HOST-MICROBE INTERACTIONS IN NON-NATIVE ESTUARINE ANEMONES:

BIOGEOGRAPHY AND TEMPERATURE

By

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ABSTRACT

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Non-native species are increasing in prevalence around the world, resulting in

negative economic and ecological impacts. However, the broad distributions of non-

native species also offer a system for investigating the response of host-associated

microbial communities to environmental factors across a range of ecological scales. At

the broadest scale, I investigated the geography of microbial communities in the non-

native estuarine anemone *Diadumene lineata* on the west coast of the United States of

America. Across latitudes, microbial community composition was very similar and

displayed a high percentage of *Klebsiella spp.* at all sites. However, the communities in

California tended to exhibit higher richness, Shannon-Wiener diversity, and beta-

dispersion than the communities in Oregon and Washington, driven by an abundance of

Desulfobacterota. In a stress experiment, where three anemone species (*Diadumene

lineata*, *Diadumene leucolena*, and *Metridium senile*) were subjected to a gradient from

0-40°C to evaluate each species' capacity for buffering their microbial community

against thermal stress, I found species-specific patterns across temperatures, with only *M.

senile* exhibiting evidence of buffering at moderate temperatures. In contrast, *D. lineata*

and *D. leucolena* did not appear to be buffering their microbial communities, with *D.*

lineata displaying unique community compositions across temperatures, while the communities on *D. leucolena* generally exhibited high beta-dispersion. Finally, I isolated anemone-associated bacteria on a novel medium made of anemone tissue and measured their growth from 30-40°C, identifying candidates for beneficial host-microbe interactions in warm environments. The anemone-based medium overwhelmingly selected for the genera *Pseudoalteromonas* and *Peribacillis,* regardless of anemone host species. *Peribacillus spp.* were particularly thermal tolerant, growing similarly from 30-40°C, while *Pseudoalteromonas spp.* grew well from 30-35°C. The remaining tested genera preferred 30°C, however one of the *Litoreibacter sp.* produced a putative melanin that may protect cells against thermal stress. This is the first study exploring microbial communities in the non-native estuarine anemone *D. lineata* and lays the foundation for an expanded global assessment of latitudinal gradients, investigating how additional abiotic factors like genotype and pH drive microbial community composition, and directly testing beneficial host-microbe interactions with isolated bacteria.

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INTRODUCTION

Host organisms generate complex habitats for microbial communities that function similarly to macro habitats (Apprill et al., 2016; Dittmer et al., 2016). Migration occurs between the host and the environment, and unique microhabitats form within the host that recruit specific microbiota. For example, in Caribbean corals, we see distinct microbial communities inhabiting the coral tissues versus the outer mucus layer (Apprill et al., 2016). Likewise, in the anemone *Metridium senile*, we find low-diversity aggregates of bacteria inhabiting pockets inside the tentacles (Schuett et al., 2007). Macro organisms often play a major role in supporting the health of their environment (Gamfeldt et al., 2015), and similarly, microbiota can perform beneficial functions supporting the resilience of their host (Baldassarre et al., 2022).

Microbes provide benefits to the host that contribute to extending the host's niche and play an important role in its ecology and evolution (Cuellar-Gempeler, 2021). Examples of microbe-derived benefits include defense against predators (Shnit-Orland et al., 2012), increased access to nutrients (Armitage, 2017), developmental cues (Cavalcanti et al., 2020), and tolerance to environmental stress (Baldassarre et al., 2022; Fontaine & Kohl, 2023). For example, transplanting bacteria from heat-acclimated *Nematostella vectensis* to bacteria-depleted anemones results in lower mortality, effectively reducing stress to the host at high temperatures (Baldassarre et al., 2022). Increasing host tolerance to environmental stress may be particularly important in marine environments, which currently face unprecedented warming (Ruela et al., 2020), ocean

acidification (Doney et al., 2009), and pollution (Jambeck et al., 2015). The ability to predict the role of microorganisms in enhancing marine invertebrate survival and shifting their distributions would allow us to make more informed decisions for managing delicate coastal ecosystems.

Temperature is a key environmental stressor that dictates geographical distributions of marine organisms (Chaudhary et al., 2016), which are expected to change in the coming decades (Donelson et al., 2019; Hellberg et al., 2001). Temperature can damage tissues (Bhaumik et al., 1995), denature proteins (Feder & Hofmann, 1999), and increase the production of radical oxygen species (Banh et al., 2016). To combat environmental stressors, microbiota may alter host gene expression by generating molecules that directly bind to host transcription factors (Nichols & Davenport, 2020) or by producing metabolites that act as ligands for host proteins (H. Chen et al., 2019). To fully understand the dynamic interactions between microbial species and the host organism within the context of temperature, I conducted studies at three levels of ecological complexity. First, I examined how geographical patterns of host-associated microbial community composition respond to temperature patterns across latitudinal gradient. Second, using a manipulative experiment, I explored the stability of microbial communities associated with hosts that may experience different thermal regimes. Lastly, I characterized the thermal profile of bacterial isolates in an effort to identify microbial candidates that could contribute to host thermal tolerance.

The anemone *Diadumene lineata* is an ideal study system for investigating the ecology of host-associated microorganisms at different scales. Inhabiting the intertidal

zone in estuaries (Ryan & Kubota, 2016) makes this species particularly tolerant to broad ranges of environmental factors such as temperature and salinity (Shick, 1976), and it has also been so widely introduced through anthropogenic activity that it is now found on nearly every temperate coastline (Hancock et al., 2017). I used two other species of anemone for comparison: *Diadumene leucolena* and *Metridium senile*. Although I focused on the west coast of the United States in this thesis, the ease of identification, collection, and preservation of *D. lineata* offers a unique opportunity to compare microbial communities across countries that are not well-represented in the literature, with the ultimate goal of advancing our understanding of the microbial contributions to thermal tolerance and marine invertebrate distributions.

Research Objectives

1. To Explore Geographical Variation in Microbial Community Composition from Field Samples of *D. lineata* - Samples were collected on the west coast of the United States of America, from Washington to central California, and their microbial communities were identified using 16S rRNA amplicon sequencing. The geographic portion represents the highest level of ecological complexity, where the microbial communities are from different regions and experience all the uncontrolled environmental factors in nature.

2. To Compare the Response Anemone-Associated Microbial Communities Across a Thermal Gradient - Three anemone species (*D. lineata*, *D. leucolena*, and *M. senile*) were subjected to a temperature gradient covering nearly the full range of sea surface temperatures. The bacterial communities were sequenced to identify if each

species is maintaining a stable community composition and if maintenance is across a discrete range of temperatures. Bringing the anemones into the laboratory in the second objective reduced the environmental complexity so that I could evaluate the effects of individual abiotic factors (like temperature) on microbial community composition.

3. To Identify Thermotolerant Anemone-Associated Microbes - An alternative growth medium was developed to target anemone-associated bacteria using sterilized anemone tissue. A diverse subset of bacterial isolates were selected for a thermal-stress assay to determine variation in temperature tolerance, identifying candidates for beneficial host-microbe interactions in warm environments. Working with isolates represents the lowest level of ecological complexity in this study, where I can begin to evaluate the functions of individual bacteria that make up the community.

By addressing field-scale distributions and experimental responses to warming (both in host and in culture), I lay the foundation for generating a complete overview of how anemone-associated bacterial communities respond to temperature. Ultimately, I aim to further develop *D. lineata* as a model system that provides insights towards the role of microbiota in promoting anemone range shifts, invasion success, and persistence in a changing planet.

CHAPTER 1: GEOGRAPHICAL VARIATION IN MICROBIOTA ON THE NON-

NATIVE SEA ANEMONE *DIADUMENE LINEATA*

Introduction

The study of biogeography reveals the large-scale environmental patterns driving differences in species diversity and distribution, with one of the major patterns being a decrease in alpha-diversity across latitudinal gradients. Latitudinal diversity gradients can occur in a unimodal pattern, where richness decreases consistently from the poles to the equator (Macpherson et al., 2010; Sharifian et al., 2020), however marine taxa more frequently display an asymmetric bimodal pattern, with peaks of richness occurring at the mid-latitudes in the Northern and Southern hemisphere (Chaudhary et al., 2016). Anemones provide a prime example, exhibiting high peaks of richness around the 30-40° latitude in both hemispheres, with a dip in richness at the equator (Fautin et al., 2013). While historic biogeography studies have focused on alpha-diversity metrics like richness, beta-diversity allows us to compare not just the number of species across latitudinal gradients, but the overall community composition between locations.

The simplest measure of beta-diversity is variation in community composition, which simultaneously compares both the abundance and identity of species between locations (Tuomisto & Ruokolainen, 2006). Beta-diversity can help identify when locations are highly inter-connected or if disturbances are leading to significant community restructuring. For example, although the presence of non-native plants often

increases alpha-diversity (Rosenzweig, 2001), this is typically due to community

restructuring in which the diversity of native plants decreases at small special scales (Vilà

et al., 2011). Although high alpha-diversity is frequently associated with a positive

increase in ecosystem functions like primary production (Vilà et al., 2011), the identity

and abundance of those species is of critical importance to ecologists and conservationists

(Socolar et al., 2016).

Beta-diversity can be further explored by analyzing beta-dispersion, which

measures the variation in beta-diversity within a location. Beta-dispersion is useful for

explaining why some communities are highly similar to each other, while other locations

show significant heterogeneity, and if that variance in community composition can be

explained by environmental or geographic differences (Tuomisto & Ruokolainen, 2006).

Environmental factors that generate pressure, like freezing temperatures, can decrease

community beta-dispersion by excluding less tolerant species (Callaghan et al., 2004).

Conversely, host-associated microbial communities can display an alternative pattern

dubbed the "Anna Karenina Principle", where environmental stressors induce higher

beta-dispersion (as well as increasing alpha-diversity), representing a stochastic response

in community composition (Zaneveld et al., 2017). This pattern is prominent in microbial

communities on corals in the Florida Keys, which exhibited higher beta-dispersion when

temperatures were above 30°C, that was exacerbated in corals experiencing tissue loss

(Zaneveld et al., 2016). Examining beta-dispersion between sites allows us to determine

how the environmental impacts the structure of host-associated microbial communities,

and if differences in community composition align with broad-scale latitudinal gradients.

While debate remains over the driving causes of latitudinal diversity gradients, Hillebrand (2004) proposes that the generality of the pattern suggests a simple causal mechanism like temperature. For organisms that respire in the water, both cold and warm extremes result in limited oxygen availability. As temperatures increase, so does the metabolism up to a threshold, resulting in a higher oxygen demand by the mitochondria than the body can meet (Pörtner, 2002). However, at cold extremes, the partial pressure of oxygen in the blood begins to fall (Frederich & Pörtner, 2000), similarly resulting in a lack of oxygen available for the mitochondria (Pörtner, 2002). Additional threats from extreme temperatures include damage to tissues (Bhaumik et al., 1995), denaturing proteins (Feder & Hofmann, 1999), and increased production of radical oxygen species (Banh et al., 2016). To cope with these challenges, organisms may develop adaptations towards more extreme temperatures. For example, the estuarine anemone *Diadumene lineata* responds to higher temperatures by reducing energetic demands by reducing body size through asexual reproduction (W. H. Ryan et al., 2019), however at cooler temperatures it reduces metabolic demand and secretes a protective mucus cyst (Sassaman & Mangum, 1970). Microorganisms experience the same environmental stressors as macroorganisms but may not adhere to the same geographic patterns.

Many epipelagic bacterial phyla occur across latitudinal gradients and in bipolar distributions. Some phyla display clear relationships with temperature, where bacteria found in cooler environments tolerate a broader range of temperatures than those found towards the equator (Sul et al., 2013). Near the coastline, bacterial communities are shaped by habitat heterogeneity, where regionally rare microbiota may be abundant

locally (Amend et al., 2013). For microbiota that closely associate with marine hosts, dispersal is often tied to the dispersal of the host organism (Dickey et al., 2021). Although it appears that many regional patterns of microbial geography align with macroecological patterns (Amend et al., 2013), it remains unclear if this applies equally to host-associated microbial communities, or if they are driven primarily by local environmental conditions and host-associated factors.

The geography of host-associated microbial community composition represents the combined impact of host genotype and all environmental variables (Mortzfeld et al., 2016). In the anemone *Nematostella vectensis*, some bacterial taxa were ubiquitous across genotypes, while others would only recruit to hosts originating from particular sites (Mortzfeld et al., 2016). Similarly, in the invasive ascidian *Didemnum vexillum*, a difference in microbial community due to genotype was detected, however temperature also played a role in structuring community composition (Casso et al., 2020). However, for Red Sea corals, the bacterial communities inhabiting the surface mucus layer were strongly correlated with geographic latitude, in contrast with their dinoflagellate endosymbionts which were species-specific regardless of latitude (Osman et al., 2020). Like epipelagic communities, the strength of the relationship with latitude varies for different bacterial taxa. For example, in the anemone *Anthopleura elegantissima*, some core bacterial orders like *Legionellales* showed a strong latitudinal gradient where relative abundance increased towards cooler temperatures, while *Bacillales* were more evenly distributed (Morelan et al., 2019).

To investigate a relationship between latitude and host-associated microbial community composition, samples of the non-native anemone *Diadumene lineata* were collected at sites along the west coast of the United States from central California up into Washington. Although the population densities of *D. lineata* typically do not show a latitudinal trend, this is not necessarily true for the microbiota (Ryan & Miller, 2019). Using latitude as a proxy for regional sea surface temperature, I expected to see a gradient of increasing alpha-diversity and beta-dispersion from North to South, following an increase in temperature in alignment with the Anna Karenina Principle. Exploring microbial communities across large geographic regions informs whether these relationships are shaped by larger regional patterns or are responding to variation in local conditions.

Methods

Collection of Sea Anemones

Diadumene lineata were collected by Dr. Will Ryan (Towson University) at seven

sites along the west coast of the United States of America between June 28th 2018 and

July 21st 2018 (*Table 1*). They were identified morphologically by their small size (less

than three centimeters in height) and grey-green column adorned with orange/white

vertical stripes (Uchida, 1932). Although there are four primary morphotypes (Ryan &

Kubota, 2016), they still appear distinct from other anemone species in this region. The

latitudes ranged from 35.372°N to 46.862°N, which covers the 10° minimum suggested

by Hillebrand (2004) to capture a regional pattern. The mean annual water temperature

(MAWT) at each site was estimated by averaging the monthly average sea surface water

temperature (from the closest NOAA buoy recording environmental data) and reflects the

overall climate that year. A transect was laid out parallel to the tideline, with a quadrat

placed every five meters, attempting to collect a few anemones from each quadrat to

maximize genetic variation whenever possible (since *D. lineata* are clonal and these

populations are introduced, some sites may contain little to no genetic variation (Ryan et

al., 2021)). Anemones were collected from hard surfaces using flame-sterilized forceps.

They were rinsed briefly in sterile deionized water to dislodge mud and debris, then

preserved immediately in 1.5mL microcentrifuge tubes containing 80% ethanol. Color

morphology and the presence of visible gametes were recorded when possible (gametes

were not verified by dissection). Upon return to the laboratory, samples were frozen at -

20°C.

Table 1. Summary of collection sites for Diadumene lineata long the west coast of the United States of America. Sites will be referred to by the three-letter code in further figures/tables. Mean annual water temperature (MAWT) was estimated by averaging the monthly average sea surface water temperature from the closest buoy recording environmental data. n = total number of individual anemones collected from all quadrats (per site). n*= number of individuals represented in the results after processing the 16S data.

Site	Code	Latitude	Longitude	MAWT	n	n*
Morro Bay Rock, CA	ROK	35.37212	-120.86086	12.20	20	**0**
Azevado Pond, CA	AZO	36.83451	-121.73851	12.71	20	20
Bracut Industrial Park, CA	BGC	40.82784	-124.08720	11.79	10	0
Arcata Bird Sanctuary, CA	ABS	40.85608	-124.09853	11.79	20	18
Coal Bank Slough, OR	CBS	43.35619	-124.20964	11.66	20	16
Willapa Bay, WA	WIL	46.40258	-123.95243	11.63	20	0
Brady's Oysters, WA	BOB	46.86202	-124.07145	11.63	20	19
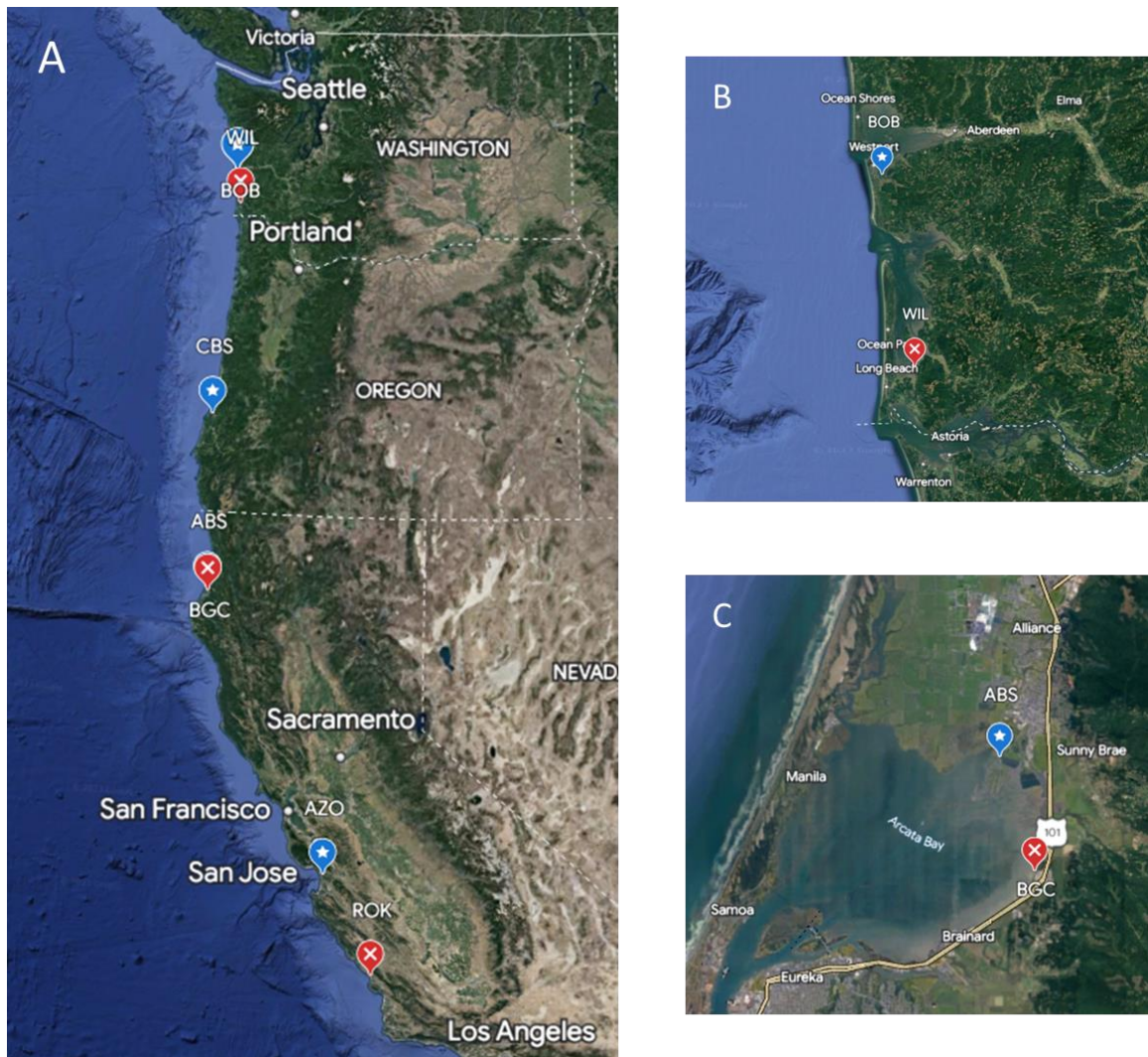
Figure 1. (A) Map of sites where D. lineata was collected from June 28th, 2018, through July 21ˢᵗ, 2018. (B) shows a close-up of the BOB and WIL sites, while (C) shows a close-up of the ABS and BGC sites. Sites with white stars within blue pins were included in the final dataset, while sites with white "X" symbols within red pins were excluded. Map generated via Google Earth version 9.182.0.1. (February 18, 2023). https://earth.google.com/

DNA Extraction

After thawing anemones and decanting off the ethanol, they were transferred whole into bead bashing tubes containing 150 uL of Ultrapure water and 750 uL of the Zymo lysis buffer. The bead bashing tubes were vortexed for 15 minutes using a tube

assembly affixed to the Vortex-Genie 2. The rest of the extraction followed the protocol outlined in the ZymoBIOMICS DNA Miniprep Kit (cat. #D4300), and DNA concentrations were quantified using the Nanodrop One (Thermo Fisher Scientific, US). Samples were shipped to Argonne National Laboratory for 16S rRNA amplicon sequencing focusing on the V4 region following the Earth Microbiome Guidelines and using Illumina MiSeq.

Bioinformatics and Data Analysis

Out of 130 samples, 34 were excluded post-sequencing due to a labeling error, where it was unclear if the samples came from this project or a separate project (including all samples from the ROK population). The 16S rRNA data for 96 samples were processed in R version 4.2.1 using *dada2* (Callahan et al., 2016) and *phyloseq* (McMurdie & Holmes, 2013). Taxa was assigned using version 138 of the SILVA database (Quast et al., 2013). All amplicon sequence variants (ASVs) identified as chloroplasts and mitochondria were removed, as well as 603 ASVs identified as the genus *Escherichia-Shigella*, which represented 23.5% of all ASVs. The removal of *Escherichia spp.* largely did not change the results, though the differences between sites were more pronounced. While *Escherichia spp.* can be found in marine sediment and biofilms, they are more frequently associated with endothermic vertebrate gut communities (Jang et al., 2017) and I suspect such a large number of ASVs are likely contamination. *metagenomeSeq* was used to normalize the sampling depth through cumulative-sum scaling (Paulson et al., 2013) and exclude samples with less than 1000 reads. A cut-off of 1000 reads minimum per sample left the BGC population with only

two samples and the WIL population with three samples, so they were excluded from the final analysis. Two more samples were removed after filtering that had zero or only one ASV, which could not be normalized. One BOB sample was also removed as it was a major outlier when comparing microbial community composition. The final number of samples for statistical analysis was 48 (AZO n = 13, ABS n = 13, CBS n = 9, BOB n = 10).

Differences in overall community composition between sites was assessed using an NMDS (Bray-Curtis distances, k=3) with the *microbiome* package (Lahti & Shetty, 2017) and PERMANOVA with the *vegan* package (Oksanen et al., 2022). Beta-dispersion was calculated with the *vegan* betadisper() function and analyzed using a pairwise permutation test of multivariate homogeneity, while richness, diversity, and evenness used a Kruskal-Wallis rank sum test followed by a pairwise comparison with a Wilcoxon rank sum test. To identify potential sources of contamination, the basic local alignment search tool (BLAST) was used to compare our *Klebsiella* ASVs to sequences of *Klebsiella* in the National Center for Biotechnology Information (NCBI) database, however each ASV matched equally with 100% accuracy to sequences from multiple sources, including humans, soil, chickens, and beetles. Differential abundance of bacterial taxa between sites was analyzed with DESeq2 as part of the *MicrobiomeExplorer* package (Reeder et al., 2021)

Results

The microbial communities showed substantial overlap in community
composition across sites (*Figure 2*), however there was still a significant difference in
composition among sites (df=44, *F*=1.778, *P*=0.005) likely due to differences in
dispersion. The Californian sites AZO and ABS generally displayed higher beta-
dispersion among samples (particularly in the first and second dimension in (*Figure 2A*),
while the Oregon site CBS and Washington site BOB clustered together. The beta-
dispersion between CBS and AZO is not significantly different, however this is likely due
to the large variation in distance to centroid of CBS (the mean is noticeably lower) (*Figure
3*). Difference in community composition by color was also evaluated, since *D. lineata* is
clonal and color can be a rough proxy for genotype, however the majority of anemones
were the same color and no significant differences among color morphs were found.
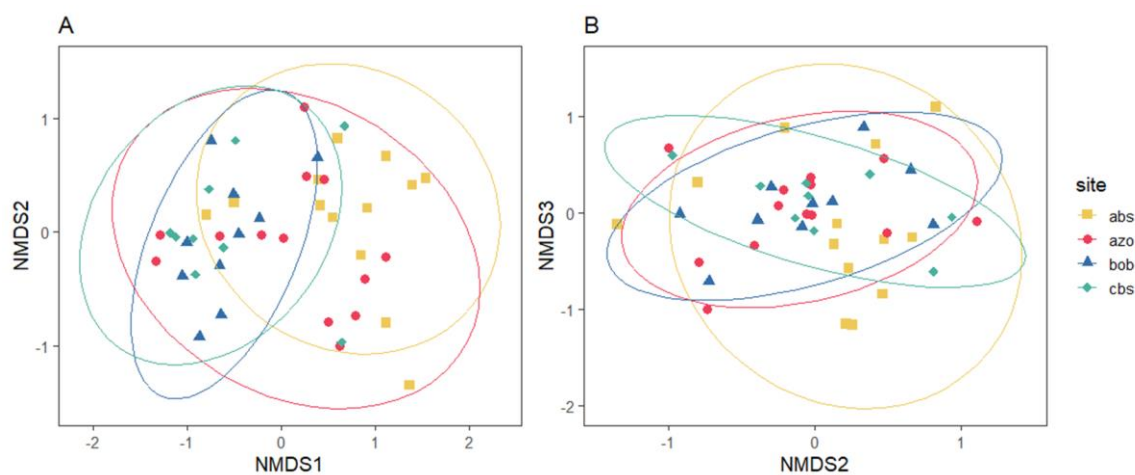
*Figure 2. Microbial community composition ordination performed via NMDS with k = 3 and stress = 0.14
using a Bray-Curtis distance matrix; (A) displays dimensions 1 and 2, (B) displays dimensions 2 and 3. The
number of k dimensions was determined by generating a screen plot showing stress from k=1 to k=10.*

Difference in community composition among sites was assessed via PERMANOVA, with 999 permutations (df=44 F=1.778, P=0.005).
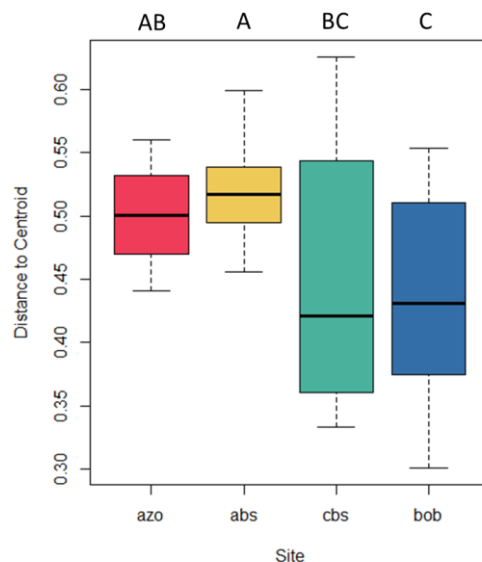
Figure 3. Beta-dispersion within sites (distance to centroid) extracted from a Bray-Curtis distance matrix. Significance assessed using a pairwise permutation test of multivariate homogeneity with 999 permutations (df=44, F=3.853, P=0.008).

The pattern of differences in beta-dispersion is also reflected in both the richness (*Figure 4A*) and the diversity (*Figure 4B*), where both the number of ASVs (df=3, X^2=12.367, P=0.006) and diversity (df=3, X^2=12.060, P=0.007) at the ABS site is significantly higher than the CBS and BOB sites. Evenness was also assessed but was not significant (df=3, X^2=6.031, P=0.110), which was not unexpected as the CSS normalization method tends to result in high evenness values across all anemones (*Figure 4C*).
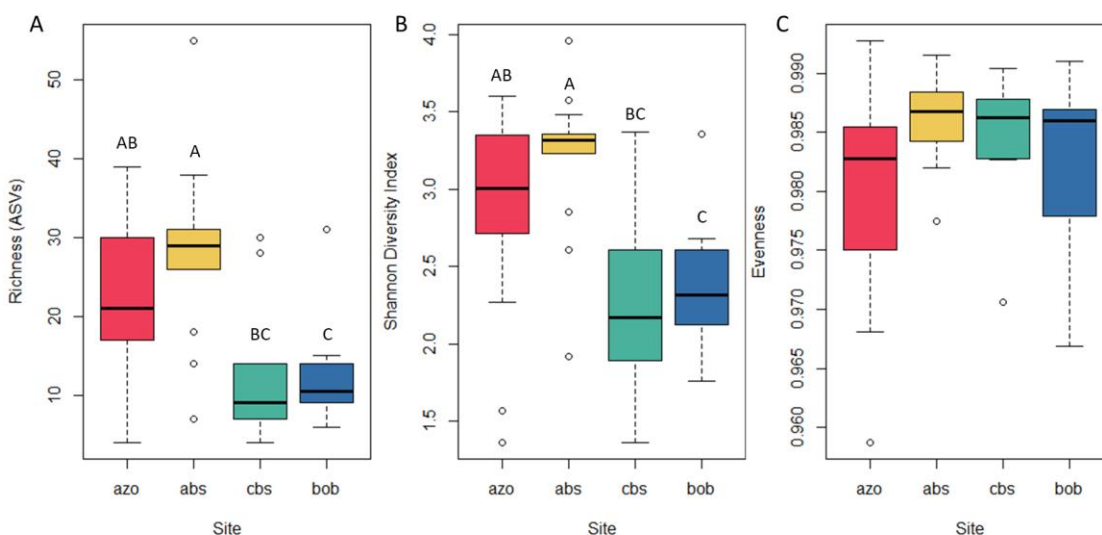
Figure 4. (A) Richness, (B) diversity, and (c) evenness of microbial communities between field sites across the west coast of the United States of America. Sites are listed in order of latitude (from South to North). Non-normality was verified for all via Shapiro-Wilk test; significant between sites was assessed with a Kruskal-Wallis rank sum test followed by a pairwise comparison with a Wilcoxon rank sum test. Both the Bonferroni and Benjamini–Hochberg corrections yielded the same results.

