not Optional', *Frontiers in*
700 *microbiology*, 8, p. 2224. Available at: https://doi.org/10.3389/fmicb.2017.02224.

701 Grossman, A. (2016) 'Nutrient Acquisition: The Generation of Bioactive Vitamin B 12 by Microalgae', *Current*
702 *Biology*, pp. R319–R321. Available at: https://doi.org/10.1016/j.cub.2016.02.047.

703 Grüning, B. *et al.* (2018) 'Bioconda: sustainable and comprehensive software distribution for the life sciences',
704 *Nature methods*, 15(7), pp. 475–476. Available at: https://doi.org/10.1038/s41592-018-0046-7.

705 Gupta, S. *et al.* (2021) 'Alleviation of salinity stress in plants by endophytic plant-fungal symbiosis: Current
706 knowledge, perspectives and future directions', *Plant and Soil*, pp. 219–244. Available at:
707 https://doi.org/10.1007/s11104-020-04618-w.

708 Hahn, M.W. *et al.* (2017) 'Silvanigrella aquatica gen. nov., sp. nov., isolated from a freshwater lake, description of
709 Silvanigrellaceae fam. nov. and Silvanigrellales ord. nov., reclassification of the order Bdellovibrionales in the class
710 Oligoflexia, reclassification of the families Bacteriovoracaceae and Halobacteriovoraceae in the new order
711 Bacteriovoracales ord. nov., and reclassification of the family Pseudobacteriovoracaceae in the order Oligoflexales',
712 *International journal of systematic and evolutionary microbiology*, 67(8), pp. 2555–2568. Available at:
713 https://doi.org/10.1099/ijsem.0.001965.

714 Hartman, L.M., van Oppen, M.J.H. and Blackall, L.L. (2020) 'Microbiota characterization of Exaiptasia diaphana
715 from the Great Barrier Reef', *Animal microbiome*, 2(1), p. 10. Available at: https://doi.org/10.1186/s42523-020-
716 00029-5.

717 Helliwell, K.E. *et al.* (2011) 'Insights into the evolution of vitamin B12 auxotrophy from sequenced algal genomes',
718 *Molecular biology and evolution*, 28(10), pp. 2921–2933. Available at: https://doi.org/10.1093/molbev/msr124.

719 Herrera, M. *et al.* (2017) 'Laboratory-Cultured Strains of the Sea Anemone Exaiptasia Reveal Distinct Bacterial
720 Communities', *Frontiers in Marine Science*. Available at: https://doi.org/10.3389/fmars.2017.00115.

721 Herrera, M. *et al.* (2021) 'Temperature transcends partner specificity in the symbiosis establishment of a cnidarian',
722 *The ISME journal*, 15(1), pp. 141–153. Available at: https://doi.org/10.1038/s41396-020-00768-y.

723 Hoegh-Guldberg, O. *et al.* (2007) 'Coral reefs under rapid climate change and ocean acidification', *Science*,
724 318(5857), pp. 1737–1742. Available at: https://doi.org/10.1126/science.1152509.

725 Jinkerson, R.E. *et al.* (2022) 'Cnidarian-Symbiodiniaceae symbiosis establishment is independent of
726 photosynthesis', *Current biology: CB*, 32(11), pp. 2402–2415.e4. Available at:
727 https://doi.org/10.1016/j.cub.2022.04.021.

728 Kalyaanamoorthy, S. *et al.* (2017) 'ModelFinder: fast model selection for accurate phylogenetic estimates', *Nature*
729 *methods*, 14(6), pp. 587–589. Available at: https://doi.org/10.1038/nmeth.4285.

730 Kitchen, S.A. and Weis, V.M. (2017) 'The sphingosine rheostat is involved in the cnidarian heat stress response but
731 not necessarily in bleaching', *Journal of Experimental Biology* [Preprint]. Available at:
732 https://doi.org/10.1242/jeb.153858.

733 Kleypas, J.A.A. and Kleypas, J.A.K. (2019) 'Climate change and tropical marine ecosystems: A review with an
734 emphasis on coral reefs', *UNED Research Journal*, pp. S24–S35. Available at:
735 https://doi.org/10.22458/urj.v11i1.2317.

736 Knowles, R. (1982) 'Denitrification', *Microbiological Reviews*, pp. 43–70. Available at:
737 https://doi.org/10.1128/mr.46.1.43-70.1982.

738 Knowlton, N. and Rohwer, F. (2003) 'Multispecies microbial mutualisms on coral reefs: the host as a habitat', *The*
739 *American naturalist*, 162(4 Suppl), pp. S51–62. Available at: https://doi.org/10.1086/378684.