Enterobacteriaceae was a dominant taxa across all sites, making up large percentage of the total reads in the dataset (***Figure 5***), most of which were in the genus *Klebsiella* (***Figure 27***, ***Appendix A***). Very few taxa were differentially abundant between sites at any taxonomic level (***Figure 6***). At the level of phylum, *Desulfobacterota* were more abundant at the ABS site than the CBS site. For order, only *Syntrophobacteriales* (a member of *Desulfobacterota*) was significantly different, with higher abundance at the ABS site than the BOB site. The CBS site also had low abundance, but the comparison between ABS and CBS was not significant after the p-value was adjusted with the Benjamini-Hochberg correction. Lastly, at the genus level, *Chromobacterium spp.* were in higher abundance at ABS than at both AZO and CBS. Although overall communities

are similar across sites, there are a handful of taxa significantly contributing to the higher

diversity at ABS.
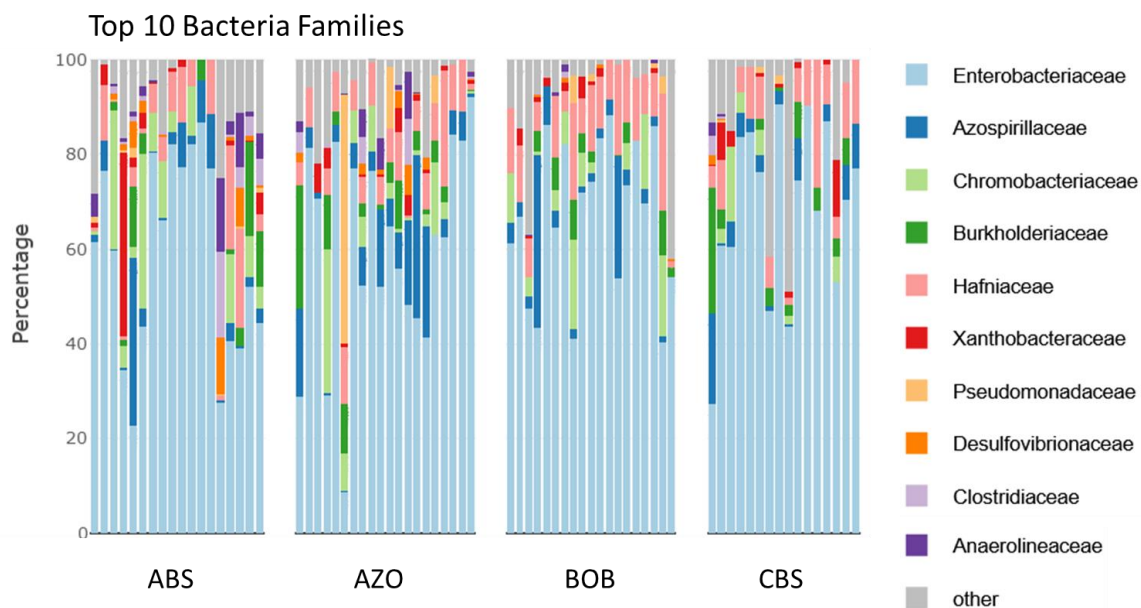
Figure 5. Relative abundance of the top 10 bacteria families across all sites. Each bar represents the microbial community on an individual anemone. Reads were normalized with CSS before converting to relative abundance. Relative abundance figure generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).
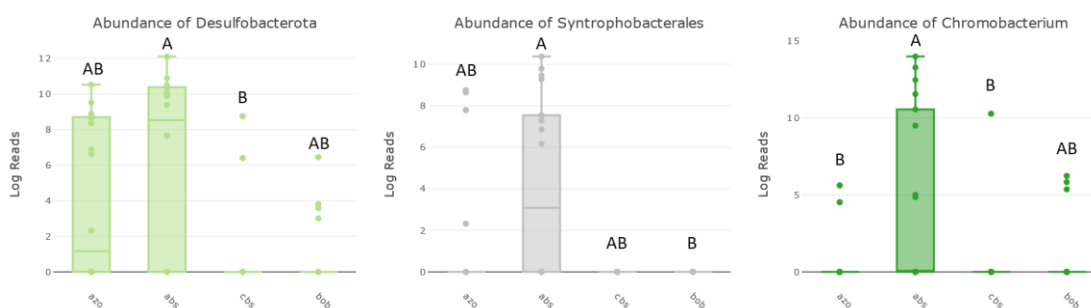
Figure 6. Differential abundance of bacterial taxa between sites, using DESeq2 with a Benjamini–Hochberg correction for pairwise comparisons. Taxa and sites that were more abundant at the ABS site include the phylum Desulfobacterota (STAT=-4.01, P=0.001), the order Syntrophobacterales (STAT=-3.597, P=0.015), and the genus Chromobacterium in comparison with the AZO (STAT=-4.512, P=0.001) and CBS (STAT=-4.11, P=0.003 sites). DESeq2 analysis and figures generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).

Discussion

Significant differences in beta-dispersion, richness, and diversity were detected between the sites, delineating the Californian sites from Oregon and Washington, however they did not strictly correspond to latitude. The greatest richness and diversity were recorded at ABS, but this site did not correspond with the highest MAWT which was at AZO (11.79°C versus 12.71°C). In contrast, the CBS and BOB sites both have a MAWT of 11.63°C (*Table 1*) and displayed the lowest mean richness and diversity. Although both Californian sites displayed high beta-dispersion, aligning with our expectation that higher regional water temperatures result in communities less similar to each other, all estimated temperatures are in the middle of the range experienced by *D. lineata* (Sassaman & Mangum, 1970; Shick, 1976) and the host is likely not experiencing thermal stress that would lead to dysbiosis. In addition, the buoys in the bay are not subject to tidal fluctuations and likely measure conditions that are more stable than those experienced by the anemones on the mudflat, where they live in the intertidal (Ryan & Kubota, 2016) and are regularly exposed to both water and air temperatures. There are also other local environmental factors that could be driving these differences, including site-specific differences salinity fluctuations or anthropogenic activity (i.e., boat dock habitat versus oyster reef habitat). Additional sites should be added, expanding the latitudinal gradient to encompass the edges of *D. lineata*'s range, and dataloggers should be employed to capture the actual environmental variation experienced by these populations.

The microbial communities at all sites were dominated by *Enterobacteriaceae*, particularly *Klebsiella spp.*, and a single *Klebsiella* ASV was responsible for 48.6% of all reads. While *Klebsiella* is a common genus that associates widely across a variety of hosts and environments (Wareth & Neubauer, 2021), including marine water and sediments (Håkonsholm et al., 2022), the dominance of this taxa in every sample at every site was still surprising and lead us to question whether contamination had occurred. Because the primary *Klebsiella* ASV matched equally with ASVs from a variety of sources I cannot entirely rule-out contamination, however the high percentage of *Klebsiella spp.* may simply be a product of the environment, since *D. lineata* can frequently be found encased in marine sediment and debris (*Figure 7*). Although a negative control was included during DNA extraction, an additional negative control during sampling would have better resolved whether the *Klebsiella spp.* were contaminants.
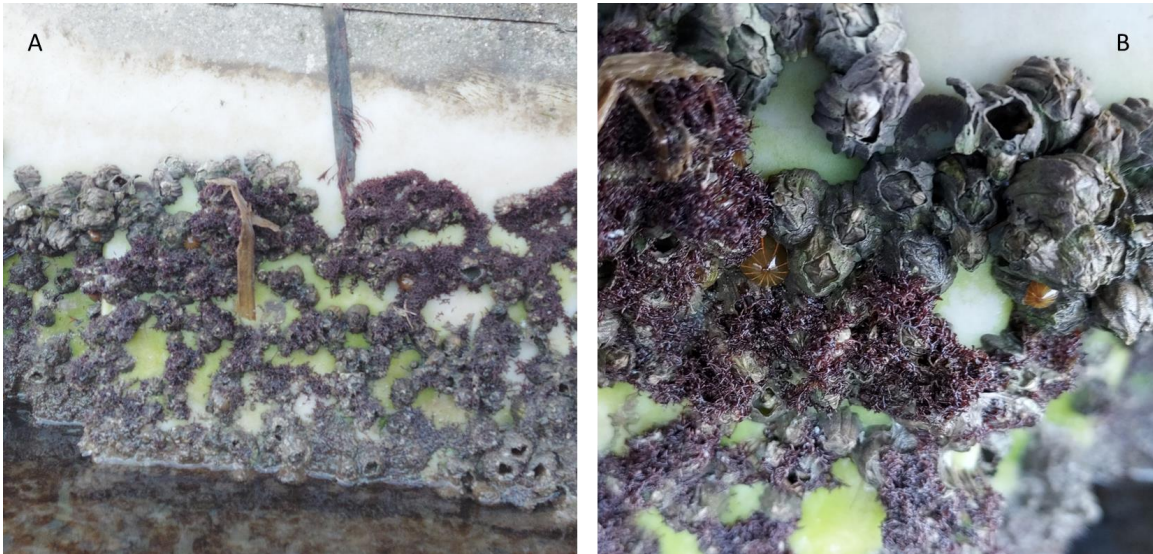
Figure 7. The side of the dock at the ABS site where D. lineata is abundant. When the tide is low, the floating dock rests right on the mudflat (A) and D. lineata can be found inside old barnacle shells and surrounded by debris (B). Towards the bottom of the dock, the anemones are frequently covered in mud, with a small hole for their tentacles to feed when the tide is high.

Currently, there no other studies on microbial communities in *D. lineata* for comparison, however there are a handful of studies on similar anemones like *Nematostella vectensis* and *Exaiptasia diaphana* (previously described as *Exaiptasia pallida* or *Aiptasia pallida*). Regardless of whether they were field or lab populations, *Enterobacteriaceae* was not a dominant taxon in *N. vectensis* (Har et al., 2015), and in *E. diaphana* samples with an excessive amount of *Enterobacteriaceae* were assumed to be contaminated and removed from the dataset (Hartman et al., 2020b). One of the major challenges of drawing comparisons between other published studies is that authors often report microbial community composition at higher taxonomic levels like phylum or class. For example, *Klebsiella* is in the highly diverse class *Gammaproteobacteria*, which in this study represented 67 unique genera and 14.11% of all ASVs. Another challenge is that many of these studies were performed on lab populations instead of field

populations, which can completely shift the identity of dominant anemone-associated bacterial taxa (Baldassarre et al., 2023). Although *Klebsiella* is dominant in this field dataset, it is not a dominant bacterial genus of our laboratory populations in Chapter 2. I was unable to draw any firm conclusions from comparing our results with similar studies on different species, and it would be highly beneficial to the field to publish supplementary files with full taxonomic tables.

Only three bacterial groups across all taxonomic levels were differentially abundant between sites: *Desulfobacterota*, *Syntrophobacterales*, and *Chromobacterium*. The phylum *Desulfobacterota* was in highest abundance at the ABS site, which did not follow a latitudinal gradient. *Desulfobacterota* is comprised of many species that use the DsrAB-dissimilatory sulfite reduction pathway (Waite et al., 2020). This phylum includes the order *Syntrophobacterales,* named for their capacity for syntrophy (i.e., where one species consumes the metabolic products of another species). Many species of *Syntrophobacterales* produce H_2 and CO_2 through the oxidation of fatty acids (Plugge et al., 2011), which can be used by methanogenic archaea in marine sediment (Chauhan et al., 2004) and may originate from the mudflat habitat surrounding *D. lineata* at the ABS site. Although methanogenesis typically occurs in layers of marine sediment where sulfate is depleted, when the substrate H_2 is in abundance (such as through the presence of *Syntrophobacterales*) both sulfate-reducers and methanogens co-exist (Kristjansson et al., 1982). Bacteria from the phylum *Desulfobacterota* are responsible for the majority of sulfate reduction in coastal marine sediments (Chen et al., 2022), where sulfate reduction drives the 65% to 85% of organic carbon decomposition (Mackin & Swider, 1989).

The last bacteria differentially abundant across sites was the genus *Chromobacterium*. *Chromobacterium* is poorly understood in host-associated marine systems, however isolates (particularly *Vibrio*) from sea anemones have been shown to inhibit cell-to-cell communication via quorum sensing in *Chromobacterium violaceum* (Reina et al., 2019). Since quorum sensing often regulates virulence factors in pathogenic bacteria (LaSarre & Federle, 2013), a reduction of *Chromobaterium spp.* at the AZO and CBS sites may indicate these anemone populations are less likely to be hosting pathogens. The abundance of *Desulfobacterota*, *Syntrophobacterales,* and *Chromobaterium spp.* at the ABS site may reflect a taxa-specific response to local conditions, as seen in the anemone *Anthopleura elegantissima*, where some bacterial taxa were more strongly correlated with particular sites (Morelan et al., 2019).

Site-specific differences in community composition could represent responses to habitat, pH, salinity, and host genotype. Although temperature was the dominant factor in determining microbial community composition for the starlet anemone *Nematostella vectensis*, host genotype still had a notable affect (Baldassarre et al., 2023). In contrast, genotype has little effect in the sponge *Pitrosia ficiformis*, where microbial community composition is more similar between sponges of different genotypes from the same location, than between sponges of the same genotype from different locations (Burgsdorf et al., 2014). For *D. lineata*, I would expect more genotypic variation at sites that also experience a substantial amount of anthropogenic activity, since introductions of marine species predominately occur through the shipping and aquaculture industries (Molnar et al., 2008). The dock at ABS is far from the main inlet and marinas in Humboldt Bay,

therefore the population is more likely to consist of clones and display low genotypic variation, while the BOB site occurs near an oyster farm and may have experienced multiple introductions of *D. lineata* attached to non-native oysters (Hancock et al., 2017). Although I did not investigate genotype in this study, populations of *D. lineata* in its invasive range often display low genotypic diversity (Ryan et al., 2021).

In this study I found differences in richness, diversity, and beta-dispersion of microbial communities associated with *D. lineata* among sites, however site-specific differences did not strictly coincide with latitude. There was significant overlap in microbial community composition across sites along the west coast of North America and differences between sites were primarily seen in beta-dispersion (resulting in higher diversity driven by an increased richness of ASVs). This supports prior studies on *N. vectensis*, where dissimilarity in microbial community composition increases in sites that were farther from each other (Baldassarre et al., 2023) and the signatures of population biogeography persisted even after the anemones adjusted to laboratory conditions (Baldassarre et al., 2023; Mortzfeld et al., 2016). Future studies should expand sampling efforts to include a wider latitudinal range that captures the edges of *D. lineata*'s temperature tolerance and falls outside of the temperate latitudes where high diversity is often present. Latitudinal gradient studies in microbial ecology are currently biased towards temperate regions, particularly in the Northern hemisphere (Dickey et al., 2021), and the broad distribution of *D. lineata* (Newcomer et al., 2019) offers a system for disentangling the relative contributions of multiple environmental and habitat characteristics at a global scale.

CHAPTER 2: TEMPERATURE SHIFTS MICROBIAL COMMUNITY

COMPOSITION IN NON-NATIVE ESTUARINE ANEMONES

Introduction

The Northern Californian ecoregion displays high numbers of documented marine

introductions, which can pose major threats to ecosystem health and function. As of

2008, there were 85 introduced marine species identified in Northern California, 66% of

which were considered ecologically harmful (Molnar et al., 2008). To prevent subsequent

invasions, it is critical to fully understand which factors impact the success of non-native

species. When non-native species are first introduced, they face a gauntlet of biotic and

abiotic obstacles that determine whether they successfully establish a population (Crowl

et al., 2008). Of the abiotic factors, temperature is often considered the most limiting

(Somero, 2002), particularly for organisms that are ectothermic. Not only do invasive

species typically tolerate a broad range of temperatures, a wider geographic range is

correlated with a higher upper thermal limit, and invasive species are more likely to

increase their upper thermal limit through acclimation (Kelley, 2014). While abiotic

tolerance and asexual reproduction are frequently cited as important traits for invasion

success in anemones (Gimenez & Brante, 2021), the potential for anemone-associated

microbial communities to mitigate temperature stress is largely unexplored.

Broadly, microbial communities may include both obligate and transient

members, forming a network of symbiotic relationships that can be pathogenic,

beneficial, or commensal in nature (Ayres, 2016). Mutualist community members can provide beneficial services to their host, such as enhancing disease resistance (Lawley et al., 2012; Rosado et al., 2019), improving nutrient acquisition (Roeselers & Newton, 2012), and facilitating environmental acclimation. For example, green frog tadpoles that were depleted of their microbiota showed a substantial increase in gene expression under heat stress, suggesting the tadpole microbial community was effectively alleviating the stress response of the host (Fontaine & Kohl, 2023). Extensive research on the model anemone *Exaiptasia diaphana* has also highlighted the importance of host-microbe interactions in host resilience during bleaching events (Cziesielski et al., 2018). Similarly, transplanting bacteria from heat-acclimated *Nematostella vectensis* to bacteria-depleted anemones reduces mortality at high temperatures (Baldassarre et al., 2022). Microbiota may alter host gene expression by generating molecules that directly bind to host transcription factors (Nichols & Davenport, 2020) or by producing metabolites that act as ligands for host proteins (Chen et al., 2019). Although hosts may develop mechanisms to recruit and maintain beneficial bacteria from the environment (Nyholm & McFall-Ngai, 2004), these mechanisms are not well understood in anemones.

Once the anemone has recruited bacteria from the environment, it is advantageous to maintain and "buffer" their microbial community. Buffering is the anemone's ability to guard their microbiota against environmental stress, maintaining community compositions markedly different from the surrounding water and preserving beneficial microbial community members (Hartman et al., 2020a; Muller et al., 2016). Hartman et al. (2020a) proposed that there may be a temperature stress threshold at which significant

community changes start to occur. In a two-year experiment, *Exaiptasia diaphana* reared

at 32°C displayed higher richness and beta-diversity in their microbial community than

anemones reared at 25°C (Ahmed et al., 2019). This pattern has been dubbed the 'Anna

Karenina Principle', where beta-diversity is used as a proxy for host health and the

stability of the community decreases under stressed conditions (Zaneveld et al., 2017).

Although prior work has largely focused on host resilience towards warming waters,

using beta-diversity to identify microbial community stress could be further applied

towards investigating temperature tolerance in invasive species during range expansion.

This chapter aims to identify the temperature thresholds under which three species

of sea anemone (*Diadumene lineata, Diadumene leucolena,* and *Metridium senile*) buffer

their microbial communities. These species are closely related, broadly tolerant to

variation in temperature and salinity, and are commonly found in the intertidal and as part

of fouling communities on docks and pilings. Although they inhabit similar estuary

environments, all three anemones display varying propensities for invasion at different

temperatures (Glon et al., 2020). While *D. lineata* is thought to be endemic to East Asia,

it is now one of the most widespread anemones in the world and has been identified on

nearly every temperate coastline (*Figure 8A*) (Hancock et al., 2017). Although *D.*

leucolena is closely related to *D. lineata*, it is found predominantly on the eastern coast of

North America with far fewer introduced populations discovered in the Eastern North

Pacific, Eastern Atlantic, and Indian oceans (*Figure 8B*). In contrast, *M. senile* has a

widespread native range across the Northern hemisphere (Glon et al., 2020), with few

introduced populations discovered in the Southern hemisphere (*Figure 8C*) (Glon et al.,

2020; Laird & Griffiths, 2016). *D. lineata* has successfully established across an incredibly broad range of sea surface temperatures, while *D. leucolena* has primarily been introduced to warmer regions and *M. senile* in colder regions (***Figure 8***).

Here, I explore whether host-associated microbial communities have the capacity to enhance invasion success by investigating the anemone's ability to maintain a stable microbial community through buffering. If the ability to maintain a distinct microbial community contributes to host resilience and increases invasion success, the most wide-spread invasive anemone (*D. lineata*) may buffer its microbial community under the broadest range of temperatures. Alternatively, a less regulated microbial community may allow non-native anemones to take advantage of microbes in new environments, in which case *D. lineata* will display less maintenance of their microbial community than *D. leucolena* or *M. senile*. If microbial communities are not involved, I expect to see idiosyncratic maintenance regardless of their range or invasiveness in response to extreme temperatures. This work highlights the potential for *D. lineata* to be further developed as a model organism for investigating host selection mechanisms on microbial communities under a variety of abiotic stressors.
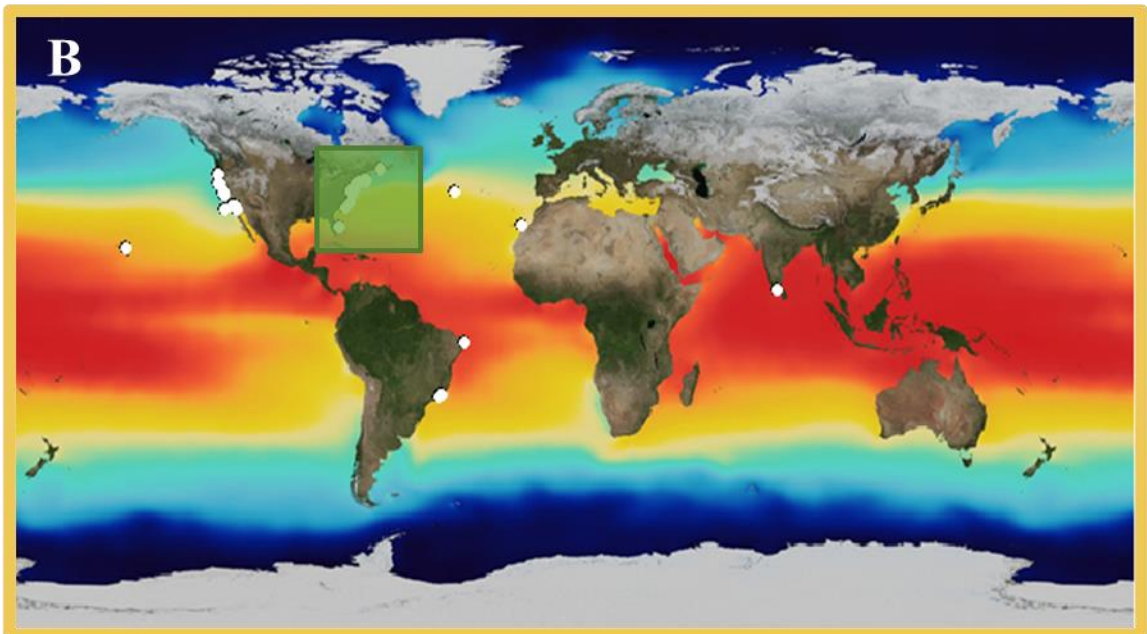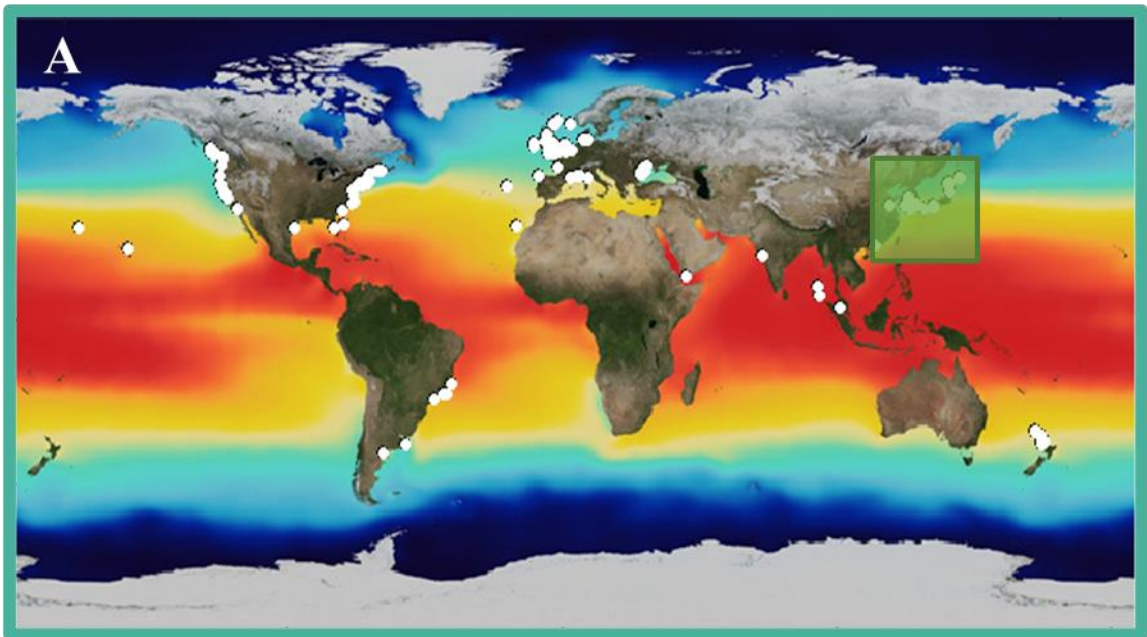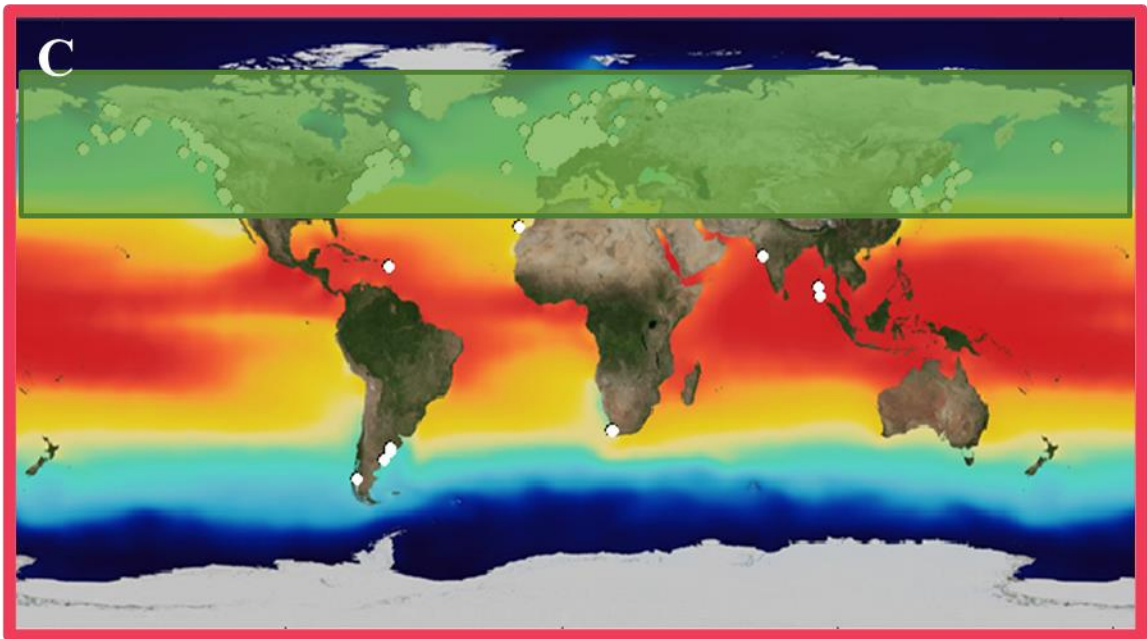
Figure 8. Documented distribution of (A) D. lineata, (B) D. leucolena, and (C) M. senile marked by white points, with occurrence data sourced from the Ocean Biodiversity Information System (OBIS) database. Green squares highlight approximate native range. Average sea surface temperatures sourced from NASA Scientific Visualization Studio. OBIS dataset citations are listed in Appendix A; maps created in R with ggmap (Kahle & Wickham, 2013).

Methods

Preparation of Artificial Seawater

Artificial seawater was prepared by mixing Instant Ocean Reef Crystals with deionized water and measuring the salinity with a calibrated refractometer. It was sterilized in two-liter batches by autoclaving at 121°C. Since autoclaving releases dissolved gases, fresh batches of artificial seawater were aerated on a stir plate for a minimum of four hours. Autoclaving also caused calcium to precipitate in the artificial seawater, which needed to be removed by filtration through a 0.22-micron Nalgene filter apparatus. Precipitation was likely exacerbated by using Reef Crystals instead of the regular Instant Ocean formula, which contains extra calcium for reef-building organisms. The salinity was not substantially affected by autoclaving or filtering, however over time it would increase by a one to two ppt due to evaporation within the bottle.

Collection of Sea Anemones

Diadumene lineata were identified morphologically by their small size (less than three centimeters in height) and grey-green column adorned with orange/white vertical stripes (Uchida, 1932). Although there are four primary morphotypes (Ryan & Kubota, 2016), they still appear distinct from other anemone species in this region. *Diadumene leucolena* are similar in size to *D. lineata*, but can be distinguished by a transparent or pink column color, without any stripes and with prominent cinclides (Ma et al., 2020). *Metridium senile* are larger and average five cm in height, with a smooth column that is commonly white, but can also appear grey, orange, or brown in color (Fautin et al.,

1987). At the top of the column in a collar-like parapet, followed by extensions of feathery tentacles with a frilled margin (Fautin & Hand, 2007). The *D. leucolena* in this study included pink and transparent morphotypes (mostly pink), and the *M. senile* in were predominately white and grey morphotypes. *D. lineata* were collected from the dock at the Arcata Marsh in Arcata, CA (40.856253, -124.098625), *M. senile* were collected from the docks at the marinas in Eureka, CA (40.803866, -124.176253) and at Woodley Island, CA (40.807440, -124.164895), and *D. leucolena* were shipped from wild populations on the East Coast of the United Stated. Anemones were collected in 50 mL tubes using a spatula and jeweler's forceps (sterilized in 70% ethanol) to carefully pry them from the sides of the docks. Small *Metridium* were selected so that the anemones were of comparable size for all species. They were transported back to the lab within an hour and stored in the refrigerator until they could be separated into individual sterile 15 mL tubes with fresh artificial seawater. Separation occurred within 48 hours to prevent the deaths of damaged anemones from fouling the tubes. Anemones were rinsed to remove as much debris as possible, particularly from the pedal disc, then stored sideways in 15 mL tubes at 4°C. The sterile artificial seawater was changed every three weeks, with *D. leucolena* kept in 20 ppt seawater and *D. lineata* and *M. senile* kept in 35 ppt seawater (similar to their areas of origin). They were fed *Artemia* nauplii sporadically and were allowed to feed for a minimum of four hours, giving them time to egest waste before changing the water. This helped further minimize fouling during storage in the refrigerator.

Cryopreservation of Inoculate

Ocean microbiota from each anemone's origin were homogenized and cryopreserved to reduce variability between the inoculated water across temperature treatments. Cryopreservation of inoculate was initially performed incorrectly by collecting 600 mL of natural sweater from each anemone's environment of origin in 50 mL tubes, then combining each tube with 2.70 mL of DMSO and freezing at -20°C. The final concentration of DMSO in the water sample was approximately 5%, however the samples should have been mixed in a 50:50 ratio of natural seawater to 5% DMSO solution (Kerckhof et al., 2014). To correct this error, the water samples were thawed and centrifuged at 7000 xg for 15 minutes, the supernatant was poured off, and the microbiota were resuspended in sterile artificial seawater (20 ppt or 35 ppt, depending on the ppt of the collected water samples). All samples from the same origin were combined with more artificial seawater for a total of 1000 mL and homogenized thoroughly with a magnetic stir bar. 1000 mL of inoculated water supplied enough water for 10 mL per individual anemone for five temperature treatments over two temperature experiments (the second experiment is unpublished). The water was separated into 100 mL aliquots (for each treatment), centrifuged at 7000 xg for 15 minutes, resuspended in 20 mL of artificial seawater, which was combined with 20 mL of 5% DMSO (chilled to 4°C to minimize cytotoxicity). The aliquots were frozen at -20°C.

Thermal Stress Experiment

Prior to running the experiment, a trial was performed to ensure that most individuals of *D. lineata* could survive the experimental conditions at 30°C (they all died in the 40°C trial, which was expected). The experiment was performed at the Telonicher

Marine Laboratory, using a single incubator for all treatments. Temperature treatments

could only be run one at a time due to incubator availability, however they were run

consecutively over the course of five weeks to attempt to minimize variation. There were

five temperature treatments total, from 0°C to 40°C in 10-degree increments. Anemones

(*D. lineata* N=10, *M. senile* N=10, *D. leucolena* N=7 per temperature treatment) were fed

Artemia in the morning and allowed to eat throughout the afternoon. Before placing them

in fresh artificial seawater, the salinity was measured with a refractometer and the water

was diluted as needed to the correct ppt (20 ppt for *D. leucolena*, 35 ppt for *D. lineata*

and *M. senile*). The frozen microbial inoculate was thawed and centrifuged at 7000 xg for

15 minutes. The supernatant containing DMSO was poured off, followed by the addition

of 100 mL of artificial seawater and vigorous vortexing for one minute. Sea anemones

and inoculated water were transferred to a sterile glass beaker (one per species), which

were covered and placed in a dark cabinet at room temperature to acclimate for three

days. After the acclimation period, each anemone were transferred into an individual 15

mL tubes with 10 mL of the inoculated water, then placed into an incubator for another

three days. Wet weight and mortality were recorded, then five individuals from each

species (per treatment) were selected for 16S rRNA amplicon sequencing. Samples were

processed immediately at the end of the temperature trial by rinsing the anemones twice

in sterile water, submerging them in 500 uL Zymo Research DNA/RNA Shield (cat.

#R1100) and macerating them gently with a sterile plastic pestle. 300 uL of DNA/RNA

Shield was added for a total volume of 800 uL. Water samples for each species (per

treatment) were pooled, centrifuged at 7000 xg for 15 minutes, and resuspended in 1000

uL of Ultrapure water. The samples were transferred to a microcentrifuge tube and

centrifuged again at 10,000 xg for one minute, then the pellet was resuspended in 1200

uL of Zymo Research DNA/RNA Shield (cat. #R1100). All anemone and water samples

were frozen at -20°C until they could be extracted using the ZymoBIOMICS DNA

Miniprep Kit (cat. #D4300). Because they were stored in DNA/RNA Shield, the lysis

buffer was omitted and the samples in DNA/RNA Shield were transferred directly to the

bead bashing lysis tubes. Vortexing time during the lysis phase was 40 minutes, per the

kit recommendations for the Vortex-Genie 2. DNA concentrations were quantified using

the Nanodrop One (Thermo Fisher Scientific, US) and samples were shipped to Argonne

National Labs for 16S rRNA amplicon sequencing focusing on the V4 region following

the Earth Microbiome Guidelines and using Illumina MiSeq (*Figure 9*).
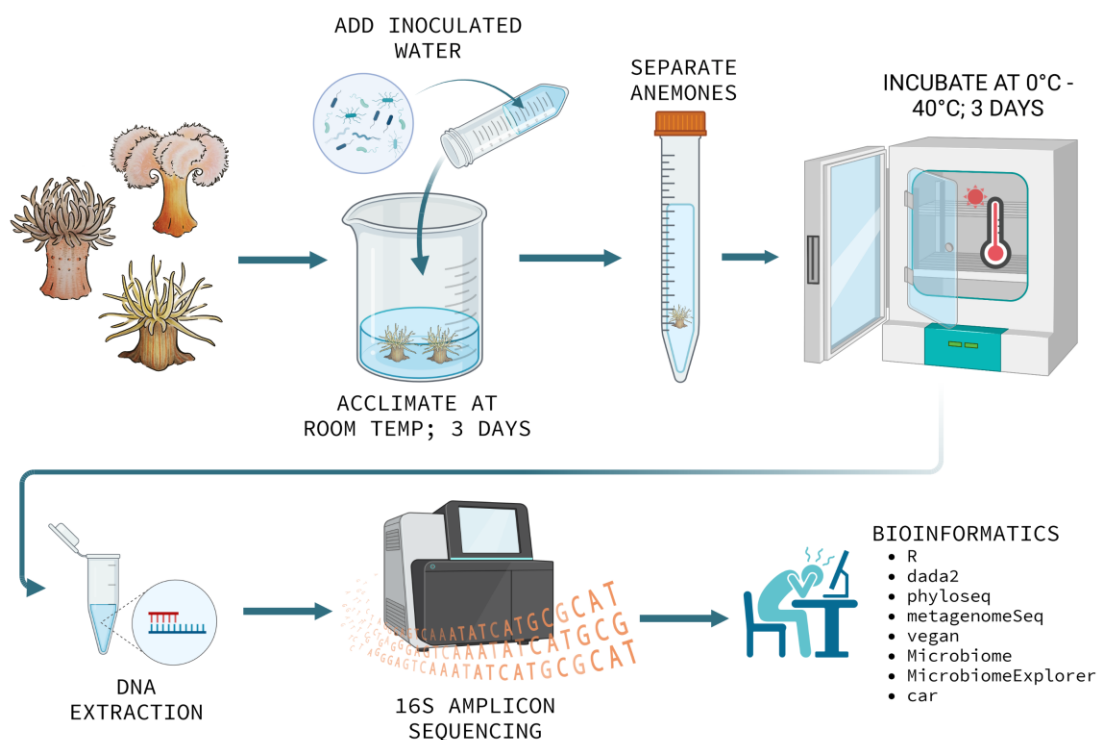
Figure 9. Flowchart of methods for the anemone thermal stress experiment. Created with BioRender.com.

Bioinformatics and Data Analysis

16S rRNA gene sequence data was processed in R version 4.2.1 using *dada2* (Callahan et al., 2016) and *phyloseq* (McMurdie & Holmes, 2013). Forward and reverse reads were trimmed to 150 bp, with maxEE=(8,8) and truncQ=2, resulting in a loss of 29.47% of all reads. After merging and forward/reverse reads and removing chimeras, taxa was assigned using version 138 of the SILVA database (Quast et al., 2013). *Phyloseq* was used to remove mitochondria, chloroplasts, and all other non-bacteria from the dataset. *metagenomeSeq* was used to normalize the sampling depth through

cumulative-sum scaling (Paulson et al., 2013) and exclude samples with less than 1000 reads.

Differences in overall community composition between temperature treatments was assessed using an NMDS (Bray-Curtis distances, k=3) with the *microbiome* package (Lahti & Shetty, 2017) and PERMANOVA with the *vegan* package (Oksanen et al., 2022). Beta-dispersion was calculated with the *vegan* betadisper() function and analyzed using a pairwise permutation test of multivariate homogeneity.

Richness and diversity were assessed using general linear models (GLM) that included temperature treatment and anemone wet weight as factors. Evenness was assessed, but excluded from the results since it was consistently high across treatment groups. During GLM analysis, four samples were removed from the *D. lineata* dataset as outliers that fell outside of an otherwise normal distribution (determined by Q-Q plot). Three of the samples had very high richness values and one sample had a very high wet weight. Two of these samples were from the 10°C treatment, one was from the 20°C treatment, and one was from the 40°C treatment. With the removal of those samples, a gaussian distribution was selected for all variables for all species. The models were evaluated with the Akaike information criterion and a Type II ANOVA using the likelihood-ratio from the *car* package in R (Fox & Weisberg, 2019). In *M. senile*, there were five large outliers for wet weight variable, and in *D. leucolena* there was two large outliers. Instead of removing the samples entirely, the wet weight variable was log transformed for these anemone species.

If the host anemones are maintaining their microbial communities, I expect to see a low proportion of ASVs shared between the anemones and the water samples. To calculate the percentage of shared ASVs between the anemone and water samples in each temperature treatment, the tables of ASVs per sample were exported from the *phyloseq* object. Since the water samples from each treatment were pooled during the experiment, the ASVs for the anemones were also pooled (per species, per temperature treatment). All ASVs that do not appear in any of the samples were removed from the table. The number of unique ASVs found in anemone, water, and shared between the two groups of samples were counted, then divided by the total number of ASVs in all temperature treatments to get the proportions. These results could not be analyzed statistically because the water samples in the experiment were pooled under the assumption there would be low densities of bacteria.

Results

Diadumene lineata

Overall microbial community composition was not impacted by anemone wet weight (df=18, F=1.428, P=0.186), but was significantly different among temperature treatments (df=18, F=7.670, P=0.001). The 0°C and 10°C treatment cluster close to each other in the NMDS space; although the 40°C treatment overlaps with them in the first and second dimension, it was distinctly separate in the second and third dimension (*Figure 10*). In contrast, the 30°C treatment is distinct in the first and second dimension but overlaps with the 0°C and 10°C treatments in the second and third dimension. The 20°C clusters separately from the other groups in all dimensions of the NMDS. Overall, the beta-dispersion of the communities was not significantly different among temperature treatments, however in a pairwise comparison the 30°C treatment is significantly more dispersed than the 10°C and 20°C treatments (*Figure 11A*).
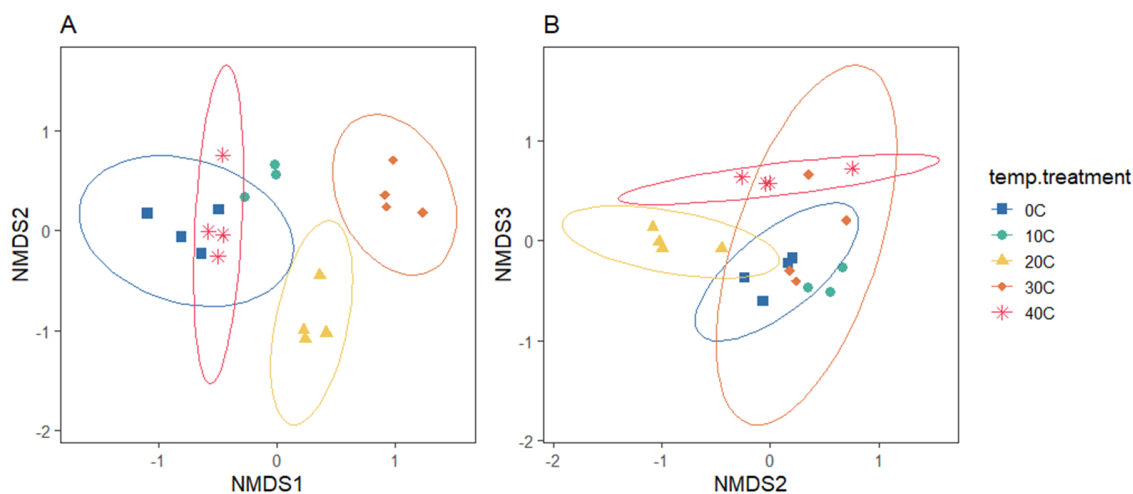
Figure 10. Microbial community composition ordination performed via NMDS with k = 3 and stress = 0.09 using a Bray-Curtis distance matrix; (A) displays dimensions 1 and 2, (B) displays dimensions 2 and 3. The number of k dimensions was determined by generating a screen plot showing stress from k=1 to k=10. Difference in community composition was assessed via two-way PERMANOVA including temperature (df=18, F=7.6699, P=0.001) and anemone wet weight (df=18 F=1.4276, P=0.186) with 999 permutations. Ellipses could not be calculated for treatments with three samples or less.

The GLM results indicate anemone wet weight is not a significant factor and that temperature is driving ASV richness, with the 10°C (*t-value*=-2.742, *P*=0.016), 30°C (*t-value*=-4.573, *P*<0.001), and 40°C (*t-value*=-2.178, *P*=0.047) treatments all having significantly less ASVs than the 20°C baseline (***Figure 11B***). Likewise, temperature was also the best explanation for differences in diversity (Shannon-Wiener index), with the 10°C (*t-value*=-2.475, *P*=0.027) and 30°C (*t-value*=-5.256, *P*<0.001) treatments lower than 20°C. 40°C was also lower, however this result was not significant (*t-value*=-2.079, *P*=0.056) (***Figure 11C***). For the complete results of all tested models, see Table 8 in Appendix B.
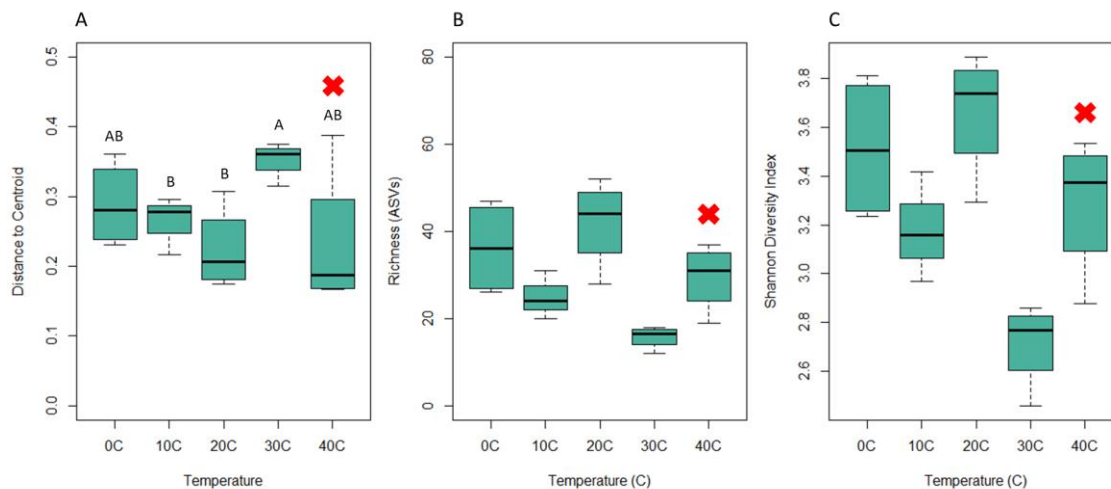
Figure 11. (A) Beta-dispersion, (B) richness, and (C) diversity across temperature treatments for Diadumene lineata. Beta-dispersion (distance to centroid) was extracted from a Bray-Curtis distance matrix and significance was assessed using a pairwise permutation test of multivariate homogeneity with 999 permutations (df=14, F=2.5008, P=0.093). The impact of temperature treatment and wet weight on richness and diversity were evaluated using a GLM with a gaussian distribution in conjunction with a Wald test. The 20°C category was selected as the baseline for the temperature GLM, since it is in the middle of the range this species is typically found in. The red X indicates temperature treatments with 100% mortality at the end of the experiment.

Underlying the observed patterns of diversity is a shift in the relative abundance of major taxa across temperature treatments, particularly *Vibrionaceae*, *Arcobacteraceae*, and *Nitrincolaceae*. At the extremes, the 0°C treatment displays quite a lot of variation among individual anemones, while the 40°C treatment where 100% mortality occurred has communities dominated by *Vibrionaceae*. Between the 20°C and 30°C treatment, *Arcobacteraceae* is overtaken by *Nitrincolaceae* as the dominant family (*Figure 12*), and the abundances of both appear to peak at 20°C (as well as *Marinamonadaceae*). Out of the top 12 bacterial families, *Marinobacteraceae* and *Stappiaceae* are only found in the 40°C treatment after mortality has occurred. *Rhodobacteraceae* is prevalent across the treatments but is in higher abundance at high temperatures. *Terasakiellaceae* is also

associated with higher temperatures, appearing only at 20°C and 30°C, with increasing

abundance at 40°C. In contrast, *Fusobacteriaceae* and *Shewanellaceae* appear to be

associated with lower temperatures. Notably, while Vibrionaceae are common across all

temperature treatments, they are in highest abundance at extreme temperatures (***Figure 13***).
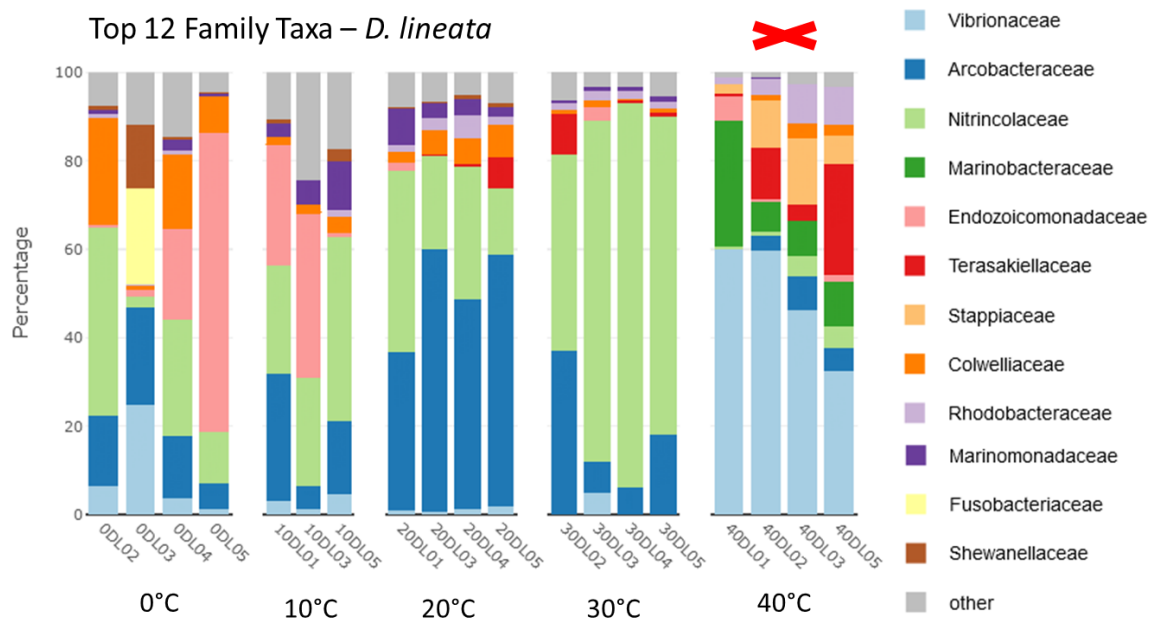
Figure 12. Relative abundance of the top 12 bacteria families across temperature treatments. Reads were normalized with CSS before converting to relative abundance. Each column represents the community of an individual anemone, with the sample ID listed below. The red X indicates temperature treatments with 100% mortality at the end of the experiment. Relative abundance figure generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).
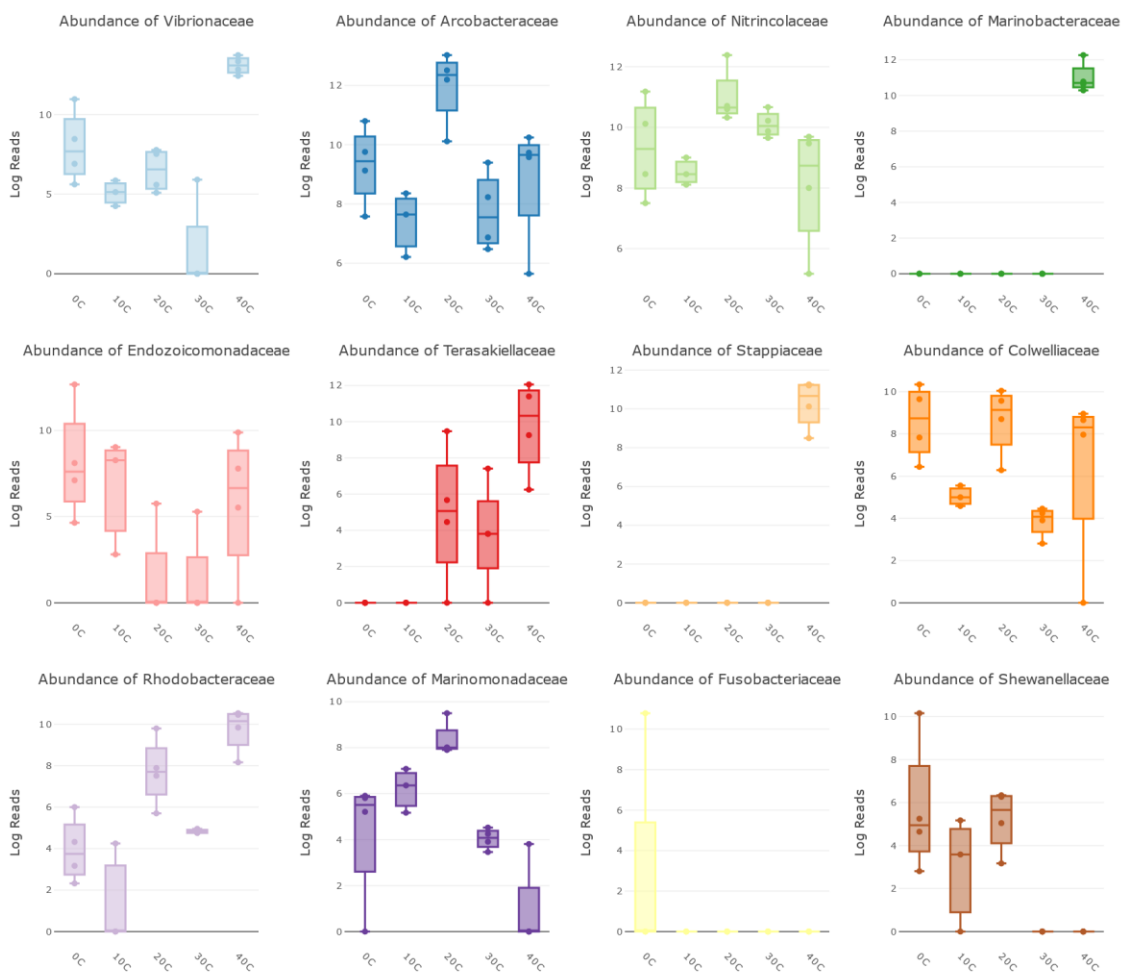
Figure 13. Differential abundance of top 12 bacterial family taxa across temperature treatments. Figures generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).

Diadumene leucolena

Microbial community composition was not impacted by the wet weight of the anemones (df=22, *F*=1.082, *P*=0.368) but was significantly different among temperature treatments (df=22, *F*=4.288, *P*=0.001) (***Figure 14***). All the temperature treatments tend to cluster together except for the 40°C treatment, particularly in the first and second dimensions. Beta-dispersion tends to be high at all temperatures. There is a significant difference in beta-dispersion (df=18, *F*=7.727, *P*=0.001), with the 40°C treatment

displaying the highest dispersion, however dispersion fluctuates among treatments.

Surprisingly, the 30°C treatment has the lowest beta-dispersion, which is also the

treatment where partial mortality occurred (*Figure 15A*).
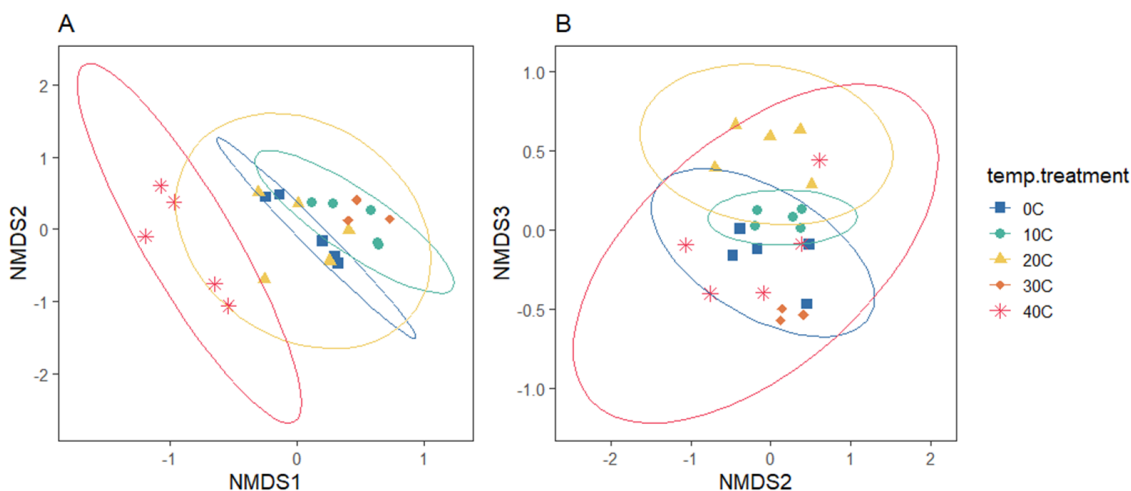
Figure 14. Microbial community composition ordination performed via NMDS with k = 3 and stress = 0.10 using a Bray-Curtis distance matrix; (A) displays dimensions 1 and 2, (B) displays dimensions 2 and 3. The number of k dimensions was determined by generating a screen plot showing stress from k=1 to k=10. Difference in community composition was assessed via two-way PERMANOVA including temperature (df=22, F=4.2875, P=0.001) and anemone wet weight (df=22, F=1.0819, P=0.368) with 999 permutations. Ellipses could not be calculated for treatments with three samples or less.