740 Lahti, L., Shetty S., *et al.* (2017). 'Tools for microbiome analysis in R.' Available at:
741 https://microbiome.github.com/microbiome

742 Lam, V.W.Y. *et al.* (2020) 'Climate change, tropical fisheries and prospects for sustainable development', *Nature*
743 *Reviews Earth & Environment*, pp. 440–454. Available at: https://doi.org/10.1038/s43017-020-0071-9.

744 Lehnert, E.M. *et al.* (2014) 'Extensive differences in gene expression between symbiotic and aposymbiotic
745 cnidarians', *G3* , 4(2), pp. 277–295. Available at: https://doi.org/10.1534/g3.113.009084.

746 Louca, S. *et al.* (2018) 'Function and functional redundancy in microbial systems', *Nature ecology & evolution*,
747 2(6), pp. 936–943. Available at: https://doi.org/10.1038/s41559-018-0519-1.

748 Maire, J., Blackall, L.L. and van Oppen, M.J.H. (2021) 'Microbiome characterization of defensive tissues in the
749 model anemone Exaiptasia diaphana', *BMC microbiology*, 21(1), p. 152. Available at:
750 https://doi.org/10.1186/s12866-021-02211-4.

751 Mather, J. (2013) 'Marine Invertebrates: Communities at Risk', *Biology*, pp. 832–840. Available at:
752 https://doi.org/10.3390/biology2020832.

753 Matthews, J.L. *et al.* (2016) 'Menthol-induced bleaching rapidly and effectively provides experimental

aposymbiotic sea anemones (Aiptasia sp.) for symbiosis investigations', *The Journal of experimental biology*, 219(Pt 3), pp. 306–310. Available at: https://doi.org/10.1242/jeb.128934.

Matthews, J.L. *et al.* (2020) 'Symbiodiniaceae-bacteria interactions: rethinking metabolite exchange in reef-building corals as multi-partner metabolic networks', *Environmental microbiology*, 22(5), pp. 1675–1687. Available at: https://doi.org/10.1111/1462-2920.14918.

McCauley, E.P., Haltli, B. and Kerr, R.G. (2015) 'Description of Pseudobacteriovorax antillogorgiicola gen. nov., sp. nov., a bacterium isolated from the gorgonian octocoral Antillogorgia elisabethae, belonging to the family Pseudobacteriovoracaceae fam. nov., within the order Bdellovibrionales', *International Journal of Systematic and Evolutionary Microbiology*, pp. 522–530. Available at: https://doi.org/10.1099/ijs.0.066266-0.

McMurdie, P.J. and Holmes, S. (2013) 'phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data', *PLoS ONE*, p. e61217. Available at: https://doi.org/10.1371/journal.pone.0061217.

Mieog, J.C. *et al.* (2009) 'Quantification of algal endosymbionts (Symbiodinium) in coral tissue using real-time PCR', *Molecular ecology resources*, 9(1), pp. 74–82. Available at: https://doi.org/10.1111/j.1755-0998.2008.02222.x.

Minh, B.Q. *et al.* (2020) 'IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era', *Molecular Biology and Evolution*, pp. 1530–1534. Available at: https://doi.org/10.1093/molbev/msaa015.

Miransari, M. (2011) 'Arbuscular mycorrhizal fungi and nitrogen uptake', *Archives of Microbiology*, pp. 77–81. Available at: https://doi.org/10.1007/s00203-010-0657-6.

Nakai, R. *et al.* (2014) 'Oligoflexus tunisiensis gen. nov., sp. nov., a Gram-negative, aerobic, filamentous bacterium of a novel proteobacterial lineage, and description of Oligoflexaceae fam. nov., Oligoflexales ord. nov. and Oligoflexia classis nov', *International Journal of Systematic and Evolutionary Microbiology*, pp. 3353–3359. Available at: https://doi.org/10.1099/ijs.0.060798-0.

Nakai, R. *et al.* (2016) 'Genome sequence and overview of Oligoflexus tunisiensis Shr3T in the eighth class Oligoflexia of the phylum Proteobacteria', *Standards in Genomic Sciences*. Available at: https://doi.org/10.1186/s40793-016-0210-6.

Olson, N.D. *et al.* (2009) 'Diazotrophic bacteria associated with Hawaiian Montipora corals: Diversity and abundance in correlation with symbiotic dinoflagellates', *Journal of Experimental Marine Biology and Ecology*, pp. 140–146. Available at: https://doi.org/10.1016/j.jembe.2009.01.012.

van Oppen, M.J.H. and Blackall, L.L. (2019) 'Coral microbiome dynamics, functions and design in a changing world', *Nature Reviews Microbiology*, pp. 557–567. Available at: https://doi.org/10.1038/s41579-019-0223-4.