Temperature treatment and wet weight had a significant impact on richness

(*Figure 15B*), particularly for the 40°C treatment alone (*t-value*=-3.220, *P*=0.007) and

when both variables are interacting (*t-value*=3.408, *P*=0.005). The richness in the 10°C

treatment also appears slightly higher than the 20°C treatment but the difference was not

significant (*t-value*=-1.805, *P*=0.094). Diversity followed a similar pattern (*Figure 15C*),

where results were insignificant for all models except when temperature interacting with

weight for the 40°C treatment alone (*t-value*=3.051, *P*=0.009) and the 40°C interaction

(*t-value*=2.706, *P*=0.018). Although this was the best model, none of the diversity models

performed better than the null model. For both richness and diversity, we see a decrease in variance at 30°C and an increase in variance at 40°C which aligns with decreasing and increasing beta-dispersion (

Table 2).
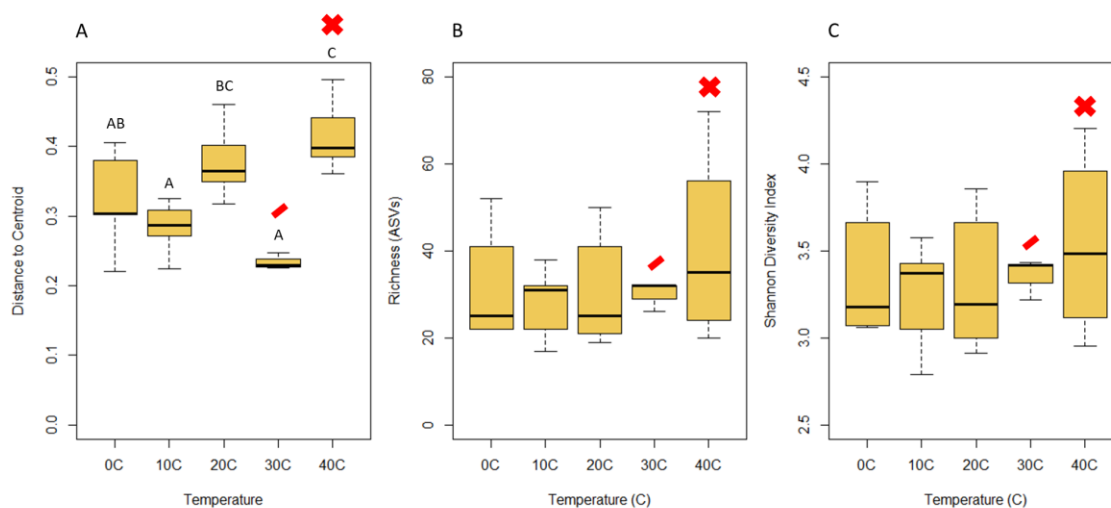
Figure 15. (A) Beta-dispersion, (B) richness, and (C) diversity across temperature treatments for Diadumene lineata. Beta-dispersion (distance to centroid) was extracted from a Bray-Curtis distance matrix and significance was assessed using a pairwise permutation test of multivariate homogeneity with 999 permutations (df=18 F=7.7268, P=0.001). The red X indicates temperature treatments with 100% mortality at the end of the experiment, while a red / indicates partial mortality.

Table 2. General linear models were used to evaluate the impact of temperature treatment and Diadumene leucolena wet weight (log-transformed) on richness, using a gaussian distribution. Est. = Estimate, SE = Standard Error, and t = t-value. The 20°C category was selected as the baseline for the temperature GLM, since it is in the middle of the range this species is typically found in. The last three columns are the results of a Type II ANOVA evaluating the entire model. The t-row is for temperature treatment, the w-row is for wet weight, and the tw-row is a combination of the two. The ANOVA was calculated using a likelihood-ratio test (LR X^2). Only the null model and the best model for richness are displayed here, results of all models listed in Table 9 in Appendix B.

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
null: richness ~ 1					189.31			
intercept	32.826	2.898	11.33	<0.001				
richness ~ temp. * weight					188.54	t: 4	3.619	0.460
						w: 1	0.120	0.729
						tw:4	12.670	**0.013**
intercept	109.44	45.21	2.420	**0.031**				
temp.0C	-112.87	78.56	-1.437	0.174				
temp.20C	-128.51	71.18	-1.805	0.094				
temp.30C	-69.61	54.13	-1.286	0.221				
temp.40C	-234.26	72.74	-3.220	**0.007**				
weight	-18.15	10.42	-1.743	0.105				
temp.0C : weight	27.53	19.73	1.395	0.186				
temp.20C : weight	33.11	20.26	1.634	0.126				
temp.30C : weight	14.73	14.49	1.017	0.328				
temp.40C : weight	66.44	19.50	3.408	**0.005**				

The relative abundance of dominant taxa shifts across temperature treatments. Lower temperatures are dominated by *Nitrincolaceae* and *Marinomonadaceae*, while the community post-mortality at 40°C is largely comprised of *Stappiaceae* and *Thalassospiraceae* (*Figure 16*). The latter two taxa are only found in the 40°C community, however we also see increasing abundance of *Arcobacteraceae* and *Rhizobiaceae* as temperature increases. In contrast, the abundance of *Nitrincolaceae* and *Marinomonadaceae* tend to decrease with temperature. *Colwelliaceae* and

Pseuodoalteromonaceae are abundant at moderate temperatures but decrease at the

extremes. *Rhodobacteriaceae*, *Flavobacteriaceae*, and *Devosiaceae* exhibit very little

change across all temperatures (***Figure 17***).
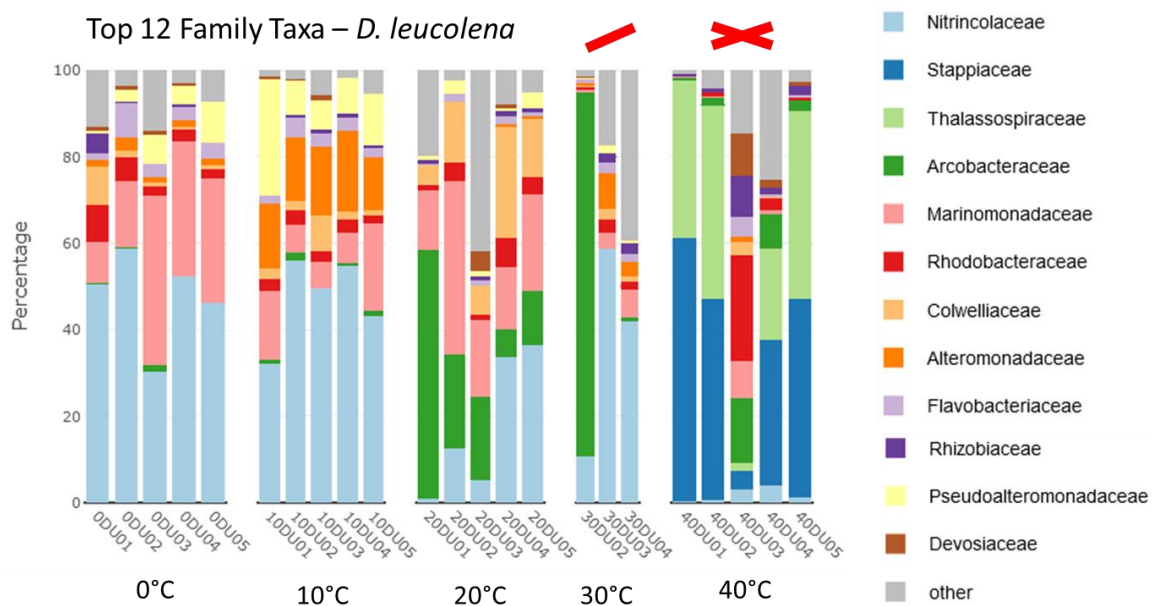
Figure 16. Relative abundance of the top 12 bacteria families across temperature treatments. Reads were normalized with CSS before converting to relative abundance. Each column represents the community of an individual anemone, with the sample ID listed below. The red X indicates temperature treatments with 100% mortality at the end of the experiment, while a red / indicates partial mortality. Relative abundance figure generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).
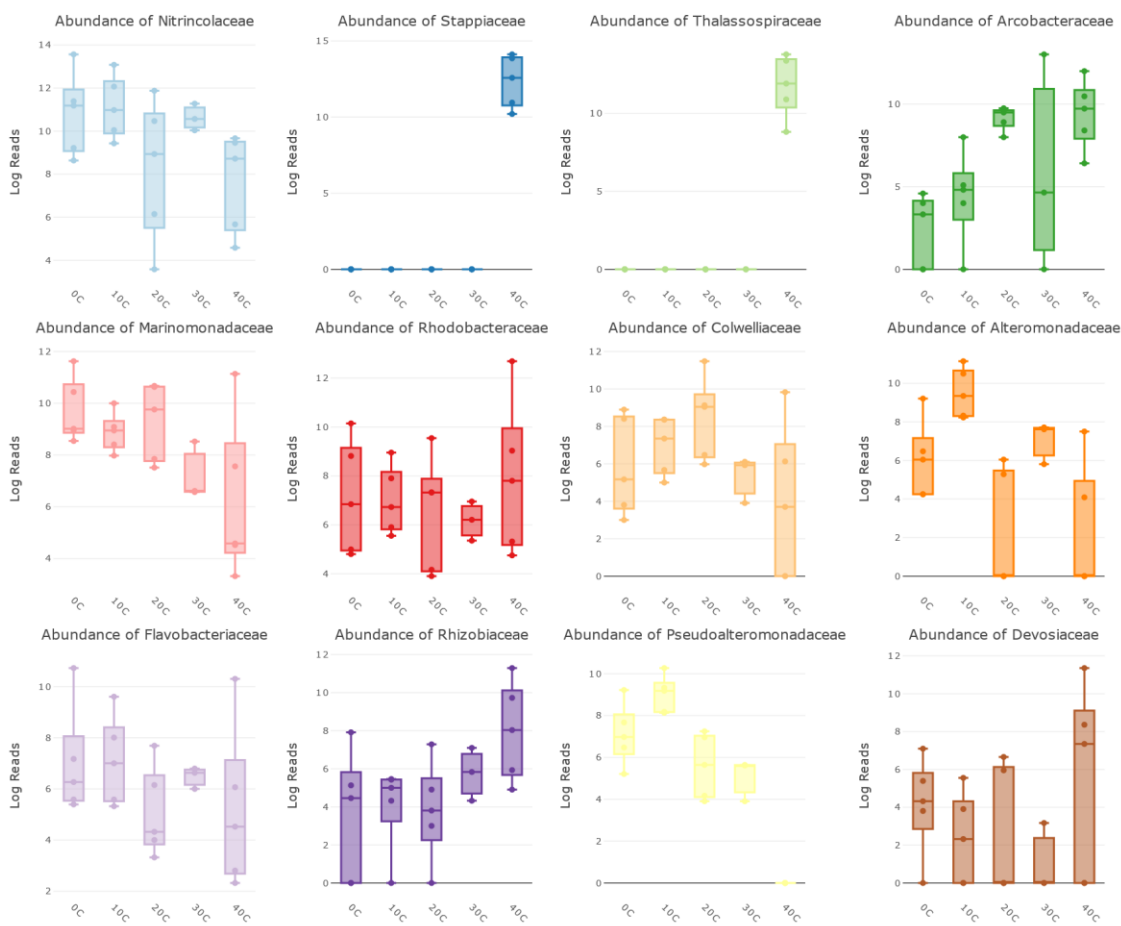
Figure 17. Differential abundance of top 12 bacterial family taxa across temperature treatments. Figures generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).

Metridium senile

Like the *Diadumene* species, temperature (df=22, *F*=9.194, *P*=0.001) drives community composition more than the anemone wet weight (df=22, *F*=1.210, *P*=0.282) in *M. senile*. The microbial community composition shows the 0°C, 10°C, and 20°C treatments clustering together, with some overlap of the 30°C treatment in the second and third dimension (*Figure 18*). The 40°C community clusters separately from the other treatments in all dimensions. Beta-dispersion is quite low, particularly for the 10°C and

30°C treatments (df=18, *F*=7.181, *P*=0.001). Although the 20°C treatment did not differ significantly from the 0°C and 40°C treatments, the temperatures at the extremes appear to have greater beta-dispersion, particularly the 0°C treatment (***Figure 19A***).
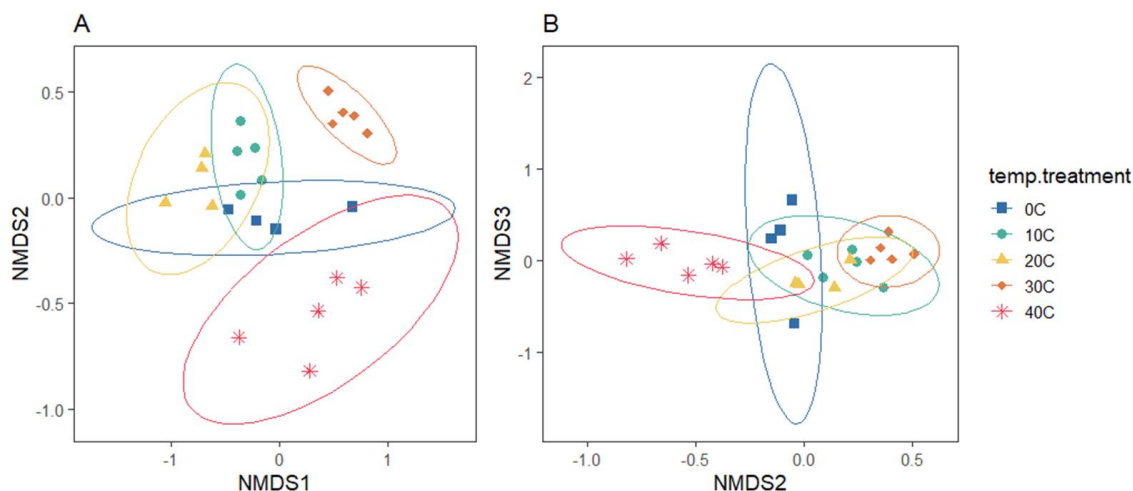
Figure 18. Microbial community composition ordination performed via NMDS with k = 3 and stress = 0.08 using a Bray-Curtis distance matrix; (A) displays dimensions 1 and 2, (B) displays dimensions 2 and 3. The number of k dimensions was determined by generating a screen plot showing stress from k=1 to k=10. Difference in community composition was assessed via two-way PERMANOVA including temperature (df=22, F=9.1943, P=0.001) and anemone wet weight (df=22 F=1.2103, P=0.282) with 999 permutations.

The effects of temperature treatment on richness (***Figure 19B***) are confounded by anemone wet weight because the three lowest temperature treatments also coincidentally ended up with the smallest anemones (***Figure 28***, ***Appendix B***). Although all models performed better than the null, when both factors are included none of the categories are significant. When assessed separately, both temperature and wet weight are significant, however the model with wet weight exhibits the lowest AIC score (***Table 3***). Diversity (***Figure 19C***) displayed a similar pattern to richness, with both temperature treatment and weight wet only significant when tested separately (***Table 10, Appendix B***).
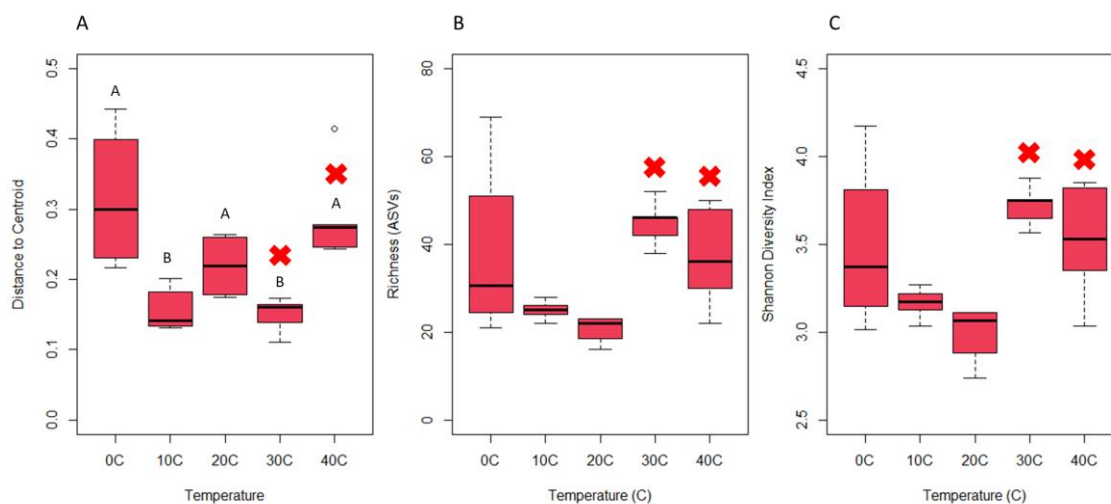
Figure 19. (A) Beta-dispersion, (B) richness, and (C) diversity across temperature treatments for Diadumene lineata. Beta-dispersion (distance to centroid) was extracted from a Bray-Curtis distance matrix and significance was assessed using a pairwise permutation test of multivariate homogeneity with 999 permutations (df=18 F=7.1807, P=0.001). The red X indicates temperature treatments with 100% mortality at the end of the experiment, while a red / indicates partial mortality.

Table 3. General linear models were used to evaluate the impact of temperature treatment and Metridium senile wet weight (log-transformed) on richness, using a gaussian distribution. Est. = Estimate, SE = Standard Error, and t = t-value. The 10°C category was selected as the baseline for the temperature GLM, since it is in the middle of the range this species is typically found in. The last three columns are the results of a Type II ANOVA evaluating the entire model. The t-row is for temperature treatment, the w-row is for wet weight, and the tw-row is a combination of the two. The ANOVA was calculated using a likelihood-ratio test (LR X^2). Only the results for richness are displayed here, the complete results for all tested models are found in Table 10 in Appendix B.

Model	Est.	SE	T	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
null: richness ~ 1					187.29			
intercept	33.435	2.773	12.06	<0.001				
richness ~ temp. + weight					180.94	t: 4	2.978	0.562
						w: 1	1.685	0.194
intercept	-0.016	19.845	-0.001	0.999				
temp.0C	9.574	7.522	1.273	0.220				
temp.20C	-0.207	7.765	-0.027	0.979				
temp.30C	11.825	9.095	1.300	0.211				
temp.40C	4.435	8.986	0.494	0.628				
weight	7.152	5.517	1.298	0.212				

Model	Est.	SE	T	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
<u>richness ~ temp. * weight</u>					180.8	t: 4	3.2435	0.518
						w: 1	1.8357	0.176
						tw:	5.5165	0.2383
						4		
intercept	12.699	80.581	0.158	0.877				
temp.0C	-175.222	114.181	-1.535	0.149				
temp.20C	34.946	95.806	0.365	0.721				
temp.30C	9.634	88.825	0.108	0.915				
temp.40C	-5.039	90.226	-0.056	0.956				
weight	3.522	23.032	0.153	0.881				
temp.0C : weight	47.354	30.840	1.535	0.149				
temp.20C : weight	-12.705	28.993	-0.438	0.668				
temp.30C : weight	1.356	24.399	0.056	0.957				
temp.40C : weight	2.932	24.660	0.119	0.907				
<u>richness ~ temp.</u>					181.11	t: 4	15.34	**0.004**
intercept	25.000	4.832	5.174	**<0.001**				
temp.0C	12.750	7.248	1.759	0.096				
temp.20C	-4.250	7.248	-0.586	0.565				
temp.30C	19.800	6.833	2.898	**0.010**				
temp.40C	12.200	6.833	1.785	0.091				
<u>richness ~ weight</u>					176.65	w: 1	15.38	**<0.001**
intercept	-10.939	11.518	-0.950	0.353				
weight	11.235	2.865	3.922	**<0.001**				

The relative abundance in lower temperatures is dominated by *Nitrincolaceae* and *Marinomonadaceae*, with *Colwelliaceae* in the 10°C treatment. As the temperature increase, Vibrionaceae eventually becomes the dominate family in the 40°C treatment (***Figure 20***). However, in terms of absolute abundance, many of the top taxa show a decrease in abundance in the 20°C treatment right before mortality occurs, followed by

an increase in the post-mortality 30°C treatment. This includes *Vibrionaceae*,

Nitrincolaceae, *Marinomonadaceae*, *Colwelliaceae*, *Rhodobacteraceae*, and

Flavobacteraceae. Taxa found only at the cold and hot extremes include

Thalassospiraceae, *Rhizobiaceae*, and *Marinobacteriaceae*. Surprisingly,

Shewanellaceae was only found in the 30°C treatment and it was in very high abundance.

In contrast, *Oceanospirillaceae* is only found in the cold 0°C-20°C treatments (*Figure 21*).
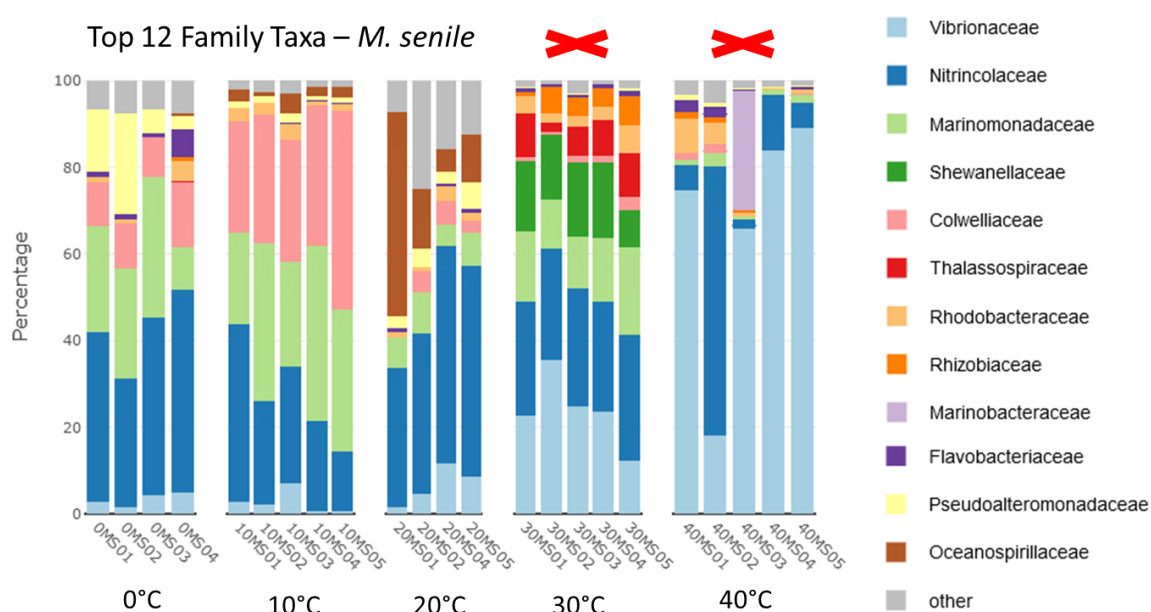
Figure 20. Relative abundance of the top 12 bacteria families across temperature treatments. Reads were normalized with CSS before converting to relative abundance. Each column represents the community of an individual anemone, with the sample ID listed below. The red X indicates temperature treatments with 100% mortality at the end of the experiment. Relative abundance figure generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).
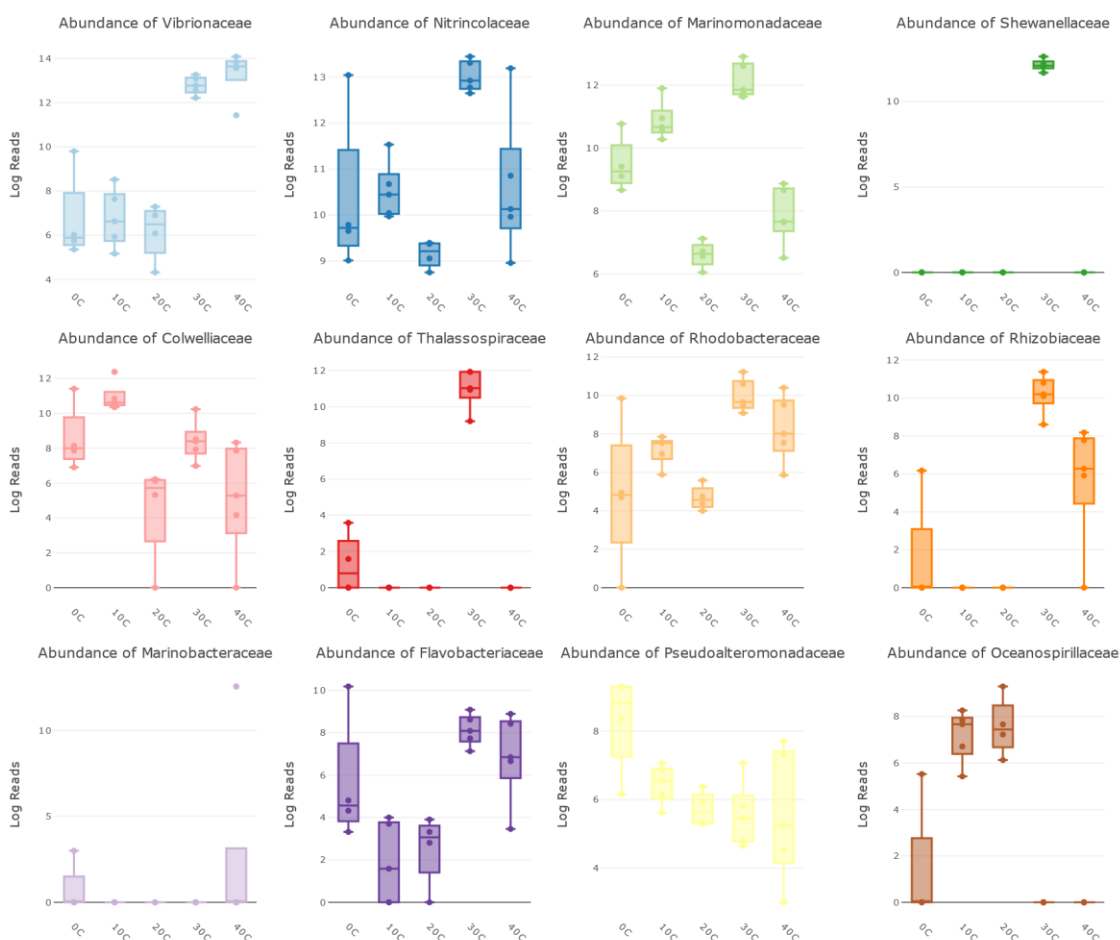
Figure 21. Differential abundance of top 12 bacterial family taxa across temperature treatments. Figures generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).

The proportion of unique ASVs on *D. lineata* is consistently low across

temperature treatments, up until mortality at the 40°C treatment. In contrast, *D. leucolena*

exhibits high proportions of unique ASVs on the anemone and shared ASVs. This shifts

at the 30°C treatment, where the amount of unique ASVs on the anemone decreases and

ASVs in the water increase. In *Metridium*, we see a similar pattern at 20°C as we see in

D. leucolena at 30°C, where there is a distinct decrease in unique ASVs inhabiting the

anemone in the treatment right before mortality occurs. For all species, mortality (above

30°C for *Metridium* and above 40°C for *Diadumene*) is marked by a notable increase in unique ASVs inhabiting the dead anemone tissue. The amount of unique ASVs in the water drops substantially, suggesting bacteria may have shifted from the water to the dead tissue (***Figure 22***).
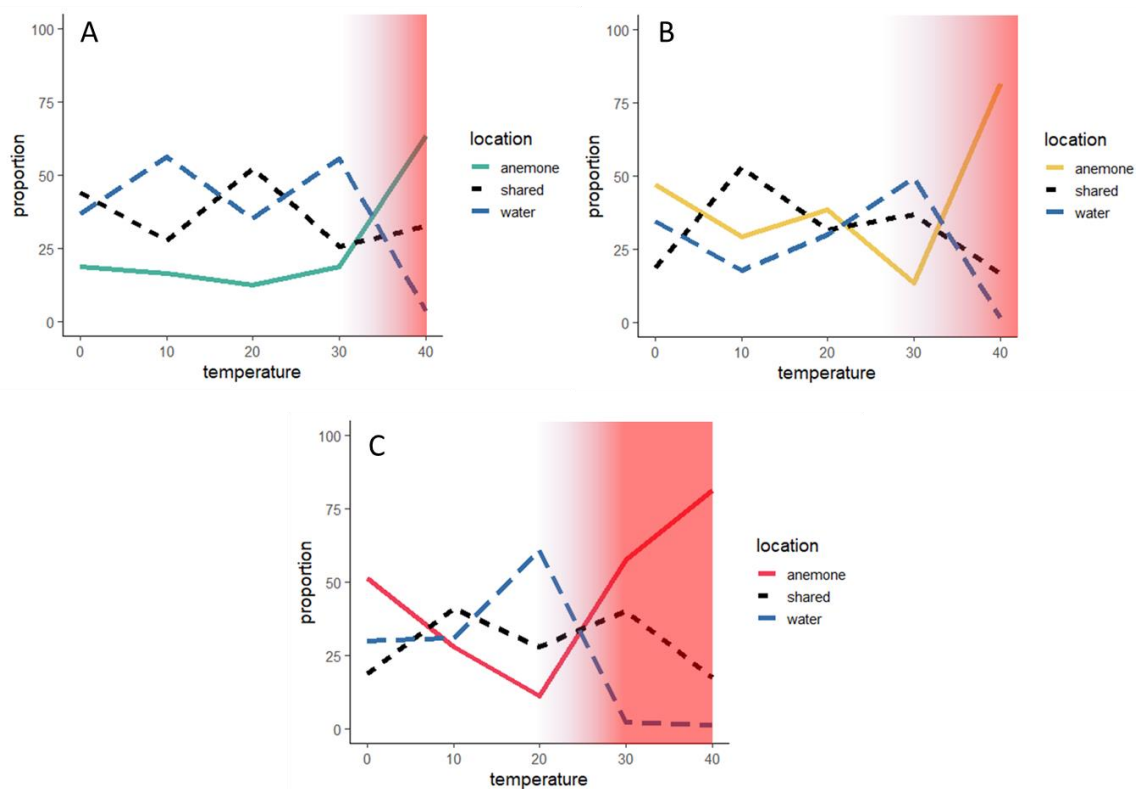
Figure 22. Proportion of ASVs found only in anemone samples, only in the water samples, or found in both. For each temperature treatment, ASVs were pooled among all anemones and all water samples (per species). (A) Diadumene lineata, (B) Diadumene leucolena, and (C) Metridium senile. The red gradient highlights when mortality begins to occur for each species.

Discussion

This is the first attempt to evaluate the buffering capacity of anemones within the context of invasion propensity, where the buffering response was species-specific and unrelated to the distributions of introduced anemones. The microbial communities of the three anemone species exhibited a different pattern of response to changes in temperature, except for post-mortality where the identities of top taxa in the communities shifted substantially for all species (40°C for the *Diadumene sp.* and above 30°C for *M. senile*). In *D. lineata*, the microbial community composition was distinct for most temperature treatments, suggesting buffering is not occurring between adjacent treatments even though richness decreases in an interesting pattern from 20°C towards 10°C and 30°C. In contrast, *D. leucolena* had highly variable communities across most temperatures with a substantial overlap in community composition, however the high richness and high beta-dispersion did not follow the expected pattern if buffering were occurring. *M. senile* is the only species that displayed some evidence for buffering, where richness was very low at adjacent moderate temperatures that also overlapped in microbial community composition.

Microbial communities on *D. lineata* varied in composition among temperature treatments, suggesting the composition of its microbiota is less regulated by the host. The microbial community within the 20°C treatment was particularly distinct from the other temperatures, even though richness and diversity were also high. This coincides with an increase in shared ASVs between the anemone and surrounding water, further supporting

a lack of buffering at this temperature. However, the host may be exerting some control at 10°C and 30°C, where the percentage of shared ASVs drops and the percentage of unique ASVs in the water increases, which coincides with a decrease in richness and diversity. This might indicate the anemones are expelling bacteria at these temperatures, particularly because multiple taxa in high abundance in the 20°C treatment decreased at 10°C and 30°C. Although *D. lineata* can survive at 30°C for at least two weeks (Shick, 1976), this temperature may be too warm for many of the microbial community members typically associated with this host (as seen by the coinciding decrease in richness). An alternative explanation might be that taxa in high abundance are particularly competitive at 20°C, however the same taxa often increase in abundance again at 0°C and 40°C. This may contrast with the pattern observed in most corals, where an increase in heat stress is often associated with increased microbial richness (McDevitt-Irwin et al., 2017), however host stress was not measured directly in this study and cold stress in anemone-associated microbial communities is not well-studied.

The microbial community composition in *D. leucolena* exhibits substantial overlap across temperature treatments, including when the anemone is likely to be experiencing stress. There was a dip in dispersion between 0°C and 20°C, which may indicate that 10°C is when the microbial community is the most stable, however this treatment also exhibited the highest number of shared ASVs between the anemone and the water which may indicate that the anemone is not maintaining this community. Overall, the community exhibits a notable lack of stability, with consistently high variation in richness and diversity up until the 30°C treatment. The Ocean Biodiversity

Information System (OBIS) reports this anemone is found most often from 10°C to 30°C,

and the partial mortality in the 30°C treatment suggests this is at the upper limit of its

range. The lack of community stability in *D. leucolena* may suggest microbe-microbe

interactions play a bigger role in shaping the community than host-microbe interactions.

Alternatively, *D. leucolena* may be maintaining a highly diverse community, but the

beta-dispersion is so high and there is so much overlap among treatments that it is

difficult to determine if buffering is occurring without comparing the specific ASVs

between the anemone and water samples.

From these results, I might predict *D. leucolena* to be a more successful invader

than *D. lineata*, however this difference may be the result of host physiology working in

conjunction with host-microbe interactions. *D. lineata* readily reproduces asexually

(Bocharova & Kozevich, 2011; Ryan et al., 2019), allowing for the proliferation of a

small founding population of introduced anemones, while *D. leucolena* appears to only

reproduce sexually (Shick & Lamb, 1977). This would not necessarily impact the initial

introduction, but substantially reduces the chance of an established population forming

since it requires the presence of compatible individuals of different sexes and the right

environmental conditions to induce reproduction (Shick & Lamb, 1977). Nevertheless,

introductions of *D. leucolena* are much more likely to be under-reported due to less

research interest and a greater difficulty in identification, whereas *D. lineata* is one of the

most well-studied invasive anemone species (Gimenez & Brante, 2021). There are far

fewer reports of *D. leucolena* on iNaturalist than *D. lineata* (predominately from the

West and East coast of the United States), however the OBIS database reveals additional

reports of *D. leucolena* from South America, Northern Africa, and India, suggesting they may be more widespread than they appear. Unfortunately, an over-representation of North American reports hinders a complete distribution record for both species (Gimenez & Brante, 2021).

The microbial community on *M. senile* appears to be more regulated by the host, maintaining similar communities between adjacent 10°C and 20°C treatments that exhibited low richness and diversity. Beta-dispersion increased in the 20°C treatment, which coincides with an increase in unique ASVs in the water and a decrease in unique ASVs on the anemone. Although 20°C is not outside of the normal range for this species, the increase in beta-diversity may reflect prior cold-acclimation of the anemones. In an acute temperature experiment, Sassaman and Magnum (1970) proposed that the upper thermal limits for *M.senile* were more dependent on previous temperature exposure than the upper thermal limits of *D. lineata* and *D. leucolena*. *M.senile* maintained at 10°C showed 100% mortality when exposed to 27.5°C, while those maintained at 22.5°C showed 0% mortality (only up to 4 hours). The *Metridium* in this experiment were stored at 4°C in the laboratory for several months prior to the start of the experiment, possibly making 20°C more stressful than usual. This is less of a concern in the 30°C treatment, where they likely would not have survived the full three-day incubation even if they were acclimated to warmer temperatures (Glon et al., 2019).

In the 0°C treatment, the microbial community on *M. senile* displays a major increase in beta-diversity, richness, and diversity. These results may suggest stress is occurring in the host as they align with my expectations for dysbiosis from the Anna

Karenina Principle, however since no mortality was observed host stress at this temperature requires confirmation. There were also multiple top taxa that were in low abundance at 10°C and 20°C, but high abundance at the extreme temperatures, further supporting the possibility that *M. senile* buffering its microbial community at moderate temperatures. Alternatively, if microbe-microbe interactions are driving community structure in *M. senile*, the abundance of these taxa at moderate temperatures may be suppressed by the presence of competitors.

Although the results for *M. senile* align with our hypothesis that having a more regulated community may coincide with being less successful invader, differences in reproduction strategy likely also play a role. The upper thermal limit for *M. senile* is above the upper limit at which asexual reproduction occurs (Glon et al., 2019), suggesting at warmer temperatures introduced *M. senile* face similar challenges to *D. leucolena* in expanding their population. In contrast, the asexual fission rate of *D. lineata* typically increases with temperature (Ryan et al., 2019). Unfortunately, the differences in richness and diversity for *M. senile* were confounded by anemone wet weight, lending uncertainty to these results (though it was not a confounding factor for overall microbial community composition).

One of the bacterial families in low abundance at moderate temperatures in *M. senile* is *Vibrionaceae*, which is often associated with stress and disease in marine hosts (Austin & Zhang, 2006; Ben-Haim & Rosenberg, 2002; Prado et al., 2005; Tout et al., 2015). *Vibrionaceae* is found in all treatments of *D. lineata*, however its abundance is lower in the 10°C and 30°C treatments, whereas *Vibrionaceae* is not a top family in *D.*

leucolena and is predominately found in the 0°C and 40°C treatments. For both

Diadumene sp. the host may be exerting control in treatments where *Vibrionaceae*

decreases or is absent, however it is equally possible that members of their microbial

communities are out-competing *Vibrionaceae*. In the coral *Acropora palmata*,

commensal bacteria living in the mucus were capable of inhibiting the growth of two

known *Vibrio* pathogens in co-culture, however the abundance of *Vibrio* increased when

exposed to higher temperatures (Frydenborg et al., 2014). It may be that the dynamic

microbial community (particularly in *D. leucolena*) outcompetes *Vibrionaceae* at

moderate temperatures.

The microbial community patterns proposed for all three species are also reflected

in the abundance of *Rhodobacteriaceae* (in the order *Rhodobacteriales*). Bacteria in the

order *Rhodobacteriales* have been described as opportunistic in corals (McDevitt-Irwin et

al., 2017), increasing in abundance to fill an open niche in response to the presence of

Vibrionales (Welsh et al., 2017). In *D. lineata*, we see a high abundance of

Rhodobacteriaceae in the 20°C treatment, which decreases at 10°C and 30°C in the same

pattern as *Vibrionaceae* (it is in highest abundance in the 40°C treatment). In *D.

leucolena*, there is a similar abundance of *Rhodobacteriaceae* across all temperature

treatments (though it appears to be highest in the 40°C treatment like *D. lineata*). In *M.

senile*, *Rhodobacteriaceae* is in low abundance in the colder temperatures and high

abundance starting at 30°C, which aligns with the proposed pattern of maintenance due to

buffering.

Ubiquitous across all anemone samples is a singular ASV from the genus *Neptuniibacter* (in the family *Nitrincolaceae*), which makes up 9% of all reads for *D. lineata*, 17% of read for *D. leucolena*, and 14% of reads for *M. senile*. Although the abundance of reads decreases in the 30°C treatment of *D. lineata*, in terms of relative abundance the community is dominated by this ASV. It is also highly prevalent from 0°C to 30°C in both *D. leucolena* and *M. senile*, suggesting a very broad temperature tolerance. *Neptuniibacter* is not well-studied or widely reported in coral and anemone literature, however many microbial community studies do not report taxonomic resolution at the level of genus. It appears to be an abundant community member in three species of Brazilian *Mussismilia* corals, and it was one of the major taxa preserved in an experiment using probiotics to mitigate the negative impact of oil-exposure on the microbiota of *Mussismilia harttii* (Fragoso ados Santos et al., 2015). In a study sampling multiple species of coral and whale, *Neptuniibacter spp.* occurred in a large percentage of samples regardless of host species (Keller et al., 2021). In addition, *Neptuniibacter spp.* associated with crustose coralline algae has also been positively linked with inducing coral larval settlement (Siboni et al., 2020). To gain species-level identification of this ASV and investigate its functional potential for beneficial host-microbe interactions, metagenomics should be performed on a subset of anemone samples.

Potential mechanisms for host buffering include antimicrobial peptides (Logashina et al., 2017), nutrient regulation (Matthews et al., 2017), and mucosal shedding (Costa et al., 2021). Peptides are major components of anemone toxins that are often cytolytic or neurotoxic, though some exhibit additional antimicrobial properties

(Logashina et al., 2017). Nutrient provisioning in algal symbiosis presents another potential mechanism, where anemones hosting unproductive algae species switch from providing nutrients to an increased catabolism of their nutrient stores (Matthews et al., 2017). Alternatively, nutrient regulation can also occur through microbe-microbe interactions, like when microbiota compete with over the same substrate. For example, as nutrients in the mucus become scarce, the coral pathogen *Serratia marcescens* becomes a more aggressive competitor through the upregulation of multiple glycosidases (Krediet et al., 2009). Mucus may also play a key role in maintaining anemone microbial communities for the host (Rosenberg et al., 2007) through a distinct separation between the mucus layer and the ectoderm by the column cilia, allowing for colonizing bacteria to be expelled through mucosal shedding (Costa et al., 2021). Mucus production was not recorded in this study, however sloughed mucus rings were observed in the water samples, and there is evidence that *D. lineata* generates more mucus at both low (Sassaman & Mangum, 1970) and high temperatures (Cahill et al., 2021).

The communities presented in this study are not expected to be identical the communities in field populations. In *Nematostella vectensis*, the richness of ASVs decreased notably after one month in the laboratory and there were different taxa in high abundance, however population-specific differences in beta-diversity were still evident (Baldassarre et al., 2023). Anemones for this study were stored for several months in the refrigerator at 4°C, which may have artificially selected for bacteria that prefer colder conditions. I aimed to remediate this by inoculating the water with microbiota from the anemone's origin and having a three-day acclimation period at room temperature, but

there may have been priory effects from the cold-tolerant community that cannot be accounted for. A positive trade-off is that anemones in poor health or otherwise damaged during collection had time to perish, resulting in no unexpected mortality throughout the experiment. In addition, the buffering mechanism employed by the host should be unaffected, even if the microbial taxa are different, and I would expect to see similar patterns in beta-diversity.

This is the first study comparing the microbiota of three invasive estuarine anemones across a temperature gradient, where I found that the microbial community responded with a unique pattern for each species and *M. senile* was the only species exhibiting any evidence of buffering. While it appears the more invasive anemone *D. lineata* has a less regulated microbial community than the less invasive *M. senile*, the species-specific results make generalizations about invasion success challenging at this stage. In addition to the prior study recommendations, it would be revealing to compare the results of *D. lineata* to another highly invasive heat-tolerant clonal anemone like *Exaiptasia diaphana* to see if the pattern holds (which would also account for reproductive strategy and genotype). Future studies should also consider the impacts of different abiotic factors to better predict the success of introductions in new environments. Although temperature often plays a larger role in shaping microbial communities than salinity (Baldassarre et al., 2023), pH may also affect community composition as it does in terrestrial environments (Fierer et al., 2011). Finally, direct measurements of stress like oxygen consumption or the expression of superoxide dismutase should be performed in any future experiments to confirm the host is

experiencing stress. Host-associated microbial communities add a new layer of complexity to invasion ecology, where success is not just driven by the environment and physiology of the host, but also by the physiology and interactions between the microbial taxa inhabiting it. The wide geographic spread and broad physiological tolerances of invasive estuarine anemones make them an excellent system for disentangling the complex network of factors that drive invasion success.

CHAPTER 3: NOVEL MICROBIOLOGICAL MEDIUM DEVELOPED FOR THE

ISOLATION OF BACTERIA ASSOCIATED WITH ESTUARINE SEA ANEMONES

Introduction

Out of the nearly one trillion species of microbiota estimated to inhabit Earth, only ten thousand have been cultured in the laboratory (Locey & Lennon, 2016). Recent diversity estimates are driven by the advance of molecular methods for differentiating taxa (Locey & Lennon, 2016), however culturing continues to play a vital role in determining the physiology and ecologic function of individual bacteria (Joint et al., 2010). This is important not just for understanding large-scale microbial contributions to ecosystems, like biogeochemical cycling (Hayatsu et al., 2008; Raina et al., 2009; Tortell et al., 1999), but also for applied research like combating pathogens (Hu et al., 2016; Rosado et al., 2019; Santoro et al., 2021), pharmaceutical exploration (Debbab et al., 2010; Joint et al., 2010), and bioremediation (Muriel-Millán et al., 2021). Through culturing, we can identify the beneficial functions of bacteria in host-associated microbial communities, which produce secondary metabolites (Chen et al., 2019) that can enhance host resilience towards stressors like disease (Lawley et al., 2012; Rosado et al., 2019) and temperature (Cunning & Baker, 2020; Cziesielski et al., 2018; Rosado et al., 2019). Targeted probiotic therapies from cultured bacteria have been employed successfully enhanced coral health by mitigating the effects of temperature stress on bleaching and combating known pathogens (Rosado et al., 2019). While probiotics offer substantial

potential, generating microbe-based therapies first requires identifying the beneficial functions that individual microbiota contribute to the host-microbe system.

Though we can investigate the functional capacity of whole communities through direct measurement (like chlorophyll production) or measuring gene expression via transcriptomics, without culturing it is impossible manipulate the community to determine the role of individual bacteria. Many studies identify that positive host-microbe interactions are occurring, but it is unclear which bacteria are responsible without bringing them into culture. For example, in the diatom *Cylindrotheca fusiformis*, a mixed microbial consortia from the diatom's native environment rapidly reverses growth inhibition induced by *Marinobacter* (Stock et al., 2019). Likewise, in the Hawaiian bobtail squid *Euprymna scolopes*, the accessory nidamental gland becomes stunted or fails to develop entirely without the presence of sediment microbiota from its natural environment (McAnulty et al., 2023). In contrast, a high-throughput culturing assay used combinations of different bacteria in conjunction with five different carbon sources to identify strains that facilitated the growth of the plant symbiont *Herbaspirillum frisingense*. Notably, while one strain of *Burkholderia sp.* was a consistent facilitator regardless of community composition, a *Bacillius sp.* and *Rahnella sp.* only facilitated symbiont growth when they were together (Kehe et al., 2019). Because interactions between different microbial taxa can affect their function within the community, these relationships can be extremely complex to disentangle.

One of the major challenges of culturing is creating media that mimics the bacteria's natural environment enough to promote growth. Microbiological media can be

developed to target host-associated microbes by using host tissue as the sole source of nutrients. For example, a medium produced from yellow onion was used to culture onion-associated pathogens (Zaid et al., 2012). Another medium used fish peptones purified from fish viscera as a less expensive alternative to marine agar to target putative pathogens and beneficial microbiota (Vázquez et al., 2004). A medium made out of alginate, the dominate polysaccharide produced by many kelp species (like *Macrocystis sp.)*, successfully cultured kelp-associated bacteria (Lin et al., 2018).

In this study, bacteria were isolated from anemones and subjected to a thermal stress assay, identifying heat tolerant individuals that may benefit the host under temperature stress. For targeting anemone-associated bacteria, I used autoclaved anemone tissue as the sole source of nutrients in an agar-seawater medium and attempted to culture bacteria from two species of sea anemone: *Metridium senile* and *Diadumene lineata* (anemones were stored as described in the collection section of Chapter 2). Samples from the same anemones were plated on both anemone medium and marine agar to determine if unique host-associated taxa could be recovered with anemone nutrients alone. Sanger sequencing the isolates allowed for a comparison between the two media. To identify individual candidates that could contribute to host resilience in warm environments, isolated bacteria were subjected to a heat stress assay measuring their growth over time across a temperature gradient. Although the anemones are closely related and inhabit similar environments, *D. lineata* displays a greater heat tolerance than *M. senile* (Glon et al., 2019; Shick, 1976). If microbiota are mitigating heat stress in the

host, I expect isolates from *D. lineata* to display greater tolerance at high temperatures

than isolates from *M. senile*.

Methods

<u>Summary</u>

A microbiological medium was developed using whole tissue from anemones and artificial seawater. Bacteria from *Diadumene lineata* and *Metridium senile* were cultured on both the anemone-based medium and marine agar. Isolates were selected from both media based on morphology and cryopreserved at -80C using glycerol. For each isolate, the 16S rRNA gene was amplified using single-colony PCR so that they could be identified to genus via Sanger sequencing (in conjunction with the BLAST algorithm). Finally, a subset of isolates were selected for a thermal stress assay to measure their growth at 30°C, 35°C, and 40°C, identifying bacteria that were particularly thermal tolerant. Although 40°C is above the upper temperature limit in which the hosts survive, I am also interested in defining the upper limits for these isolates.

<u>Development of Anemone Medium</u>

A microbiological medium was developed using anemone tissue as the sole source of nutrients, to target host-associated bacteria for isolation. Large *Metridium senile* were collected from the marina at Woodley Island in Humboldt Bay (Eureka, California) using a palette knife to gently dislodge them from the bottom of fouled plastic buoys. The anemones were rinsed of excess debris and mucus, before retiring under high pressure at 121°C (to breakdown the tissue). The tissue was further homogenized using a coffee grinder and frozen in 50 mL aliquots at -20°C. An initial trial was run testing anemone:water ratios of 1:1, 1:2, 1:3, and 1:4, which all exhibited robust bacterial

growth. A ratio of one part anemone tissue for every three parts 35 ppt artificial seawater (by volume) was ultimately selected because the bacterial colonies were moderately sized after 24 hours of incubation. The anemone medium was prepared as both a broth and in solid agar plates, with the solid medium requiring the addition of 20 g of agar per 1000 mL. Marine agar was prepared for comparison, following the product instructions for BD Difco Marine Broth (product #2216) and adding 15 g/mL of agar (the same concentration as BD Difco Marine Agar).

Isolation of Bacteria

Two small anemones (one *Diadumene lineata* and one *Metridium senile*) were rinsed with sterile deionized water and then transferred to a 1.5 mL microcentrifuge tube filled with 1000 mL 35 ppt artificial seawater. They were macerated using a sterile plastic pestle and then vortexed vigorously. 1:10 serial dilutions were prepared from each macerated anemone using 100 uL sample in 900 uL of 35 ppt artificial seawater. 100 uL from the 10^0, 10^{-1}, and 10^{-2} dilutions were plated on both anemone agar and marine agar in duplicate. Marine agar is a commercially-prepared medium commonly used for culturing marine bacteria. Negative controls for both media were prepared using 100 uL of sterile 35 ppt artificial seawater. The plates were incubated for approximately 48 hours at 30°C, which is on the high end of temperature tolerance for *Diadumene lineata*. While a longer incubation period of one week was initially planned to account for slower growing bacteria, the plates became too overgrown to effectively isolate individual colonies. Colonies exhibiting a variety of morphologies were transferred to fresh anemone or marine medium using a T-streak. Isolates recovered on anemone medium

were also plated on marine agar to see if they could grow without the presence of

anemone tissue (*Figure 23*).
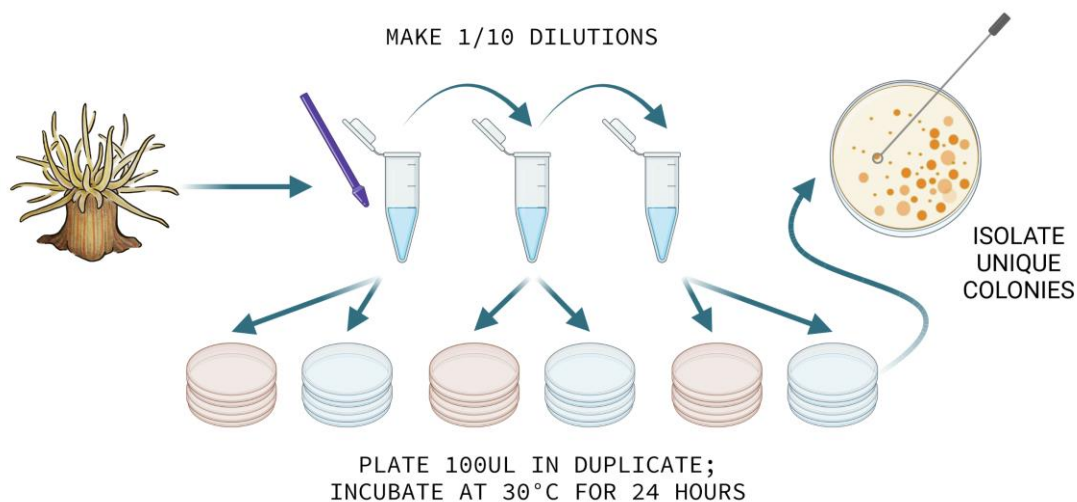
Figure 23. Process of bacterial isolation from Diadumene lineata and Metridium senile. Although only D. lineata is shown in this diagram, the same procedure was performed for both anemone species. The brown plates represent the anemone medium, while the blue plates represent marine agar. Created with BioRender.com.

Cryopreservation

All isolates were cryopreserved at -80°C using 50% v/v glycerol. Cultures were

grown in 10mL of either marine or anemone broth for approximately 48 hours, along

with 10 mL of each broth as a negative control. 500 uL of broth was combined with 500

uL of sterile 50% v/v glycerol in Nalgene Cryogenic Tubes (cat. #5000-1012). Both the

48-hour cultures and the frozen isolates were plated on their respective media and

incubated at 30°C for 24 hours, to verify no contamination was introduced at any step of

the cryopreservation process.

Sanger Sequencing

Single colony PCR was performed on fresh 24-hour cultures using Thermo DreamTaq Green PCR Master Mix (cat. #K1081). The 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') universal bacteria primers were used to target the 16S rRNA gene. PCR reactions were 50 uL total, including 0.5 uL dimethyl sulfoxide added to encourage lysis. PCR reaction: 95℃ for 4:00 minutes, followed by 35 cycles of 94℃ for 1:00 minute, 68℃ for 1:00 minute, and 72℃ for 1:00 minute, then 72℃ for 7:00 minutes before holding at 4℃ indefinitely. PCR was checked for successful amplification using gel electrophoresis. The amplified products were purified using PureLink kit (cat. #K310002; a few modifications were made to the manufacturer protocol to increase the concentration of DNA, including increasing incubation times by two to three minutes and eluting the DNA in 25 uL instead of 50 uL. DNA concentrations were measured using the ND-1000 Nanodrop, and then shipped for sequencing via the Eurofins Sanger Sequencing service. Sequences were assessed for quality using Applied Biosystems Sequence Scanner 2, trimming regions of poor quality on the ends and checking for errors, then identified to genus using NCBI BLAST.

<u>Isolate Thermal Stress Assay</u>

An initial trial was conducted to make sure the marine broth did not interfere with measuring the optical density. For the trial, a single isolate (DL-MA-07) was grown over the course of six hours, measuring growth at hours three through six by both optical density and plating on marine agar. Some of the plates were difficult to analyze, so DNA from frozen samples at each time point were extracted using the ZymoBiomics Miniprep

Kit and qPCR was run on the 16S rRNA gene to estimate the change in cell abundance over time. The growth measured via OD600 and qPCR were close enough to continue with the experiment (only one time point was abnormally low due to a low yield of extracted DNA) (*Figure 29*, *Appendix C*).

For the thermal stress assay, 10 isolates were grown for 24 hours in 5 mL of marine broth at 30°C on an orbital shaker. Sterile marine broth was used as a negative control at all stages of the assay. A wide representation of genera from both anemones were selected for the assay, with the exclusion of an *Arcobacter sp.* which failed to grow from the -80°C stock (and was replaced by an additional *Pseudoalteromonas sp.)*. Three replicate one mL aliquots were cryopreserved at -20°C with one mL of sterile 50% v/v glycerol for each isolate, so that each of the three temperature treatments would start with a similar concentration of bacteria (per isolate, all isolates were not diluted to the same concentration). Isolates were plated on marine agar at the time of cryopreservation to ensure no contamination was introduced during culturing (*Figure 24*). Each one mL aliquot was used to inoculate 50 mL of marine broth, and the flasks were placed on an incubating orbital shaker to ensure the cultures stayed homogenized and aerated during growth. Nine time points were sampled over the course of approximately nine hours. At each sampling time point, 100 uL samples were transferred in triplicate to a 96-well plate after every hour of growth and the OD600 was measured using the SpectraMax i3 plate reader. There were three temperature treatments total: 30°C, 35°C, and 40°C. Each treatment was run on a separate day so that the same incubating orbital shaker could be

used, and a HOBO datalogger was placed in the incubating orbital shaker to track any fluctuations in temperature.

Before analysis, one isolate (MS-MA-04) was excluded due to poor recovery from cryopreservation; although it grew in the initial culturing step from the -80°C freezer it did not grow substantially in any temperature treatment. The absorbance values of all replicates were averaged, then the average of the negative control replicates was subtracted. Very small negative values that occurred when measuring optical densities close to zero were converted to zero for plotting growth over time.
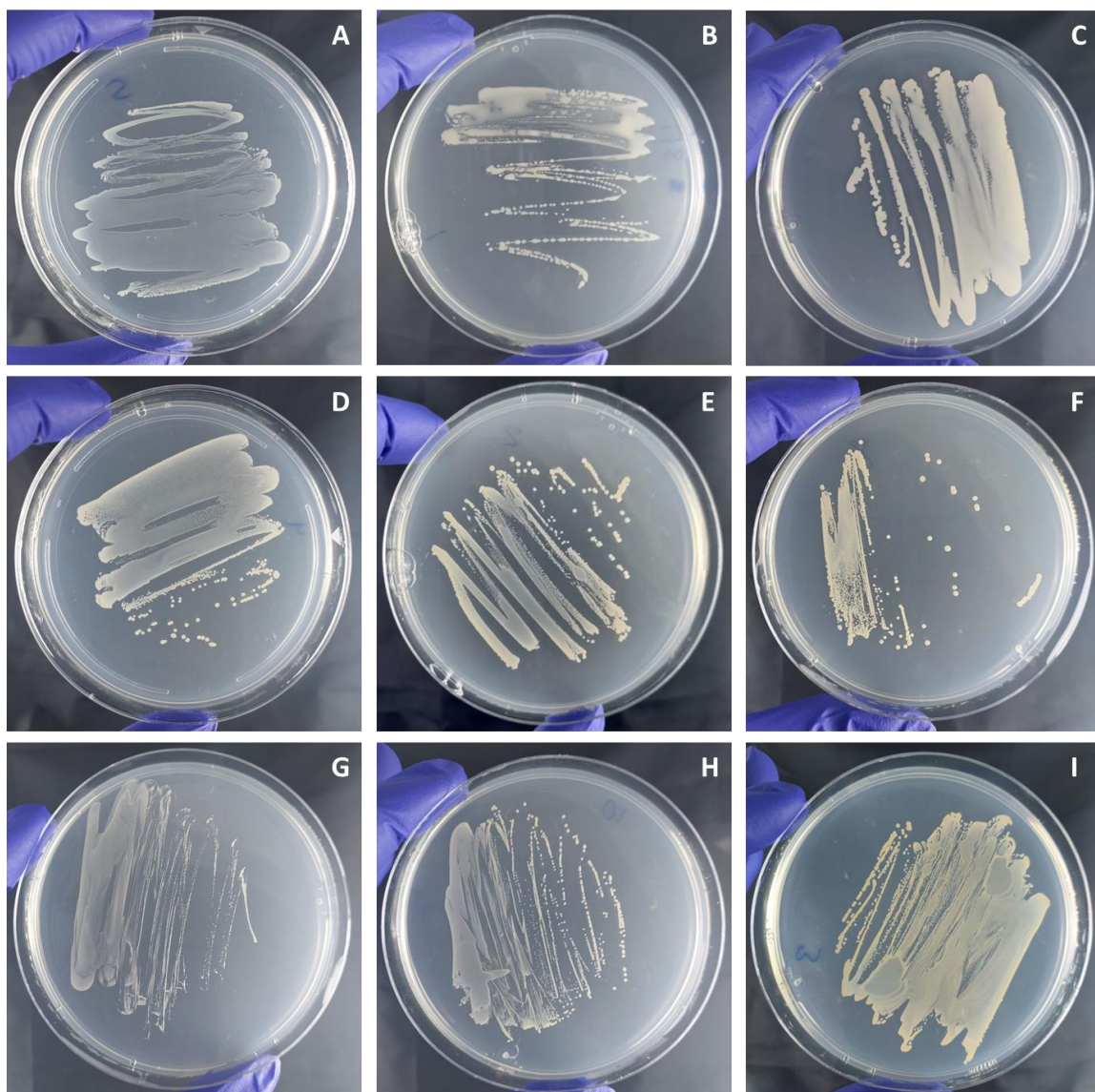
Figure 24. Subset of the anemone-associated bacteria isolates that were selected for the thermal stress assay. (A) DL-AM-10 Pseudoalteromonas, (B) MS-AM-01 Pseudoalteromonas, (C) MS-MA-06 Pseudoalteromonas, (D) DL-AM-06 Peribacillus, (E) DL-MA-07 Vibrio/Peribacillus, (F) MS-MA-02 Peribacillus, (G) MS-MA-01 Litoreibacter, (H) MS-MA-05 Colwellia, and (I) DL-MA-08 Shewanella. There was a mix-up and DL-MA-07 needs to be re-sequenced to determine if it is Vibrio or Peribacillus.