Ortiz, J.C., Gomez-Cabrera, M. del C. and Hoegh-Guldberg, O. (2009) 'Effect of colony size and surrounding substrate on corals experiencing a mild bleaching event on Heron Island reef flat (southern Great Barrier Reef, Australia)', *Coral reefs*, 28(4), pp. 999–1003. Available at: https://doi.org/10.1007/s00338-009-0546-0.

Palacio-Castro, A. M. (2019). 'Abiotic controls on endosymbiotic algal communities and their implications for coral bleaching susceptibility and recovery', (Doctoral dissertation, University of Miami).

Pandolfi, J.M. *et al.* (2003) 'Global trajectories of the long-term decline of coral reef ecosystems', *Science*, 301(5635), pp. 955–958. Available at: https://doi.org/10.1126/science.1085706.

Peixoto, R.S. *et al.* (2017) 'Beneficial Microorganisms for Corals (BMC): Proposed Mechanisms for Coral Health and Resilience', *Frontiers in microbiology*, 8, p. 341. Available at: https://doi.org/10.3389/fmicb.2017.00341.

Perez, S.F., Cook, C.B. and Brooks, W.R. (2001) 'The role of symbiotic dinoflagellates in the temperature-induced

bleaching response of the subtropical sea anemone Aiptasia pallida', *Journal of experimental marine biology and ecology*, 256(1), pp. 1–14. Available at: https://doi.org/10.1016/s0022-0981(00)00282-3.

Pernice, M. *et al.* (2015) 'A nanoscale secondary ion mass spectrometry study of dinoflagellate functional diversity in reef-building corals', *Environmental microbiology*, 17(10), pp. 3570–3580. Available at: https://doi.org/10.1111/1462-2920.12518.

Pinheiro J., Bates D. (2023). 'nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-162, R Core Team'. Available at https://CRAN.R-project.org/package=nlme.

Poole, A.Z., Kitchen, S.A. and Weis, V.M. (2016) 'The Role of Complement in Cnidarian-Dinoflagellate Symbiosis and Immune Challenge in the Sea Anemone Aiptasia pallida', *Frontiers in microbiology*, 7, p. 519. Available at: https://doi.org/10.3389/fmicb.2016.00519.

Rädecker, N. *et al.* (2021) 'Heat stress destabilizes symbiotic nutrient cycling in corals', *Proceedings of the National Academy of Sciences of the United States of America*, 118(5). Available at: https://doi.org/10.1073/pnas.2022653118.

Randle, J.L. *et al.* (2020) 'Salinity-Conveyed Thermotolerance in the Coral Model Aiptasia Is Accompanied by Distinct Changes of the Bacterial Microbiome', *Frontiers in Marine Science*. Available at: https://doi.org/10.3389/fmars.2020.573635.

Reshef, L. *et al.* (2006) 'The coral probiotic hypothesis', *Environmental microbiology*, 8(12), pp. 2068–2073. Available at: https://doi.org/10.1111/j.1462-2920.2006.01148.x.

Ritchie, K.B. (2012) 'Bacterial Symbionts of Corals and Symbiodinium', *Beneficial Microorganisms in Multicellular Life Forms*, pp. 139–150. Available at: https://doi.org/10.1007/978-3-642-21680-0_9.

Röthig, T. *et al.* (2016) 'Distinct Bacterial Communities Associated with the Coral Model Aiptasia in Aposymbiotic and Symbiotic States with Symbiodinium', *Frontiers in Marine Science*. Available at: https://doi.org/10.3389/fmars.2016.00234.

Rouzé, H. *et al.* (2016) '*Symbiodinium* clades A and D differentially predispose *Acropora cytherea* to disease and *Vibrio* spp. colonization', *Ecology and Evolution*, pp. 560–572. Available at: https://doi.org/10.1002/ece3.1895.

Sachs, J.L. *et al.* (2004) 'The evolution of cooperation', *The Quarterly review of biology*, 79(2), pp. 135–160. Available at: https://doi.org/10.1086/383541.

Santoyo, G. *et al.* (2022) *Mitigation of Plant Abiotic Stress by Microorganisms: Applicability and Future Directions*. Academic Press. Available at: https://play.google.com/store/books/details?id=V6BMEAAAQBAJ.

Sayers, E.W. *et al.* (2023) 'Database resources of the National Center for Biotechnology Information in 2023', *Nucleic acids research*, 51(D1), pp. D29–D38. Available at: https://doi.org/10.1093/nar/gkac1032.

Smith, J.M. and Szathmary, E. (1997) *The Major Transitions in Evolution*. Oxford University Press. Available at: https://books.google.com/books/about/The_Major_Transitions_in_Evolution.html?hl=&id=Qts6FAcGGaIC.