Results

Thirty-three bacteria were isolated in total: 19 from *D. lineata* and 14 from *M. senile*. From *D. lineata*, eight were from marine agar and 11 were from anemone medium; from *M.senile* eight were from marine agar and six were from anemone medium. All bacteria originally isolated on anemone medium also grew on marine agar without issue. Eight unique genera were recovered on marine agar, while only three were recovered on anemone medium. Although most of the bacteria recovered on both media were *Pseudoalteromonas spp.* or *Peribacillus spp.*, they were heavily selected for on the anemone medium and the only other genus isolated was *Colwellia* (*Table 4*). There was not a substantial difference between the number of genera recovered from *D. lineata* versus *M. Senile*, however different rare taxa were represented.

Table 4. Summary of 33 bacteria isolated and identified to genus using sanger sequencing and NCBI BLAST. The first column shows the percentage of each genus isolated out of the total number of isolates. The second column breaks down the isolated by which medium they were cultured on, while the third column breaks down the same isolates by which anemone species they came from.

Genus	All Isolates (N=33)	Marine Agar (N=16)	Anemone Medium (N=17)	*D. lineata* (N=19)	*M. senile* (N=14)
Pseudoalteromonas	51.52% (17/33)	68.75% (11/16)	64.71% (11/17)	57.89% (11/19)	42.86% (6/14)
Peribacillus	27.27% (9/33)	25.00% (4/16)	29.41% (5/17)	21.05% (4/19)	35.71% (5/14)
Arcobacter	3.03% (1/33)	6.26% (1/16)	0	5.26% (1/19)	0
Shewanella	3.03% (1/33)	6.26% (1/16)	0	5.26% (1/19)	0
Colwellia	6.06% (2/33)	6.26% (1/16)	5.88% (1/17)	5.26% (1/19)	7.14% (1/14)

Genus	All Isolates (N=33)	Marine Agar (N=16)	Anemone Medium (N=17)	*D. lineata* (N=19)	*M. senile* (N=14)
Vibrio	3.03% (1/33)	6.26% (1/16)	0	5.26% (1/19)	0
Litoreibacter	3.03% (1/33)	6.26% (1/16)	0	0	7.14% (1/14)
Macrococcus	3.03% (1/33)	6.26% (1/16)	0	0	7.14% (1/14)
Total Richness	**8**	**8**	**3**	**6**	**5**

Isolates of *Pseudoalteromonas* exhibited robust growth at both the 30°C and 35°C temperatures, with very little growth at 40°C (*Figure 25*). One *Pseudoalateromonas* isolate in particular from *M. senile* showed a strong preference for the 35°C treatment. Isolates of *Peribacillus* had the broadest range of temperature tolerance, following a similar growth pattern from 30-40°C. In contrast, *Litoreibacter*, *Colwellia*, and *Shewanella* isolates grew best in the 30°C treatment, with minimal growth at 35°C (for *Litoreibacter* only) and no growth at 40°C. HOBO dataloggers reported average temperatures of 30.40°C (st.deviation of 0.14°C), 35.61°C (st.deviation of 0.28°C), and 40.95°C (st.deviation of 0.37°C).
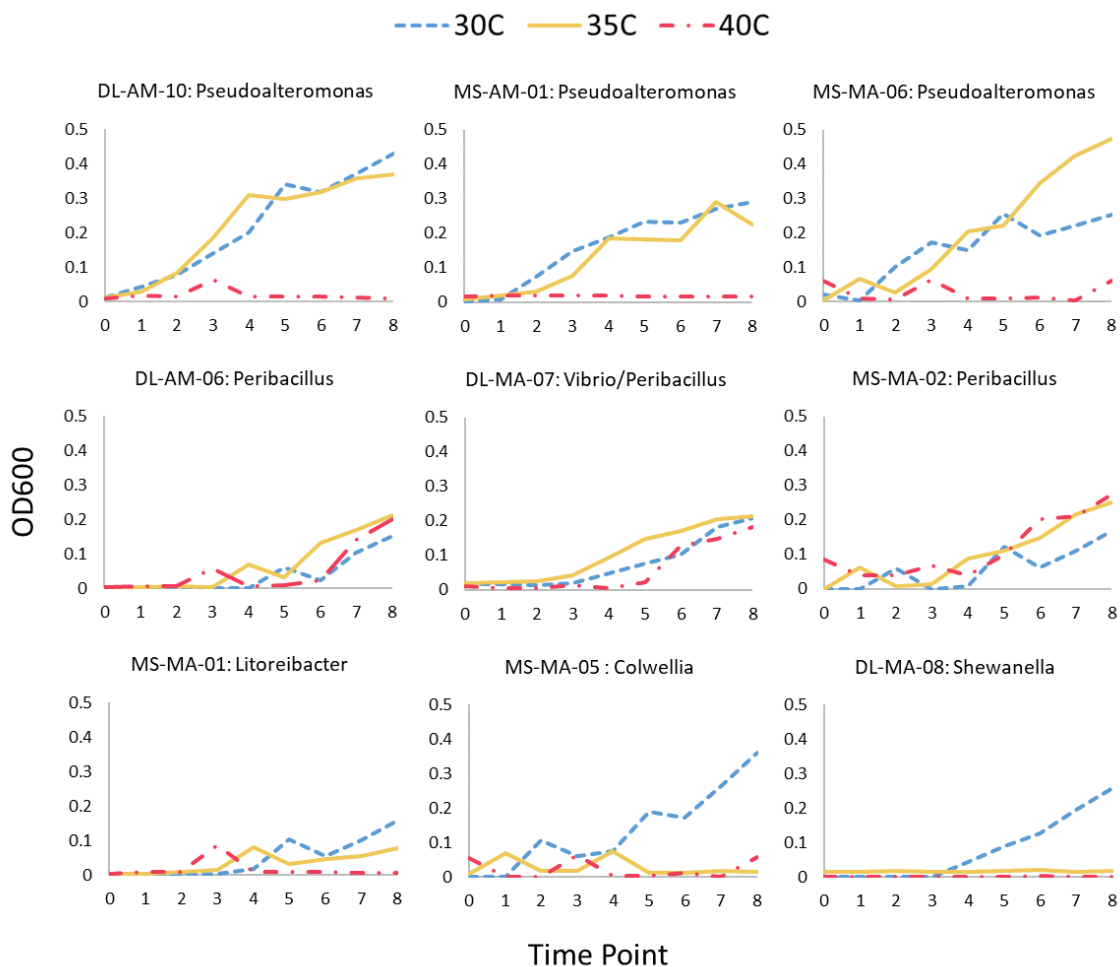
Figure 25. Growth of nine isolated bacteria from the sea anemones Diadumene lineata and Metridium senile. Growth was measured at regular intervals over the course of 9 hours using optical density at a wavelength of 600.

Discussion

The anemone medium captured a lower diversity of microorganisms than the marine agar and heavily selected for bacteria from the genera *Pseudoalteromonas* and *Peribacillus*. Lin et al., (2018) found that *Pseudoalteromonas* was also a dominant genus on medium where alginate is the sole source of nutrients, using isolated bacteria from kelp. Although the diversity was higher, marine agar may preferentially select for *Gammaproteobacteria* (Joint et al., 2010), which include the genera *Pseudoalteromonas, Shewanella*, *Vibrio*, and *Colwellia* from this set of isolates. When comparing our isolated taxa to the results of 16S rRNA amplicon sequencing in Chapter 2, only 3.6% of genera were recovered on marine agar and 1.6% on anemone medium, however these anemones were living in the laboratory for multiple months, which could have reduced the bacterial diversity.

In the thermal stress assay, *Peribacillus* isolates exhibited the broadest temperature tolerance between 30°C and 40°C, however *Pseudoalteromonas* isolates also grew well up to 35°C. In contrast, *Litoreibacter*, *Colwellia*, and *Shewanella* isoaltes all preferred lower temperatures, growing best at 30°C. *Litoreibacter spp.* have solely been isolated from marine environments and are often psychrotolerant, with optimal growth temperatures ranging from 25°C-30°C (Kanamuro et al., 2021; Kim et al., 2014; Romanenko et al., 2011). A dark diffuse pigment was observed in our isolate, which has similarly been observed in *Litoreibacter sp.* cultured from marine sediment (Romanenko et al., 2011). This may be a pyomelanin-like compound, which is associated with

protecting cells during heat stress in other bacterial taxa (Zeng et al., 2017). *Colwellia sp.* also tend to be psychrotolerant or psychrophilic (Bowman et al., 1998; Deming & Junge, 2015). Species of *Shewanella* are known pathogens in humans and aquatic organisms, with infections typically occurring in warm climates, however the majority of species in this genus are psychrophilic (Holt et al., 2005). Although the *Colwellia* and *Shewanella* isolates did not grow about 30°C, they may still be considered particularly thermotolerant representatives of their genera. While *Pseudoalteromonas spp.* may be the best candidate for beneficial host-microbe interactions, based on its thermotolerance aligning with *D. lineata*'s upper temperature limit, the possible pyomelanin production in *Literoreibacter* is also worth exploring.

The highly selective nature of the anemone medium would benefit research in marine probiotics and bioremediation by targeting novel *Pseudoalteromonas spp.* and *Peribacillus spp.* from marine environments. *Pseudoalteromonas sp.* have been noted for their capacity to readily colonize man-made surfaces through the rapid formation of biofilms (Wu et al., 2021), as well as their prolific production of bioactive secondary metabolites (Atencio et al., 2020). The BLAST results for *Pseudoaltermonas* isolate DL-AM-10 (used in the mock community in Chapter 3) shows a 99.71% match with a partial bacteria sequence from a jellyfish bell (accession # KF816501.1), as well as a 99.43% match with *Pseudoalteromonas nigrifaciens* (accession #OP344302.1), *Pseudoalteromonas carrageenovora* (accession #ON318401.1), and *Pseudoalteromonas spiralis* (accession #ON318067.1). These species are all part of a phylogenetically-related cluster of non-pigmented *Pseudoalteromonas* (Bowman, 2007; Rathgeber et al., 2006),

which are generally considered to produce less bioactive compounds than pigmented

species (many produce antibiotics and antifungals). Although non-pigmented species are

less well studied, current evidence suggests they tend to be generalists, displaying broad

physiological tolerances and unique enzymes like carrageenases and chitinases that allow

them to take advantage of a variety of nutrient sources (Bowman, 2007). Similar to many

Pseudoalteromonas sp., marine *Bacillus* also produce a variety of secondary metabolites

of interest (Mondol et al., 2013), including lipopeptides with surfactant properties that

could be used in oil spill remediation (Hentati et al., 2019), antibiotics, and antifungals

(Franks et al., 2006; Shnit-Orland et al., 2012). Both *Pseudoalteromonas* and *Peribacillus*

have the potential to combat opportunistic bacteria from the environment when the host is

experiencing heat stress. While less research has investigated *Perbacillus spp.* in marine

hosts, hemolytic *Pseudoalteromonas spp.* have been found living aggregated inside the

tentacles of the anemone *Sargatia elegans* (Schuett et al., 2007) and *Pseudoalteromonas*

has been identified as a putative beneficial genus for corals (Rosado et al., 2019).

Alternatively, the capacity for these genera to feed on anemone tissue, in

conjunction with their high temperature tolerance, may suggest the capacity for becoming

opportunistic pathogens. Both genera were abundant in samples from both anemone

species, and exhibited similar growth patterns regardless of which anemone they were

cultured from or host temperature tolerance. In an onion-based medium, known

pathogens grew better on onion tissue and non-pathogenic onion bacteria grew better on

less selective LB medium (Zaid et al., 2012). However, in the 16S rRNA data from

Chapter 2, we do not see increases in these genera in the mortality community (although

Pseudoalteromonas spp. are prevalent in both *M.senile* and *D. leucolena*). It may be that these genera are feeding on the mucus of the anemone, not directly on the tissue. *Pseudoalteromonas spp.* have been found in the mucus layer of corals, some of which had antibacterial properties that could protect the host (Shnit-Orland et al., 2012). Future research could either culture isolates from different parts of the anemone, or test if the isolates in this study grow on a medium prepared only with anemone mucus versus anemone tissue (Schuett et al., 2007 describes a washing procedure for removing external bacteria from the anemone).

When using the anemone medium in future research, one issue to consider is the variation that occurs between batches of ground anemone. The anemone tissue had varying amounts of air whipped into it during the grinding process, resulting in some 50 mL aliquots containing more air pockets than others. There were also differences in viscosity between batches, likely related to the quantity of mucus, which affected how well the anemone tissue homogenized in the medium solution. Although this never prevented the growth of the cultured isolates, it is an important consideration for reproducibility in an experiment. A final potential issue is that the anemone medium had a very short shelf life (even when refrigerated) and lasted about five days before bacteria stopped growing on it entirely. Throughout this study, the medium was prepared fresh the day before it was intended to be used, which may be inconvenient for some experimental designs.

Microbiological medium made from anemone tissue successfully cultured host-associated microbiota and was more selective than commercially available marine agar.

Similar dominant genera (*Pseudoalteromonas* and *Peribacillus*) were cultured from both estuarine anemone species *D. lineata* and *M. senile* on both media (but were preferentially selected for by the anemone medium). Although I was unable to detect a difference between bacteria recovered from two different anemone species, the robust growth at 30-35°C in conjunction with research on probiotics in corals (Rosado et al., 2019) highlights the genus *Pseudoalteromonas* as a potential candidate for beneficial anemone-microbe interactions under high temperature stress. Further research should explore the production of secondary metabolites in these isolates, particularly antimicrobial/antifungal activity towards environmental microbiota. This work supports culturing as a critical component of unraveling the individual functions of these bacteria in anemone-associated microbial communities.

CONCLUSION

I investigated the microbial communities associated with estuarine anemones, aiming to advance our understanding of microbial contributions to thermal tolerance and geographic distributions in marine invertebrate hosts. At the regional scale, I found that the richness, diversity, and beta-dispersion of microbial communities associated with *D. lineata* differed across collection sites, however differences among sites did not strictly coincide with latitude. There was significant overlap in microbial community composition across sites along the west coast of North America and differences among sites were primarily seen in beta-dispersion (resulting in higher diversity driven by an increased richness of ASVs). *Desulfobacterota*, *Syntrophobacterales,* and *Chromobaterium* were in higher abundance, particularly at the Arcata Marsh site, which may reflect a taxa-specific response to local conditions.

In the temperature gradient experiment, the microbial communities of the three anemone species exhibited a different pattern of response to changes in temperature, except for post-mortality where the identities of top taxa in the communities shifted substantially for all species (40°C for the *Diadumene sp.* and above 30°C for *M. senile*). *D. lineata* lacked evidence of buffering, displaying unique microbial communities between adjacent temperatures and high richness at moderate temperature. In contrast, *D. leucolena* had a highly variable communities across most temperatures, with high richness and high beta-dispersion that did not follow an expected pattern of stress. *M. senile* was the only anemone species where low richness in adjacent temperatures

coincided with low beta-dispersion and overlapping microbial community composition, suggesting buffering may be occurring.

The anemone medium captured a lower diversity of microorganisms than the marine agar and heavily selected for bacteria from the genera *Pseudoalteromonas* and *Peribacillus*. *Peribacillus* isolates exhibited the broadest temperature tolerance between 30°C and 40°C, however *Pseudoalteromonas* isolates also grew well up to 35°C. In contrast, *Litoreibacter*, *Colwellia*, and *Shewanella* isolates all preferred lower temperatures, growing best at 30°C. Regardless of which host they were isolated from, the *Pseudoalteromonas* and *Peribacillus* isolates grew similarly in response to temperature.

In conjunction, these three scales of ecological complexity show a clear response to temperature in the microbial communities inhabiting estuarine anemones, however further research is needed to demonstrate whether host-microbe or microbe-microbe interactions affect invasion success. High beta-dispersion in field conditions supports less regulation in the microbial community on *D. lineata*, particularly at moderate temperatures, and its microbiota display a flexible response when exposed to a thermal gradient. Isolating thermotolerant host-associated bacteria further paves the way for manipulation experiments directly testing their capacity to increase host resilience to clarify the relative contribution of microbiota to host resilience. The wide geographic spread and broad physiological tolerances of invasive estuarine anemones make them an excellent system for disentangling the complex interplay of environmental factors, host-

microbe interactions, and microbe-microbe interactions shaping geographic distributions and facilitating invasion success.

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APPENDICES

Appendix A: Chapter 1 Supplementary Materials
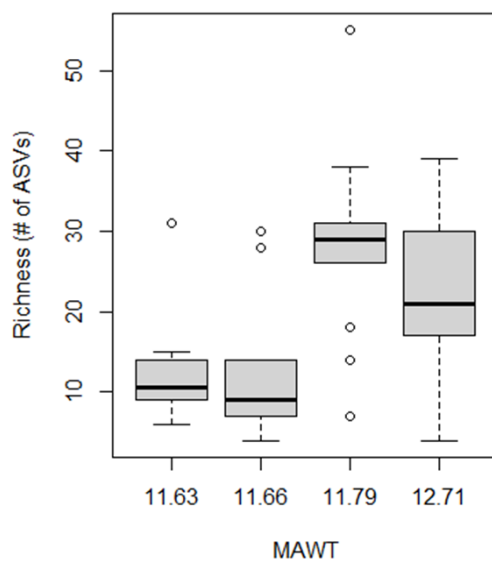
Figure 26. Richness (number of amplicon sequence variants) versus mean annual water temperature (MAWT), measured in Celsius.

Top 10 Bacteria Genera
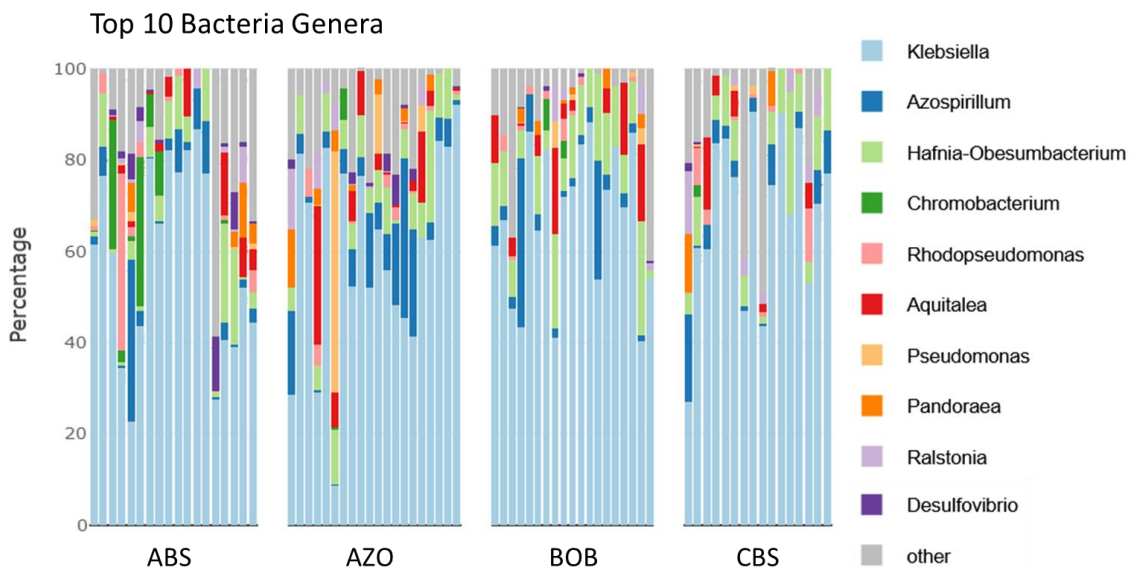
Figure 27. Relative abundance of the top 10 bacteria genera across all sites. Reads were normalized with CSS before converting to relative abundance. Relative abundance figure generated using the MicrobiomeExplorer package in R (Reeder et al. 2021).

<u>Methods considerations for future anemone-microbe projects</u>

Many anemone samples in Chapter 1 and Chapter 2 were omitted due to an excessively low number of reads, and it is not uncommon for samples containing a high percentage of host DNA to have issues with recovering microbial DNA. Differences in reads between samples tends to have a larger impact on alpha diversity than beta diversity, since dominate taxa are often still well-represented while rarer taxa are lost (Reigel et al., 2020), however future studies could improve sample retention by adjusting the tissue processing method and reducing host DNA contamination. These samples were processed mechanically with bead bashing during the lysis step, however using a tissue homogenizer would result in higher bacterial diversity and lower variance between anemone samples (Rocha et al., 2014). In addition, large concentrations of host DNA

include mitochondrial DNA from host cells that get targeted by the same 16S rRNA primers used for bacteria. This results in a high percentage of mitochondrial reads needing to be removed, which can be detrimental for samples that already have low reads. Increasing the read depth can ameliorate this effect, however it is often cost prohibitive. An alternative is inhibiting the amplification of host DNA using a species-specific peptide nucleic acid (PNA) clamps. Using a PNA clamp on the gorgonian *Eunicea flexuosa* resulted in the percentage of reads associated with microbiota increasing from 13.7% (an average of 2,469 microbial reads) to 80.8% (an average of 17,215 microbial reads) (Reigel et al., 2020). Improving tissue processing and reducing host DNA contamination would lead to higher quality results and sample retention in cnidarian hosts, without substantially increasing the cost.

Appendix B: Chapter 2 Supplementary Materials

OBIS Citations

The data used to generate the maps in Chapter 2 were compiled by the Ocean

Biodiversity Information System (OBIS) database from existing records. All datasets

were accessed on January 2nd, 2023. OBIS requires all datasets to be individually cited.

The following tables of citations were exported alongside the downloaded datasets on

January 2nd, 2023. Where citations were missing, I listed who uploaded the data and their

association.

Table 5. Citations used to create maps for the distribution of Diadumene lineata, generated by OBIS.

Dataset Name	Citation
Hexacorallians of the world	Fautin, Daphne G. (2013). Hexacorallians of the World.
Coastal Habitat Invasives Monitoring Program	Contributors to the Coastal Habitat Invasives Monitoring Program Database (2007) Coastal Habitat Invasives Monitoring Program Dataset; generated by Parker Lund; using Ocean Biogeographic Information System (OBIS) [online application]. Salem, MA: Salem Sound Coastwatch [producer], Cambridge, MA: MIT Sea Grant College Program, Massachusetts Institute of Technology [distributor], New Brunswick, NJ: OBIS, Rutgers University Institute of Marine and Coastal Science [distributor]. Http://www.iobis.org/mapper; accessed on January 2nd 2023.
The entire coast survey of Hatakejima Islands from 1983	Nakano, T. (2021) The entire coast survey of Hatakejima Islands from 1983. Available at https://doi.org/10.48518/00011. Accessed on 2023-01-02.
The south coast survey of Hatakejima Islands from 1969	Nakano, T. (2021) The south coast survey of Hatakejima Islands from 1969. Available at https://doi.org/10.48518/00012. Accessed on 2023-01-02.
Rapid Assessment Surveys of Native and Introduced Marine Organisms in the Northeast United States; Staten Island, New York to Eastport, Maine	Contributors to the Rapid Assessment Surveys Database (2007) Rapid Assessment Survey Dataset; generated by Parker Lund; using Ocean Biogeographic Information System (OBIS) [online application]. Cambridge, MA: MIT Sea Grant College Program, Massachusetts Institute of Technology [producer and distributor], New Brunswick, NJ: OBIS, Rutgers University Institute of Marine and Coastal Science [distributor]. http://www.iobis.org/mapper; accessed on January 2nd 2023.
Marine Recorder Snapshot extract of surveys entered by Natural England	Natural England (NE) (2021): Marine Recorder Snapshot extract of surveys entered by Natural England. v2.0. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/thn0xd

Dataset Name	Citation
Marine Recorder Snapshot extract of surveys entered by JNCC	Joint Nature Conservation Committee (JNCC) (2021): Marine Recorder Snapshot extract of surveys entered by JNCC. v2.0. Marine Biological Association. Dataset/Samplingevent https://doi.org/10.17031/mehqrq
Marine Recorder Snapshot extract of surveys entered by NRW	Natural Resources Wales (NRW) (2021): Marine Recorder Snapshot extract of surveys entered by NRW. v2.0. Marine Biological Association. Dataset/Samplingevent https://doi.org/10.17031/b3efts
Historical benthos data from the Romanian Black Sea Coast between 1954 and 1968	Teaca, A.; Begun, T.; Muresan, M.; National Research and Development Institute for Marine Geology and Geoecology – GeoEcoMar, Romania (2016): Historical benthos data from the Romanian Black Sea Coast between 1954 and 1968
Coleção de Cnidaria do Museu Nacional (MNRJ)	MNRJ(2015) Coleção de Cnidaria do Museu Nacional da Universidade Federal do Rio de Janeiro (Hexacorallia e Octocorallia), 7679 registros (atualizado em 15/12/2016).
Marine species citizen-science observations from NatureWatch NZ	NatureWatch NZ (2016). Marine species citizen-science observations from NatureWatch NZ. Southwestern Pacific OBIS, National Institute of Water and Atmospheric Research (NIWA), Wellington, New Zealand, 13030 records, Online http://nzobisipt.niwa.co.nz/resource.do?r=naturewatchnz released on March 26, 2017.
Marine Invader Monitoring and Information Collaborative	Contributors to the Marine Invader Monitoring and Information Collaborative Database (2010) Marine Invader Monitoring and Information Collaborative Dataset; generated by Parker Lund; using Ocean Biogeographic Information System (OBIS) [online application]. Boston, MA: Massachusetts Office of Coastal Zone Management [producer], Cambridge, MA: MIT Sea Grant College Program, Massachusetts Institute of Technology [distributor], New Brunswick, NJ: OBIS, Rutgers University Institute of Marine and Coastal Science [distributor]. http://www.iobis.org/mapper; accessed on January 2nd 2023.
Nonindigenous Aquatic Species (NAS) Database Non-freshwater Specimens	None listed, uploaded by Pam Fuller with the U.S. Geological Survey.
CAS Invertebrate Zoology (IZ)	None listed, uploaded by Christina Piotrowski with the California Academy of Sciences.
1915-2016 Department for Environment Food & Rural Affairs (Defra), Marine Strategy Framework Directive (MSFD) Collation of invasive non-indigenous species	Department for Environment Food and Rural Affairs (Defra) (2018): 1915-2016 Department for Environment Food & Rural Affairs (Defra), Marine Strategy Framework Directive (MSFD) Collation of invasive non-indigenous species. https://doi.org/10.17031/f0vfo3
Marine Recorder Snapshot extract of surveys entered by SeaSearch	SeaSearch (2021): Marine Recorder Snapshot extract of surveys entered by SeaSearch. v2.0. Marine Biological Association. Dataset/Samplingevent https://doi.org/10.17031/yq0gbg
Marine Recorder Snapshot extract of surveys entered by National Museums Northern Ireland (NMNI)	National Museums Northern Ireland (NMNI) (2021): Marine Recorder Snapshot extract of surveys entered by National Museums Northern Ireland (NMNI). v2.0. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/frdvov

Dataset Name	Citation
Taxonomic Information System for the Belgian coastal area	Flanders Marine Institute (VLIZ). Taxonomic Information System for the Belgian coastal area. 10 Aug 2004, Oostende, Belgium, Accessed on 02/01/2023.
IndOBIS Dataset (20001-22000)	Indian Ocean Biodiversity Information System (IndOBIS)- Distribution records of marine organisms from the Indian Ocean.
Ocean Genome Legacy Collection	Accession ID OGL-#####. The Ocean Genome Legacy Center. Northeastern University. Published on the web at ogl.northeastern.edu/catalog.
UF Invertebrate Zoology	None listed, uploaded by Gustav Paulay with the Florida Museum of Natural History.
IndOBIS, Indian Ocean Node of OBIS	Chavan, VIshwas and C. T. Achuthankutty (editors), IndOBIS Catalogue of Life, Available at http://www.indobis.org/, Retrived January 2nd 2023.
IOW Natural History & Archaeological Society Marine Invertebrate Records 1853- 2011	UK National Biodiversity Network: Isle of Wight Local Records Centre - IOW Natural History & Archaeological Society Marine Invertebrate Records 1853- 2011. Accessed via http://www.gbif.org/dataset/8b97e0ae-c803-4d24-8098-74874113c344 on 2023-01-02 https://doi.org/10.15468/d9amhg
Auckland Museum NZ Marine Collection	Blom W, Moriarty A (2018). Auckland Museum NZ Marine Collection. Version 1.11. Auckland War Memorial Museum. Occurrence Dataset https://doi.org/10.15468/plyefd accessed via GBIF.org on 2018-01-15.
Benthic Network	Agence pour la recherche et la Valorisation Marines (ARVAM); Cellule De Suivi Du Littoral Normand (CSLN); Creocean; Groupe d'Etude des Milieux Estuariens et Littoraux; Groupe d'étude des milieux estuariens et littoraux Normandie (GEMEL Normandie); Hemisphere Sub; Impact Mer; Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER); MAREX Expertise & Conseil en Environnement Marin (MAREX); National Natural History Museum Paris; CRESCO - Station de biologie marine de Dinard; National Natural History Museum Paris; Station de Biologie Marine de Concarneau; Pierre & Marie Curie University; Roscoff biology station (RBS); TBM Environnement; The National Center for Scientific Research; Laboratoire d'Ecogéochimie des Environnements Benthiques UMR 8222 (LECOB); The National Center for Scientific Research; Laboratoire d'Océanologie et de Géosciences - UMR 8187 LOG (LOG); Université Bordeaux 1; Environnements et Paléoenvironnements Océaniques (EPOC); Université de Bretagne Occidentale; Institut Universitaire Européen de la Mer (IUEM); Université de Bretagne Occidentale; Laboratoire d'écophysiologie et de biotechnologie des halophytes et algues Marines (IUEM-LEBHAM); Université de Bretagne Occidentale; Laboratory of Sciences of the Marine Environment CNRS-UMR6539 (LEMAR); Université de la Réunion; Laboratoire d'Ecologie Marine (ECOMAR); Université de Liège; Station de Recherche Sous-marines et Océanographiques (STARESO); Université de Nantes; Laboratoire de Biologie Marine (Bio-littoral); Université des Sciences et Technologies de Lille; Station Marine de Wimereux (INSU/CNRS); (2019): REBENT - Réseau Benthique.
Vulnerable marine ecosystems in the South Pacific Ocean region	NIWA (2016). Vulnerable marine ecosystems in the South Pacific Ocean region. National Institute of Water and Atmospheric Research (NIWA), Wellington, New Zealand, 202873 records, Online

Dataset Name	Citation
	http://nzobisipt.niwa.co.nz/resource.do?r=vme_inverts released on February 3, 2016.
National Museum of Natural History Invertebrate Zoology Collections	National Museum of Natural History, Smithsonian Institution NMNH Invertebrate Zoology Collection Database. National Museum of Natural History, Smithsonian Institution, 10th and Constitution Ave. N.W., Washington, DC 20560-0193, 2001, Version 3.2.04 (0802221).
Marine Recorder Snapshot extract of surveys entered by The archive for marine species and habitats data (DASSH)	The archive for marine species and habitats data (DASSH) (2021): Marine Recorder Snapshot extract of surveys entered by The archive for marine species and habitats data (DASSH). v2.1. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/myrqac
SOMBASE/TOTAL - Bioconstructors	GRIFFITHS, H.J., LINSE, K. & CRAME, J.A. 2003. SOMBASE – Southern Ocean Mollusc Database: A tool for biogeographic analysis in diversity and ecology. Organisms Diversity and Evolution. 3, 207–213.
Marine Recorder Snapshot extract of surveys entered by NatureScot	NatureScot (2021): Marine Recorder Snapshot extract of surveys entered by NatureScot. v2.0. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/pqhlyg
The UK Archive for Marine Species and Habitats Data	Marine Biological Association of the UK (MBA); (2016): DASSH: The UK Archive for Marine Species and Habitats Data
Canadian National Aquatic Invasive Species Database	DFO 2015. Canadian National Aquatic Invasive Species Database. Version 3 In OBIS Canada Digital Collections. Bedford Institute of Oceanography, Dartmouth, NS, Canada. Published by OBIS, Digital http://www.iobis.org/. Accessed on January 2nd 2023.
Marine Invertebrata specimen database of Osaka Museum of Natutal History	Ishida S (2016). Marine Invertebrata specimen database of Osaka Museum of Natutal History. National Institute of Genetics, ROIS. Occurrence dataset https://doi.org/10.15468/zhubgk accessed via GBIF.org on 2023-01-02.
Marine records from Pembrokeshire Marine Species Atlas	Dale Rostron. Marine records from Pembrokeshire Marine Species Atlas. Countryside Council for Wales, Gwynedd, UK. https://doi.org/10.15468/42yudm
Biodiversity of the North Sea - Helgoland	GEO-Tag der Artenvielfalt, Artenvielfalt der Nordsee - Helgoland (accessed through GBIF data portal, http://data.gbif.org/datasets/resource/2688, 2023-01-02) https://doi.org/10.15468/omx28y
Bay of Fundy Species List	Van Guelpen L, Pohle G (2014): Bay of Fundy Species List. v1.4. Canadian node of the Ocean Biogeographic Information System (OBIS Canada). Dataset/Occurrence. http://ipt.iobis.org/obiscanada/resource?r=bofetf&v=1.4
Marine Invertebrate from Argentina, Uruguay and Chile	Bigatti G (2015): Marine Invertebrate from Argentina, Uruguay and Chile. v1.4. ArOBIS Centro Nacional Patagónico. Dataset/Occurrence. http://arobis.cenpat-conicet.gob.ar:8081/resource?r=arobis-marineinvertebrate&v=1.4
Marine biological observation data from coastal and offshore surveys around New Zealand	SWPRON (2014). Marine biological observation data from coastal and offshore surveys around New Zealand. Southwestern Pacific OBIS, National Institute of Water and Atmospheric Research (NIWA), Wellington, New Zealand, Online http://nzobisipt.niwa.co.nz/resource.do?r=mbis_nz
Atlantic Reference Centre Museum of Canadian	Van Guelpen, L., 2016. Atlantic Reference Centre Museum of Canadian Atlantic Organisms - Invertebrates and Fishes Data. Version 4 In OBIS Canada Digital Collections. Bedford Institute of Oceanography,

Dataset Name	Citation
Atlantic Organisms - Invertebrates and Fishes Data	Dartmouth, NS, Canada. Published by OBIS, Digital http://www.iobis.org/. Accessed on January 2nd 2023.
Cobscook Bay Inventory: A Historical Checklist of Marine Invertebrates Spanning 162 Years (1843 - 2005)	Trott, Thomas J. (2017) Cobscook Bay Inventory: A Historical Checklist of Marine Invertebrates Spanning 162 Years (1843 - 2005). Version 1 In OBIS Canada Digital Collections. Bedford Institute of Oceanography, Dartmouth, NS, Canada. Published by OBIS, Digital http://www.iobis.org/. Accessed on January 2nd 2023.
NEFSC Benthic Database	Northeast Fisheries Science Center, National Marine Fisheries Service, NOAA, U.S. Department of Commerce. 2010. NEFSC Benthic Database. Northeast Fisheries Science Center, 166 Water Street, Woods Hole Laboratories, Woods Hole, MA 02543.Retrieved from http://www.usgs.gov/obis-usa/
Marine Invertebrate voucher specimens at the Florida Biodiversity Collection, Florida Fish and Wildlife Conservation Commission	Not listed, uploaded by Paul Larson with the Fish and Wildlife Research Institute in St. Petersburg, FL.
Marine Recorder Snapshot extract of surveys entered by Kent Wildlife Trust	Kent Wildlife Trust (2021): Marine Recorder Snapshot extract of surveys entered by Kent Wildlife Trust. v2.1. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/rkwbds
Marine species data for Scottish waters held and managed by Scottish Natural Heritage, derived from benthic surveys 1993 to 2012	Scottish Natural Heritage. Marine species data for Scottish waters held and managed by Scottish Natural Heritage, derived from benthic surveys 1993 to 2012. Scottish Natural Heritage, Edinburgh, UK. https://doi.org/10.15468/faxvgd
Marine Intertidal Phase 1 species dataset from the Countryside Council for Wales 1996-2005	UK National Biodiversity Network, Countryside Council for Wales - Marine Intertidal Phase 1 species dataset from the Countryside Council for Wales 1996-2005. https://doi.org/10.15468/kflo7m

Table 6. Citations used to create maps for the distribution of Diadumene leucolena, generated by OBIS.

Dataset Name	Citation
Nonindigenous Aquatic Species (NAS) Database Non-freshwater Specimens	None listed, uploaded by Pam Fuller with the U.S. Geological Survey.
Hexacorallians of the world	Fautin, Daphne G. (2013). Hexacorallians of the World.
UF Invertebrate Zoology	None listed, uploaded by Gustav Paulay with the Florida Museum of Natural History.
Coleção de Cnidaria do Museu Nacional (MNRJ)	MNRJ(2015) Coleção de Cnidaria do Museu Nacional da Universidade Federal do Rio de Janeiro (Hexacorallia e Octocorallia), 7679 registros (atualizado em 15/12/2016).
Rapid Assessment Surveys of Native and Introduced Marine Organisms in the Northeast United States; Staten Island, New York to Eastport, Maine	Contributors to the Rapid Assessment Surveys Database (2007) Rapid Assessment Survey Dataset; generated by Parker Lund; using Ocean Biogeographic Information System (OBIS) [online application]. Cambridge, MA: MIT Sea Grant College Program, Massachusetts Institute of Technology [producer and distributor], New Brunswick, NJ: OBIS, Rutgers University Institute of Marine and Coastal Science

Dataset Name	Citation
	[distributor]. http://www.iobis.org/mapper; accessed on January 2nd 2023.
A Biological Survey of the Waters of Woods Hole and Vacinity	Sumner, F. B., R. C. Osborn, L. J. Cole, and B. M. Davis. A biological survey of the waters of Woods Hole and vicinity. Bulletin of the U.S. Bureau of Fisheries. 1911. 31: 1-860
CAS Invertebrate Zoology (IZ)	None listed, uploaded by Christina Piotrowski with the California Academy of Sciences.
Marine data from the Bernice P. Bishop Museum	Pyle R (2016). Bernice P. Bishop Museum. Version 8.1. Bernice Pauahi Bishop Museum. Occurrence dataset https://doi.org/10.15468/s6ctus accessed via GBIF.org on 2018-11-16.
EPA'S EMAP Database	None listed, uploaded by the US Environmental Protection Agency (EPA).
National Museum of Natural History Invertebrate Zoology Collections	National Museum of Natural History, Smithsonian Institution NMNH Invertebrate Zoology Collection Database. National Museum of Natural History, Smithsonian Institution, 10th and Constitution Ave. N.W., Washington, DC 20560-0193, 2001, Version 3.2.04 (0802221).
IndOBIS Dataset (84001-86000)	Indian Ocean Biodiversity Information System (IndOBIS)- Distribution records of marine organisms from the Indian Ocean.
Diveboard - Scuba diving citizen science observations	Diveboard - Scuba diving citizen science observations. Online at http://www.diveboard.com and http://ipt.diveboard.com/resource.do?r=diveboard-occurrences. https://dx.doi.org/10.15468/tnjrgy
IndOBIS Dataset (20001-22000)	Indian Ocean Biodiversity Information System (IndOBIS)- Distribution records of marine organisms from the Indian Ocean.
Bay of Fundy Species List	Van Guelpen L, Pohle G (2014): Bay of Fundy Species List. v1.4. Canadian node of the Ocean Biogeographic Information System (OBIS Canada). Dataset/Occurrence. http://ipt.iobis.org/obiscanada/resource?r=bofetf&v=1.4

Table 7. Citations used to create maps for the distribution of Metridium senile, generated by OBIS.

Dataset Name	Citation
Marine Recorder Snapshot extract of surveys entered by SeaSearch	SeaSearch (2021): Marine Recorder Snapshot extract of surveys entered by SeaSearch. v2.0. Marine Biological Association. Dataset/Samplingevent https://doi.org/10.17031/yq0gbg
Marine Recorder Snapshot extract of surveys entered by JNCC	Joint Nature Conservation Committee (JNCC) (2021): Marine Recorder Snapshot extract of surveys entered by JNCC. v2.0. Marine Biological Association. Dataset/Samplingevent https://doi.org/10.17031/mehqrq
Marine Recorder Snapshot extract of surveys entered by The archive for marine species and habitats data (DASSH)	The archive for marine species and habitats data (DASSH) (2021): Marine Recorder Snapshot extract of surveys entered by The archive for marine species and habitats data (DASSH). v2.1. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/myrqac

Dataset Name	Citation
Marine Recorder Snapshot extract of surveys entered by National Museums Northern Ireland (NMNI)	National Museums Northern Ireland (NMNI) (2021): Marine Recorder Snapshot extract of surveys entered by National Museums Northern Ireland (NMNI). v2.0. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/frdvov
Marine Recorder Snapshot extract of surveys entered by NatureScot	NatureScot (2021): Marine Recorder Snapshot extract of surveys entered by NatureScot. v2.0. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/pqhlyg
Marine Recorder Snapshot extract of surveys entered by NRW	Natural Resources Wales (NRW) (2021): Marine Recorder Snapshot extract of surveys entered by NRW. v2.0. Marine Biological Association. Dataset/Samplingevent https://doi.org/10.17031/b3efts
Marine Life Information Network (MarLIN) marine survey data (Professional)	Parr, J. Marine Life Information Network (MarLIN) marine survey data (Professional). Marlin, Collated Marine Life Survey Datasets, Marine Biological Association of the UK, Plymouth, UK
Hexacorallians of the world	Fautin, Daphne G. (2013). Hexacorallians of the World.
Marine species data for Scottish waters held and managed by Scottish Natural Heritage, derived from benthic surveys 1993 to 2012	Scottish Natural Heritage. Marine species data for Scottish waters held and managed by Scottish Natural Heritage, derived from benthic surveys 1993 to 2012. Scottish Natural Heritage, Edinburgh, UK. https://doi.org/10.15468/faxvgd
Marine Recorder Snapshot extract of surveys entered by Natural England	Natural England (NE) (2021): Marine Recorder Snapshot extract of surveys entered by Natural England. v2.0. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/thn0xd
Marine data from Natural Resources Wales (NRW) Technical Support (Research & Monitoring) Contracts, Wales	UK National Biodiversity Network, Countryside Council for Wales - Marine data from Countryside Council for Wales (CCW) Technical Support (Research & Monitoring) Contracts, Wales https://doi.org/10.15468/az7nw3
Marine Life Survey Data (collected by volunteers) collated by MarLIN	Parr, J. Marine Life Survey Data (collected by volunteers) collated by MarLIN. MarLIN, collated Marine Life Survey Datasets, Marine Biological Association of the UK, Plymouth, UK.
Marine Intertidal Phase 1 species dataset from the Countryside Council for Wales 1996-2005	UK National Biodiversity Network, Countryside Council for Wales - Marine Intertidal Phase 1 species dataset from the Countryside Council for Wales 1996-2005. https://doi.org/10.15468/kflo7m

Dataset Name	Citation
Gwaii Haanas Invertebrates	Sloan, N.A., Bartier, P.M., Austin, W.C. 2004. Gwaii Haanas Invertebrates (Living marine legacy of Gwaii Haanas II: Marine invertebrate baseline to 2000). Parks Canada-Technical Reports in Ecosystem Science. OBIS Canada, Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada, Version 1, Digital, retrieved from http://iobis.org/.
Rapid Assessment Surveys of Native and Introduced Marine Organisms in the Northeast United States; Staten Island, New York to Eastport, Maine	Contributors to the Rapid Assessment Surveys Database (2007) Rapid Assessment Survey Dataset; generated by Parker Lund; using Ocean Biogeographic Information System (OBIS) [online application]. Cambridge, MA: MIT Sea Grant College Program, Massachusetts Institute of Technology [producer and distributor], New Brunswick, NJ: OBIS, Rutgers University Institute of Marine and Coastal Science [distributor]. http://www.iobis.org/mapper; accessed on January 2nd 2023.
Coastal Habitat Invasives Monitoring Program	Contributors to the Coastal Habitat Invasives Monitoring Program Database (2007) Coastal Habitat Invasives Monitoring Program Dataset; generated by Parker Lund; using Ocean Biogeographic Information System (OBIS) [online application]. Salem, MA: Salem Sound Coastwatch [producer], Cambridge, MA: MIT Sea Grant College Program, Massachusetts Institute of Technology [distributor], New Brunswick, NJ: OBIS, Rutgers University Institute of Marine and Coastal Science [distributor]. Http://www.iobis.org/mapper; accessed on January 2nd 2023.
NEFSC Benthic Database	Northeast Fisheries Science Center, National Marine Fisheries Service, NOAA, U.S. Department of Commerce. 2010. NEFSC Benthic Database. Northeast Fisheries Science Center, 166 Water Street, Woods Hole Laboratories, Woods Hole, MA 02543.Retrieved from http://www.usgs.gov/obis-usa/
The UK Archive for Marine Species and Habitats Data	Marine Biological Association of the UK (MBA); (2016): DASSH: The UK Archive for Marine Species and Habitats Data
Marine records from Pembrokeshire Marine Species Atlas	Dale Rostron. Marine records from Pembrokeshire Marine Species Atlas. Countryside Council for Wales, Gwynedd, UK. https://doi.org/10.15468/42yudm
National Museum of Natural History Invertebrate Zoology Collections	National Museum of Natural History, Smithsonian Institution NMNH Invertebrate Zoology Collection Database. National Museum of Natural History, Smithsonian Institution, 10th and Constitution Ave. N.W., Washington, DC 20560-0193, 2001, Version 3.2.04 (0802221).
Marine Nature Conservation Review (MNCR) and associated benthic marine data held and managed by CCW	Countryside Council for Wales. Marine Nature Conservation Review (MNCR) and associated benthic marine data held and managed by CCW. Countryside Council for Wales, Gwynedd, UK.
NaGISA Project	None listed, uploaded by EDUARDO KLEIN with the UNIVERSIDAD SIMÓN BOLÍVAR

Dataset Name	Citation
Danish benthic marine monitoring data from ODAM	Josefson, A.; Rytter, D.; Department of Bioscience - AU, Denmark; (2015): Danish benthic marine monitoring data from ODAM.
BEWREMABI dataset: Belgian Shipwreck - hotspots for Marine Biodiversity: Macrofauna on shipwrecks	None listed, uploaded by Koninklijk Belgisch Instituut voor Natuurwetenschappen; Operationele Directie Taxonomie en Fylogenie; Afdeling Malacologie
2013 University of Plymouth Thanet Coast (UK) Special Area of Conservation (SAC) towed underwater video condition assessment of epibenthic communities and biotopes	University of Plymouth (2021): 2013 University of Plymouth Thanet Coast (UK) Special Area of Conservation (SAC) towed underwater video condition assessment of epibenthic communities and biotopes. v1.1. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/ghed-7t19
Survey of North Wales and Pembrokeshire Tide Influenced Communities	UK National Biodiversity Network, Countryside Council for Wales - Survey of North Wales and Pembrokeshire Tide Influenced Communities
HELCOM/OSPAR Ballast water observations	HELCOM/OSPAR Joint Ballast Water Exemptions Decision Support Tool: https://maps.helcom.fi/website/RA_tool/ HELCOM and OSPAR, 2020
HELCOM/OSPAR Netherlands ports water sampling	HELCOM/OSPAR Joint Ballast Water Exemptions Decision Support Tool: https://maps.helcom.fi/website/RA_tool/ HELCOM and OSPAR, 2020
CAS Invertebrate Zoology (IZ)	None listed, uploaded by Christina Piotrowski with the California Academy of Sciences.
Historical benthic data from the southern Baltic Sea (1839-2001)	Zettler M.L. (2001). Historical benthic data from the southern Baltic Sea (1839-2001). Baltic Sea Research Institute Warnemünde (IOW), Germany.
DFO Quebec Region Biodiversity of the Planning for Integrated Environmental Response Coastal Survey in the St. Lawrence Estuary and Gulf (2017-2021)	Grégoire B (2022): Biodiversity of the Planning for Integrated Environmental Response Coastal Survey in the St. Lawrence Estuary and Gulf (2017-2021). v1. Fisheries and Oceans Canada. Dataset/Samplingevent. https://doi.org/10.26071/OGSL-90C40DBA

Dataset Name	Citation
Diveboard - Scuba diving citizen science observations	Diveboard - Scuba diving citizen science observations. Online at http://www.diveboard.com and http://ipt.diveboard.com/resource.do?r=diveboard-occurrences. https://dx.doi.org/10.15468/tnjrgy
Groundfish Survey Invertebrate Data	Marine Institute (2003); Groundfish Survey Invertebrate Data,
PANGAEA - Data from circulation and transfer of pollutants in the North Sea (ZISH)	None listed, uploaded by the Flanders Marine Institute (VLIZ).
Southern Maine Community College Gulf of Maine Invertebrate Data	None listed, uploaded by Robert Siegel with Southern Maine Community College.
IOW Macrozoobenthos monitoring Baltic Sea (1980-2005)	Zettler M. L., 2005: Macrozoobenthos baltic sea (1980-2005) as part of the IOW-Monitoring. Institut für Ostseeforschung Warnemünde, Germany
RSMP Baseline Dataset	Cooper et al. (2017). RSMP Baseline Dataset. Cefas, UK. V1. https://doi.org/10.14466/CefasDataHub.34
A comparison of benthic biodiversity in the North Sea, English Channel and Celtic Seas - Epifauna	Rees, H.L. et al. A comparison of benthic biodiversity in the North Sea, English Channel and Celtic Seas - Epifauna. Centre for Environment, Fisheries and Aquaculture Science; Burnham Laboratory, 12 Apr 2005, Essex, UK.
Taxonomic Information System for the Belgian coastal area	Flanders Marine Institute (VLIZ). Taxonomic Information System for the Belgian coastal area. 10 Aug 2004, Oostende, Belgium, Accessed on [02/01/2023].
Maritimes Summer Research Vessel Surveys	Regnier-McKellar C (2021): Maritimes Summer Research Vessel Surveys. v1.2. Fisheries and Oceans Canada. Dataset/Samplingevent. http://ipt.iobis.org/obiscanada/resource?r=maritimes_summer_rv_surveys&v=1.2
IOW Natural History & Archaeological Society Marine Invertebrate Records 1853-2011	UK National Biodiversity Network: Isle of Wight Local Records Centre - IOW Natural History & Archaeological Society Marine Invertebrate Records 1853-2011. Accessed via http://www.gbif.org/dataset/8b97e0ae-c803-4d24-8098-74874113c344 on yyyy-mm-dd https://doi.org/10.15468/d9amhg
Marine Recorder Snapshot extract of surveys entered by Kent Wildlife Trust	Kent Wildlife Trust (2021): Marine Recorder Snapshot extract of surveys entered by Kent Wildlife Trust. v2.1. Marine Biological Association. Dataset/Samplingevent. https://doi.org/10.17031/rkwbds
MEL: Benthic samples collected by divers with a	Hughes, R.N. and M.L.H. Thomas (2016). MEL: Benthic samples collected by divers with a suction dredge from Bedeque Bay, an estuary in Prince Edward Island, Canada in 1967. Version 1 In OBIS Canada Digital Collections. Bedford

Dataset Name	Citation
suction dredge from Bedeque Bay, an estuary in Prince Edward Island, Canada in 1967.	Institute of Oceanography, Dartmouth, NS, Canada. Published by OBIS, Digital http://www.iobis.org/. Accessed on January 2nd 2023.
Marine species recorded in Ireland during field surveys by EcoServe, Ecological Consultancy Services Ltd.	Allen D., Beckett B., Brophy J., Costello M.J., Emblow C., Maciejewska B., McCrea M., Nash R., Penk M. & Tierney A. (2009) Marine species recorded in Ireland during field surveys by EcoServe, Ecological Consultancy Services Ltd. https://dx.doi.org/10.14284/485
Norman and Florence Hammond records. Seawatch and coastal survey records	Norman and Florence Hammond records. Seawatch and coastal survey records. Cumbria Biodiversity Data Centre, UK - UK National Biodiversity Network. https://doi.org/10.15468/1u5tii
UF Invertebrate Zoology	None listed, uploaded by Gustav Paulay with the Florida Museum of Natural History.
Intertidal Biodiversity in the Gulf of Maine	None listed, uploaded by the Intergovernmental Oceanographic Commission of UNESCO.
Explore Your Shore	National Biodiversity Data Centre (NBDC), Ireland; (2022): Explore Your Shore https://dx.doi.org/10.14284/563
Maritimes Spring Research Vessel Surveys	Regnier-McKellar C (2021): Maritimes Spring Research Vessel Surveys. v1.2. Fisheries and Oceans Canada. Dataset/Samplingevent. http://ipt.iobis.org/obiscanada/resource?r=maritimes_spring_rv_surveys&v=1.2
Pacific Multispecies Small Mesh Bottom Trawl Survey	Flemming R (2013): Pacific Multispecies Small Mesh Bottom Trawl Survey. v2.4. Fisheries and Oceans Canada. Dataset/Samplingevent. http://iobis.org/mapper/?resource_id=261
Distribution of macrobenthos in the Yellow Sea in July 2010	(2020): Distribution of macrobenthos in the Yellow Sea in July 2010. v1.3. No organisation. Dataset/Occurrence. http://ipt.iobis.org/obis-china/resource?r=qualitative_analysis_of_macrobenthos_in_the_yellow_sea_in_july_2010&v=1.3
DFO Pacific Shorekeepers Intertidal Survey – a community based project	DFO (2013). DFO Pacific Shorekeepers Intertidal Survey – a community based project. Version 1 In OBIS Canada Collections. Bedford Institute of Oceanography, Dartmouth, NS, Canada. Published by OBIS. http://www.iobis.org/. (consulted on January 2nd 2023)
Epibenthos and demersal fish monitoring in function of aggregate extraction in the Belgian part of the North Sea	Bio-environmental research groups; Institute of Agricultural and Fisheries research (ILVO), Belgium; (2019): Epibenthos and demersal fish monitoring in function of aggregate extraction in the Belgian part of the North Sea. https://dx.doi.org/10.14284/197

Dataset Name	Citation
IndOBIS Dataset (20001-22000)	Indian Ocean Biodiversity Information System (IndOBIS)- Distribution records of marine organisms from the Indian Ocean.
Marine flora and fauna records from the North-east Atlantic	Marine flora and fauna records from the North-east Atlantic. Porcupine Marine Natural History Society, UK - UK National Biodiversity Network. https://doi.org/10.15468/pcmg9q
Atlantic Reference Centre Museum of Canadian Atlantic Organisms - Invertebrates and Fishes Data	Van Guelpen, L., 2016. Atlantic Reference Centre Museum of Canadian Atlantic Organisms - Invertebrates and Fishes Data. Version 4 In OBIS Canada Digital Collections. Bedford Institute of Oceanography, Dartmouth, NS, Canada. Published by OBIS, Digital http://www.iobis.org/. Accessed on January 2nd 2023.
Epibenthos and demersal fish monitoring at long-term monitoring stations in the Belgian part of the North Sea	Bio-environmental research group; Institute of Agricultural and Fisheries research (ILVO), Belgium; (2015): Epibenthos and demersal fish monitoring at long-term monitoring stations in the Belgian part of the North Sea https://dx.doi.org/10.14284/54
Study of the biotic environment in the Sluice Dock in relation to oyster farming between 1960 and 1964	Leloup E.; Van Meel L.; Polk P.; Halewyck R.; Gryson A; Ministère de l'Agriculture - Commission T.W.O.Z. Groupe de travail Ostreiculture: Belgium; (2016): Study of the biotic environment in the Sluice Dock in relation to oyster farming between 1960 and 1964. (http://www.vliz.be/en/imis?module=dataset&dasid=4583) https://dx.doi.org/10.14284/135
DFO Quebec Region MLI museum collection	Miller R (2013): DFO Quebec Region MLI museum collection. v3.6. Fisheries and Oceans Canada. Dataset/Occurrence. http://iobis.org/mapper/?resource_id=2673
Rocky reef biodiversity survey: Punta Pardelas, Argentina	Bravo G, Livore J P, Battini N, Gastaldi M, Lauretta D, Brogger M, Raffo M P, Lagger C, Bigatti G (2021): Rocky reef biodiversity survey: Punta Pardelas, Argentina. v1.10. ArOBIS Centro Nacional Patagónico. Dataset/Samplingevent. http://arobis.cenpat-conicet.gob.ar:8081/resource?r=arrs&v=1.10
BIOMAERL.Maerl Biodiversity.Functional Structure And Antropogenic Impacts (1996-1998).	Hall-Spencer J.,Grall J., Gerovasileiou V.,Mavraki D.,Paranou P., Baily N.,Nikolopoulou S.,(2018): BIOMAERL.Maerl Biodiversity.Functional Structure And Antropogenic Impacts (1996-1998).Hellenic Center for Marine Research.
Coleção de Cnidaria do Museu Nacional (MNRJ)	MNRJ(2015) Coleção de Cnidaria do Museu Nacional da Universidade Federal do Rio de Janeiro (Hexacorallia e Octocorallia), 7679 registros (atualizado em 15/12/2016).
Distribution of macrobenthos in the Yellow Sea in November 2010	(2021): Distribution of macrobenthos in the Yellow Sea in November 2010. v1.2. No organisation. Dataset/Occurrence. http://ipt.iobis.org/obis-china/resource?r=macrobenthos_in_the_yellow_sea_in_nov_2010&v=1.2
Dutch long term monitoring of macrobenthos in the Dutch Continental	Ministery of Infrastructure and Environment, The Netherlands; (2018): Dutch long term monitoring of macrobenthos in the Dutch Continental Economical Zone of the North Sea.