Starzak, D.E. *et al.* (2014) 'The influence of symbiont type on photosynthetic carbon flux in a model cnidarian–dinoflagellate symbiosis', *Marine Biology*, pp. 711–724. Available at: https://doi.org/10.1007/s00227-013-2372-8.

Stock, S.P. (2019) 'Partners in crime: symbiont-assisted resource acquisition in Steinernema entomopathogenic nematodes', *Current opinion in insect science*, 32, pp. 22–27. Available at: https://doi.org/10.1016/j.cois.2018.10.006.

Sydnor, J.R. (2020) *The Bacterial Community Associated with the Model Sea Anemone Exaiptasia Pallida: Response to Rising Ocean Temperature*. Available at: https://books.google.com/books/about/The_Bacterial_Community_Associated_with.html?hl=&id=WICfzwEACAJ.

Thomas, L. *et al.* (2018) 'Mechanisms of Thermal Tolerance in Reef-Building Corals across a Fine-Grained Environmental Mosaic: Lessons from Ofu, American Samoa', *Frontiers in Marine Science*. Available at: https://doi.org/10.3389/fmars.2017.00434.

University of Massachusetts Amherst Massachusetts Lynn Margulis, Margulis, L. and Fester, R. (1991) *Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis*. MIT Press. Available at: https://books.google.com/books/about/Symbiosis_as_a_Source_of_Evolutionary_In.html?hl=&id=3sKzeiHUIUQC.

Voolstra, C.R. and Ziegler, M. (2020) 'Adapting with Microbial Help: Microbiome Flexibility Facilitates Rapid Responses to Environmental Change', *BioEssays: news and reviews in molecular, cellular and developmental biology*, 42(7), p. e2000004. Available at: https://doi.org/10.1002/bies.202000004.

Wahida, A., Tang, F. and Barr, J.J. (2021) 'Rethinking phage-bacteria-eukaryotic relationships and their influence on human health', *Cell host & microbe*, 29(5), pp. 681–688. Available at: https://doi.org/10.1016/j.chom.2021.02.007.

Waite, D.W. *et al.* (2020) 'Proposal to reclassify the proteobacterial classes and the phylum into four phyla reflecting major functional capabilities', *International journal of systematic and evolutionary microbiology*, 70(11), pp. 5972–6016. Available at: https://doi.org/10.1099/ijsem.0.004213.

Wang, Q. *et al.* (2007) 'Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy', *Applied and environmental microbiology*, 73(16), pp. 5261–5267. Available at: https://doi.org/10.1128/AEM.00062-07.

Wei, T. and Simko, V. (2021) R Package "Corrplot": Visualization of a Correlation Matrix. Available at: https://github.com/taiyun/corrplot

Weiss, S. *et al.* (2017) 'Normalization and microbial differential abundance strategies depend upon data characteristics', *Microbiome*, 5(1), p. 27. Available at: https://doi.org/10.1186/s40168-017-0237-y.

Weis, V.M. (2008) 'Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis', *The Journal of experimental biology*, 211(Pt 19), pp. 3059–3066. Available at: https://doi.org/10.1242/jeb.009597.

Weis, V.M. (2019) 'Cell Biology of Coral Symbiosis: Foundational Study Can Inform Solutions to the Coral Reef Crisis', *Integrative and Comparative Biology*, pp. 845–855. Available at: https://doi.org/10.1093/icb/icz067.

Wichard, T. (2015) 'Exploring bacteria-induced growth and morphogenesis in the green macroalga order Ulvales (Chlorophyta)', *Frontiers in plant science*, 6, p. 86. Available at: https://doi.org/10.3389/fpls.2015.00086.

Wilkinson, L. (2011) 'ggplot2: Elegant Graphics for Data Analysis by WICKHAM, H', *Biometrics*, pp. 678–679. Available at: https://doi.org/10.1111/j.1541-0420.2011.01616.x.

Wooldridge, S.A. (2009) 'A new conceptual model for the warm-water breakdown of the coral - algae endosymbiosis', *Marine and Freshwater Research*, p. 483. Available at: https://doi.org/10.1071/mf08251.

Worthen, P.L., Gode, C.J. and Graf, J. (2006) 'Culture-independent characterization of the digestive-tract microbiota of the medicinal leech reveals a tripartite symbiosis', *Applied and environmental microbiology*, 72(7), pp. 4775–4781. Available at: https://doi.org/10.1128/AEM.00356-06.

Zan, J. *et al.* (2019) 'A microbial factory for defensive kahalalides in a tripartite marine symbiosis', *Science*. Available at: https://doi.org/10.1126/science.aaw6732.