Dataset Name	Citation
Economical Zone of the North Sea	
Senckenberg's collection management system	Türkay, M. Senckenbergisches Sammlungsverwaltungssystem, SeSam. Senckenbergische Naturforschende Gesellschaft , Frankfurt, Germany. http://sesam.senckenberg.de/
Collection Cnidaria SMF	Senckenberg, Collection Cnidaria SMF. https://doi.org/10.15468/e2vb1g
Historic data (1908-1963) of benthic macrofauna from the Limfjord, Denmark	National Institute of Aquatic Resources (Aqua) - DTU, Denmark; International Council for the Exploration of the Sea (ICES), Denmark (2016): Historic data (1908-1963) of benthic macrofauna from the Limfjord, Denmark
SHARK - Regional monitoring, recipient control and monitoring projects of zoobenthos in Sweden since 1972	Swedish county administration boards, Swedish municipalities, Swedish coalitions of water conservation, Swedish companies and Swedish Meteorological and Hydrological Institute et.al.(2021). Regional monitoring, recipient control and monitoring projects of zoobenthos in Sweden since 1972. https://doi.org/10.15468/cesssx
SOMBASE/TOTAL - Bioconstructors	GRIFFITHS, H.J., LINSE, K. & CRAME, J.A. 2003. SOMBASE – Southern Ocean Mollusc Database: A tool for biogeographic analysis in diversity and ecology. Organisms Diversity and Evolution. 3, 207–213.
Ecological study of intertidal clay-and peatbanks at Raversijde (Belgium) in 1970	Jocqué R.; Van Damme D.; Laboratorium voor Oecologie der Dieren, Zoögeografie en Natuurbehoud; Laboratorium voor Paleontologie. RUG: Belgium; (2016): Ecological study of intertidal clay-and peatbanks at Raversijde (Belgium) in 1970. (http://www.vliz.be/en/imis?module=dataset&dasid=5113) https://dx.doi.org/10.14284/138
Marine records from Skomer Marine Nature Reserve (MNR) Marine Monitoring Programme	Marine records from Skomer Marine Reserve (MNR) Marine Monitoring Programme. Countryside Council for Wales, UK - UK National Biodiversity Network. https://doi.org/10.15468/207iog
Marine Benthic Fauna List, Island of Læsø, Denmark	The Danish Biodiversity Information Facility, Marine Benthic Fauna List, Island of Læsø, Denmark. https://doi.org/10.15468/ty0smg
Marine apostome ciliates	None listed, uploaded by the Intergovernmental Oceanographic Commission of UNESCO
N3 data of Kiel bay	Rumohr, H., D. Fleischer, 2004: N3 data of Kiel Bay. Leibniz Institute of Marine Sciences, Marine Ecology Division, Germany https://dx.doi.org/10.14284/182
Literature review of the Scheldt estuary with emphasis on the biotic components	Moerland G.; Hummel H.; Bakker C.; Delta Instituut voor Hydrobiologisch Onderzoek (DIHO): The Netherlands; (2016): Literature review of the Scheldt estuary with emphasis on the biotic components. (http://www.vliz.be/en/imis?module=dataset&dasid=5181) https://dx.doi.org/10.14284/122
Coastal and marine species	National Biodiversity Data Centre: Collated by the National Biodiversity Data Centre from different sources - Coastal and marine species. Dataset/Occurrence. https://doi.org/10.15468/oynwkx

Dataset Name	Citation
SeamountsOnline	None listed, uploaded by Karen Stocks with the Geological Data Center, Scripps Institution of Oceanography,
Benthic Network	Agence pour la recherche et la Valorisation Marines (ARVAM); Cellule De Suivi Du Littoral Normand (CSLN); Creocean; Groupe d'Etude des Milieux Estuariens et Littoraux; Groupe d'étude des milieux estuariens et littoraux Normandie (GEMEL Normandie); Hemisphere Sub; Impact Mer; Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER); MAREX Expertise & Conseil en Environnement Marin (MAREX); National Natural History Museum Paris; CRESCO - Station de biologie marine de Dinard; National Natural History Museum Paris; Station de Biologie Marine de Concarneau; Pierre & Marie Curie University; Roscoff biology station (RBS); TBM Environnement; The National Center for Scientific Research; Laboratoire d'Ecogéochimie des Environnements Benthiques UMR 8222 (LECOB); The National Center for Scientific Research; Laboratoire d'Océanologie et de Géosciences - UMR 8187 LOG (LOG); Université Bordeaux 1; Environnements et Paléoenvironnements Océaniques (EPOC); Université de Bretagne Occidentale; Institut Universitaire Européen de la Mer (IUEM); Université de Bretagne Occidentale; Laboratoire d'écophysiologie et de biotechnologie des halophytes et algues Marines (IUEM-LEBHAM); Université de Bretagne Occidentale; Laboratory of Sciences of the Marine Environment CNRS-UMR6539 (LEMAR); Université de la Réunion; Laboratoire d'Ecologie Marine (ECOMAR); Université de Liège; Station de Recherche Sous-marines et Océanographiques (STARESO); Université de Nantes; Laboratoire de Biologie Marine (Bio-littoral); Université des Sciences et Technologies de Lille; Station Marine de Wimereux (INSU/CNRS); (2019): REBENT - Réseau Benthique.
Marine Recorder Snapshot extract of surveys entered by Wildlife Trusts	Wildlife Trusts (2021): Marine Recorder Snapshot extract of surveys entered by Wildlife Trusts. v2.1. Marine Biological Association. Dataset/Samplingevent https://doi.org/10.17031/zwrsjz
Macrobenthos Data from Shoreham, the Tyne and the Thames Estuaries, UK, 2000 to 2006	Rees et al.; (2020): Macrobenthos Data from Shoreham, the Tyne and the Thames Estuaries, UK, 2000 to 2006. Cefas, UK. V1. https://doi.org/10.14466/CefasDataHub.45
Vulnerable marine ecosystems in the South Pacific Ocean region	NIWA (2016). Vulnerable marine ecosystems in the South Pacific Ocean region. National Institute of Water and Atmospheric Research (NIWA), Wellington, New Zealand, 202873 records, Online http://nzobisipt.niwa.co.nz/resource.do?r=vme_inverts released on February 3, 2016.
Benthos of the White Sea - A database	Naumov, A. Benthos of the White Sea. A database. White Sea Biological Station, Zoological Institute RAS.
Tauchen und Meer 02	GEO-Tag der Artenvielfalt: Tauchen und Meer 02. Accessed via http://www.gbif.org/dataset/62b54d08-f762-11e1-a439-00145eb45e9a on 2023-01-02 https://doi.org/10.15468/utfmvr
ICES Zoobenthos Community dataset	ICES Environmental Database (DOME), Zoobenthos community. Available online at http://dome.ices.dk. ICES, Copenhagen. Consulted on 2023-01-02.
Biodiversity of the North Sea - Helgoland	GEO-Tag der Artenvielfalt, Artenvielfalt der Nordsee - Helgoland (accessed through GBIF data portal, http://data.gbif.org/datasets/resource/2688, 2023-01-02) https://doi.org/10.15468/omx28y

Dataset Name	Citation
Biodiversity of the North Sea - Sylt	GEO-Tag der Artenvielfalt, Artenvielfalt der Nordsee - Sylt (accessed through GBIF data portal, http://data.gbif.org/datasets/resource/2839, 2023-01-02) https://doi.org/10.15468/nvhjkx
A summary of benthic studies in the sluice dock of Ostend during 1976-1981	Thielemans L.K.H.; Heip C.H.R.; Van Gansbeke D.; Marine Biology Section; Zoology Institute. State University of Gent: Belgium; (2017): A summary of benthic studies in the sluice dock of Ostend during 1976-1981. (http://www.vliz.be/en/imis?module=dataset&dasid=4230) https://dx.doi.org/10.14284/273
Marine Invertebrate from Argentina, Uruguay and Chile	Bigatti G (2015): Marine Invertebrate from Argentina, Uruguay and Chile. v1.4. ArOBIS Centro Nacional Patagónico. Dataset/Occurrence. http://arobis.cenpat-conicet.gob.ar:8081/resource?r=arobis-marineinvertebrate&v=1.4
Type locality distributions from the World Register of Marine Species	WoRMS Editorial Board (2021). Type locality distributions from the World Register of Marine Species. Available from http://www.marinespecies.org at VLIZ. Accessed on 2023-01-02.
Infaunal SACFOR abundance data from underwater video footage from the selected Special Areas of Conservation (SACs), Northern Ireland, 2017.	Agri-Food & Biosciences Institute (AFBI), Northern Ireland; (2020) Infaunal SACFOR abundance data from underwater video footage from the selected Special Areas of Conservation (SACs), Northern Ireland, 2017. https://doi.org/10.17031/ufocqw
PANGAEA - Data from various sources	None listed, uploaded by the Flanders Marine Institute (VLIZ).
Irish Benthos monitoring as part of the Water framework directive since 2012	Marine Institute (2020), Irish Benthos monitoring as part of the Water framework directive since 2012
Cobscook Bay Inventory: A Historical Checklist of Marine Invertebrates Spanning 162 Years (1843 - 2005)	Trott, Thomas J. (2017) Cobscook Bay Inventory: A Historical Checklist of Marine Invertebrates Spanning 162 Years (1843 - 2005). Version 1 In OBIS Canada Digital Collections. Bedford Institute of Oceanography, Dartmouth, NS, Canada. Published by OBIS, Digital http://www.iobis.org/. Accessed on January 2nd 2023.
IndOBIS, Indian Ocean Node of OBIS	Chavan, VIshwas and C. T. Achuthankutty (editors), IndOBIS Catalogue of Life, Available at http://www.indobis.org/, Retrieved 02, 01, 2023.
2012-ongoing UK Offshore Marine Conservation Zone	Centre for Environment, Fisheries and Aquaculture Science (Cefas), United Kingdom; (2019) 2012-ongoing UK Offshore Marine Conservation Zone (MCZ) Survey Data https://doi.org/10.17031/ravlvw

Dataset Name	Citation
(MCZ) Survey Data	
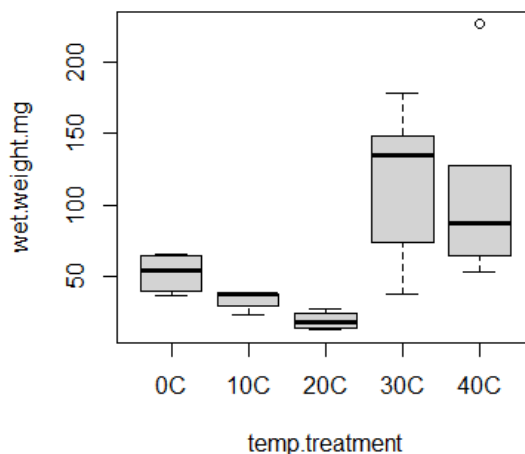
Figure 28. Temperature treatment versus the anemone wet weight (in mg) for the Metridium senile.

Complete GLM Results: All tested models are listed below, in the order the data is

presented in Chapter 2.

Table 8. For Diadumene lineata, general linear models were used to evaluate the impact of temperature treatment and anemone wet weight on richness and diversity, using a gaussian distribution. Est. = Estimate, SE = Standard Error, and t = t-value. The 20°C category was selected as the baseline for the temperature GLM, since it is in the middle of the range this species is typically found in. The last three columns are the results of a Type II ANOVA evaluating the entire model. The t-row is for temperature treatment, the w-row is for wet weight, and the tw-row is a combination of the two. The ANOVA was calculated using a likelihood-ratio test (LR X^2).

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
null: richness ~ 1					150.96			
intercept	29.947	2.727	10.98	<0.001				
richness ~ temp. + weight					140.35	t: 4	22.264	**<0.001**
						w: 1	0.951	0.329
intercept	51.180	10.253	4.992	**<0.001**				

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
temp.0C	-4.845	5.824	-0.832	0.421				
temp.10C	-15.170	6.488	-2.338	**0.036**				
temp.30C	-31.802	8.091	-3.930	**0.002**				
temp.40C	-8.175	7.261	-1.126	0.281				
weight	-0.174	0.178	-0.975	0.347				
richness ~ temp. * weight					144.57	t: 4	18.809	**<0.001**
						w: 1	0.804	0.370
						tw:4	1.983	0.739
intercept	75.617	21.959	3.443	**0.007**				
temp.0C	-44.321	45.838	-0.967	0.359				
temp.10C	-55.611	31.486	-1.766	0.111				
temp.30C	-55.444	24.706	-2.244	0.052				
temp.40C	-36.378	38.527	-0.944	0.370				
weight	-0.637	0.408	-1.563	0.152				
temp.0C : weight	0.723	0.802	0.902	0.391				
temp.10C : weight	0.716	0.536	1.337	0.214				
temp.30C : weight	0.425	0.645	0.659	0.526				
temp.40C : weight	0.512	0.574	0.892	0.396				
richness ~ temp.					**139.69**	t: 4	24.592	**<0.001**
intercept	42.000	4.059	10.348	**<0.001**				
temp.0C	-5.750	5.740	-1.002	0.333				
temp.10C	-17.000	6.200	-2.742	**0.0159**				
temp.30C	-26.250	5.740	-4.573	**<0.001**				
temp.40C	-12.500	5.740	-2.178	**0.047**				
richness ~ weight					151.31	w: 1	1.540	0.215
intercept	21.684	7.181	3.020	**0.008**				
weight	0.153	0.123	1.241	0.232				
null: diversity ~ 1					23.094			
intercept	3.277	0.094	34.77	**<0.001**				
diversity ~ temp. + weight					8.721	t: 4	27.203	**<0.001**
						w: 1	1.137	0.286
intercept	3.979	0.321	12.394	**<0.001**				
temp.0C	-0.119	0.182	-0.655	0.524				

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X²	Pr(>X²)
temp.10C	-0.421	0.203	-2.072	0.059				
temp.30C	-1.141	0.253	-4.503	**<0.001**				
temp.40C	-0.228	0.227	-1.003	0.334				
weight	-0.006	0.006	-1.066	0.306				
<u>diversity ~ temp. * weight</u>					13.07	t: 4	22.823	**<0.001**
						w: 1	0.954	0.329
						tw:4	1.907	0.753
intercept	4.536	0.690	6.585	**<0.001**				
temp.0C	-1.135	1.440	-0.788	0.451				
temp.10C	-1.603	0.989	-1.620	0.140				
temp.30C	-1.526	0.776	-1.966	0.081				
temp.40C	-0.761	1.210	-0.629	0.545				
weight	-0.017	0.013	-1.289	0.229				
temp.0C : weight	0.018	0.025	0.733	0.482				
temp.10C : weight	0.020	0.017	1.214	0.256				
temp.30C : weight	0.002	0.020	0.115	0.911				
temp.40C : weight	0.010	0.018	0.568	0.584				
<u>diversity ~ temp.</u>					**8.314**	t: 4	32.433	**<0.001**
intercept	3.665	0.128	28.648	**<0.001**				
temp.0C	-0.150	0.181	-0.831	0.420				
temp.20C	-0.484	0.195	-2.475	**0.027**				
temp.30C	-0.951	0.181	-5.256	**<0.001**				
temp.40C	-0.376	0.181	-2.079	0.056				
<u>diversity ~ weight</u>					22.173	w: 1	2.8259	0.093
intercept	2.902	0.240	12.093	**<0.001**				
weight	0.007	0.004	1.681	0.111				

Table 9. For Diadumene leucolena, general linear models were used to evaluate the impact of temperature treatment and anemone wet weight (log-transformed) on richness and diversity, using a gaussian distribution. Est. = Estimate, SE = Standard Error, and t = t-value. The 20°C category was selected as the baseline for the temperature GLM, since it is in the middle of the range this species is typically found in. The last three columns are the results of a Type II ANOVA evaluating the entire model. The t-row is for

temperature treatment, the w-row is for wet weight, and the tw-row is a combination of the two. The ANOVA was calculated using a likelihood-ratio test (LR X^2).

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
null: richness ~ 1					189.31			
intercept	32.826	2.898	11.33	<0.001				
richness ~ temp. + weight					196.19	t: 4	2.396	0.663
						w: 1	0.080	0.778
intercept	22.521	31.467	0.716	0.484				
temp.0C	2.185	9.974	0.219	0.829				
temp.10C	-0.861	12.491	-0.069	0.946				
temp.30C	1.692	14.881	0.114	0.911				
temp.40C	11.956	11.207	1.066	0.301				
weight	2.014	7.139	0.282	0.781				
richness ~ temp. * weight					**188.54**	t: 4	3.619	0.460
						w: 1	0.120	0.729
						tw:4	12.670	**0.013**
intercept	109.44	45.21	2.420	**0.031**				
temp.0C	-112.87	78.56	-1.437	0.174				
temp.10C	-128.51	71.18	-1.805	0.094				
temp.30C	-69.61	54.13	-1.286	0.221				
temp.40C	-234.26	72.74	-3.220	**0.007**				
weight	-18.15	10.42	-1.743	0.105				
temp.0C : weight	27.53	19.73	1.395	0.186				
temp.10C : weight	33.11	20.26	1.634	0.126				
temp.30C : weight	14.73	14.49	1.017	0.328				
temp.40C : weight	66.44	19.50	3.408	**0.005**				
richness ~ temp.					194.29	t: 4	2.521	0.641
intercept	32.200	6.325	4.848	**<0.001**				
temp.0C	1.200	9.101	0.132	0.897				
temp.10C	-3.200	9.101	-0.352	0.729				
temp.30C	-1.200	10.509	-0.114	0.910				
temp.40C	10.200	9.101	1.121	0.277				
richness ~ weight					191.22	w: 1	0.082	0.775
intercept	28.172	16.519	1.705	0.103				

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
weight	1.302	4.546	0.546	0.286				
null: diversity ~ 1					**23.191**			
intercept	3.369	0.078	43.03	**<0.001**				
diversity ~ temp. + weight					31.303	t: 4	1.440	0.837
						w: 1	0.026	0.873
intercept	3.187	0.873	3.650	**0.002**				
temp.0C	0.065	0.277	0.233	0.818				
temp.10C	-0.043	0.347	-0.125	0.902				
temp.30C	0.077	0.413	0.187	0.854				
temp.40C	0.246	0.311	0.792	0.439				
weight	0.032	0.198	0.161	0.874				
diversity ~ temp. * weight					25.275	t: 4	2.026	0.731
						w: 1	0.036	0.849
						tw:4	10.924	**0.027**
intercept	5.684	1.300	4.373	**<0.001**				
temp.0C	-3.611	2.259	-1.599	0.134				
temp.10C	-3971	2.046	-1.940	0.074				
temp.30C	-2.004	1.556	-1.288	0.220				
temp.40C	-6.143	2.091	-2.937	**0.012**				
weight	-0.548	0.300	-1.829	0.090				
temp.0C : weight	0.888	0.567	1.565	0.142				
temp.10C : weight	1.034	0.583	1.775	0.099				
temp.30C : weight	0.435	0.417	1.044	0.316				
temp.40C : weight	1.710	0.561	3.051	**0.009**				
diversity ~ temp.					29.338	t: 4	1.511	0.825
intercept	3.324	0.178	18.643	**<0.001**				
temp.0C	0.049	0.252	0.194	0.828				
temp.10C	-0.080	0.252	-0.319	0.754				
temp.30C	0.032	0.291	0.108	0.915				
temp.40C	0.219	0.252	0.867	0.397				
diversity ~ weight					25.173	w: 1	0.017	0.896

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
intercept	3.312	0.447	7.409	**<0.001**				
weight	0.016	0.124	0.131	0/897				

Table 10. For Metridium senile, general linear models were used to evaluate the impact of temperature treatment and anemone wet weight (log-transformed) on richness and diversity, using a gaussian distribution. Est. = Estimate, SE = Standard Error, and t = t-value. The 10°C category was selected as the baseline for the temperature GLM, since it is in the middle of the range this species is typically found in. The last three columns are the results of a Type II ANOVA evaluating the entire model. The t-row is for temperature treatment, the w-row is for wet weight, and the tw-row is a combination of the two. The ANOVA was calculated using a likelihood-ratio test (LR X^2).

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
null: richness ~ 1					187.29			
intercept	33.435	2.773	12.06	<0.001				
richness ~ temp. + weight					180.94	t: 4	2.978	0.562
						w: 1	1.685	0.194
intercept	-0.016	19.845	-0.001	0.999				
temp.0C	9.574	7.522	1.273	0.220				
temp.20C	-0.207	7.765	-0.027	0.979				
temp.30C	11.825	9.095	1.300	0.211				
temp.40C	4.435	8.986	0.494	0.628				
weight	7.152	5.517	1.298	0.212				
richness ~ temp. * weight					180.8	t: 4	3.2435	0.518
						w: 1	1.8357	0.176
						tw: 4	5.5165	0.2383
intercept	12.699	80.581	0.158	0.877				
temp.0C	-175.222	114.181	-1.535	0.149				
temp.20C	34.946	95.806	0.365	0.721				
temp.30C	9.634	88.825	0.108	0.915				
temp.40C	-5.039	90.226	-0.056	0.956				
weight	3.522	23.032	0.153	0.881				
temp.0C : weight	47.354	30.840	1.535	0.149				

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
temp.20C : weight	-12.705	28.993	-0.438	0.668				
temp.30C : weight	1.356	24.399	0.056	0.957				
temp.40C : weight	2.932	24.660	0.119	0.907				
richness ~ temp.					181.11	t: 4	15.34	**0.004**
intercept	25.000	4.832	5.174	**<0.001**				
temp.0C	12.750	7.248	1.759	0.096				
temp.20C	-4.250	7.248	-0.586	0.565				
temp.30C	19.800	6.833	2.898	**0.010**				
temp.40C	12.200	6.833	1.785	0.091				
richness ~ weight					176.65	w: 1	15.38	**<0.001**
intercept	-10.939	11.518	-0.950	0.353				
weight	11.235	2.865	3.922	**<0.001**				
null: diversity ~ 1					21.739			
intercept	3.387	0.076	44.650	**<0.001**				
diversity ~ temp. + weight					12.911	t: 4	3.667	0.453
						w: 1	1.260	0.262
intercept	2.604	0.514	5.063	**<0.001**				
temp.0C	0.245	0.195	1.257	0.226				
temp.20C	-0.079	0.201	-0.393	0.699				
temp.30C	0.375	0.235	1.592	0.130				
temp.40C	0.179	0.233	0.768	0.453				
weight	0.160	0.143	1.122	0.277				
diversity ~ temp. * weight					11.727	t: 4	4.180	0.382
						w: 1	1.436	0.231
						tw: 4	6.380	0.172
intercept	2.502	2.042	1.226	0.242				
temp.0C	-3.872	2.893	-1.339	0.204				
temp.20C	1.887	2.427	0.777	0.451				
temp.30C	0.736	2.251	0.327	0.749				
temp.40C	0.168	2.286	0.074	0.942				
weight	0.190	0.583	0.325	0.750				
temp.0C : weight	1.043	0.781	1.334	0.205				

Model	Est.	SE	t	Pr(>|t|)	AIC	df	LR X^2	Pr(>X^2)
temp.20C : weight	-0.666	0.734	-0.906	0.381				
temp.30C : weight	-0.085	0.618	-0.138	0.892				
temp.40C : weight	-0.005	0.625	-0.007	0.994				
diversity ~ temp.					12.556	t: 4	19.996	**<0.001**
intercept	3.165	0.124	25.562	**<0.001**				
temp.0C	0.316	0.186	1.703	0.106				
temp.20C	-0.170	0.186	-0.914	0.372				
temp.30C	0.554	0.175	3.164	**0.005**				
temp.40C	0.353	0.175	2.016	0.059				
diversity ~ weight					9.404	w: 1	18.166	**<0.001**
intercept	2.116	0.304	6.969	**<0.001**				
weight	0.322	0.076	4.262	**<0.001**				

Appendix C: Chapter 3 Supplementary Materials

DL-MA-05 came up as *Poseidonibacter* on BLAST, but has been labeled

Arcobacter based on recent taxonomic revisions (On et al., 2021). DL-MA-07 is marked

with a * because it was originally *Vibrio*, but may have gotten mixed up with a

Peribacillus isolate. There's no way to know for certainty if the isolate in the heat stress

assay was *Vibrio* or *Peribacillus* without re-sequencing the stock from the -80°C freezer.

DL-AM-11 is marked with an * because it appears to have been contaminated at one

point with a different bacteria (possibly *Stentrophomonas*, but the re-sequencing results

were too messy to say for sure).

Table 11. NCBI BLAST results for bacteria isolated from Diadumene lineata (DL) and Metridium senile (MS). MA indicates it was cultured on marine agar, while AM indicates it was cultures on anemone medium. Quality was assigned based on the SeqScanner2 results. A good quality sequence has a long segment of contiguous high quality basepair assignments.

Isolate ID	Genus	Quality	BP	% Identity	Accession #
DL-MA-01	Pseudoalteromonas	Good	999	99.70%	MN889141.1
DL-MA-02	Peribacillus	Good	989	99.90%	MN826155.1
DL-MA-03	Peribacillus	Good	963	99.90%	MN826155.1
DL-MA-04	Pseudoalteromonas	Good	945	100.00%	MH061228.1
DL-MA-05	Arcobacter	Good	1027	99.22%	OP709877.1
DL-MA-06	Pseudoalteromonas	Good	948	100.00%	JX525985.1
DL-MA-07	Vibrio*	Good	956	99.90%	MK720201.1
DL-MA-08	Shewanella	Good	991	100.00%	KR270269.1
DL-AM-01	Pseudoalteromonas	Good	1006	99.60%	MK439566.1
DL-AM-02	Pseudoalteromonas	*Okay*	787	99.24%	MK439566.1

Isolate ID	Genus	Quality	BP	% Identity	Accession #
DL-AM-03	Peribacillus	Good	959	99.79%	LT838048.1
DL-AM-04	Pseudoalteromonas	*Bad*	746	94.46%	KP980759.1
DL-AM-05	Pseudoalteromonas	Good	952	99.79%	P118496.1
DL-AM-06	Peribacillus	Good	961	99.90%	LT838048.1
DL-AM-07	Pseudoalteromonas	Good	938	99.89%	MN746121.1
DL-AM-08	Pseudoalteromonas	Good	916	100.00%	MK577380.1
DL-AM-09	Pseudoalteromonas	Good	1002	99.90%	MN889141.1
DL-AM-10	Pseudoalteromonas	Good	1012	100.00%	MN889157.1
DL-AM-11	Colwellia*	*Bad*	871	98.17%	JX436365.1
MS-MA-01	Litoreibacter	Good	1001	100.00%	OL662961.1
MS-MA-02	Peribacillus	Good	894	100.00%	MN826155.1
MS-MA-03	Peribacillus	Good	874	100.00%	OM281797.1
MS-MA-04	Macrococcus	Good	863	99.88%	OK103765.1
MS-MA-05	Colwellia	Good	1034	99.90%	MW580181.1
MS-MA-06	Pseudoalteromonas	Good	876	100.00%	MN889131.1
MS-MA-07	Pseudoalteromonas	Good	939	100.00%	MN889240.1
MS-MA-08	Pseudoalteromonas	Good	864	100.00%	OQ300257.1
MS-AM-01	Pseudoalteromonas	Good	940	100.00%	DQ492734.1
MS-AM-02	Pseudoalteromonas	Good	814	99.88%	OQ300257.1
MS-AM-03	Pseudoalteromonas	Good	984	99.90%	MN209950.1
MS-AM-04	Peribacillus	Good	935	100.00%	LT838048.1
MS-AM-05	Peribacillus	*Bad*	194	100.00%	MT310824.1
MS-AM-06	Peribacillus	*Okay*	750	100.00%	KX881437.1

Before running the heat stress assay on all isolates, a small six-hour trial was performed to make sure the marine broth wasn't interfering with the optical density readings. Only measurements for hours three through were measured, based on the growth patterns of previous trials. In the initial trial of optical density versus plating, the plate results were difficult to count due to high-density splotches of bacteria (particularly at the later time points). During this trial, 900 uL of culture was frozen at -20°C for each time point to use for qPCR in case the plating results were messy. The frozen culture was extracted using the ZymoBiomics Miniprep Kit and qPCR was run using the primers EUB338 (5'-ACTCCTACGGGAGGCAGCAG-3') and EUB518 (5'-ATTACCGCGGCTGCTGG-3'). The results of qPCR follow a similar pattern of growth to optical density, except for time point six. This is likely due to an issue in the extraction process, since time point five had a higher DNA concentration than time point six (8.5 versus 6.5 ng/uL). The results for OD600 versus qPCR were clear enough for me to continue with the heat stress assay on the isolates.
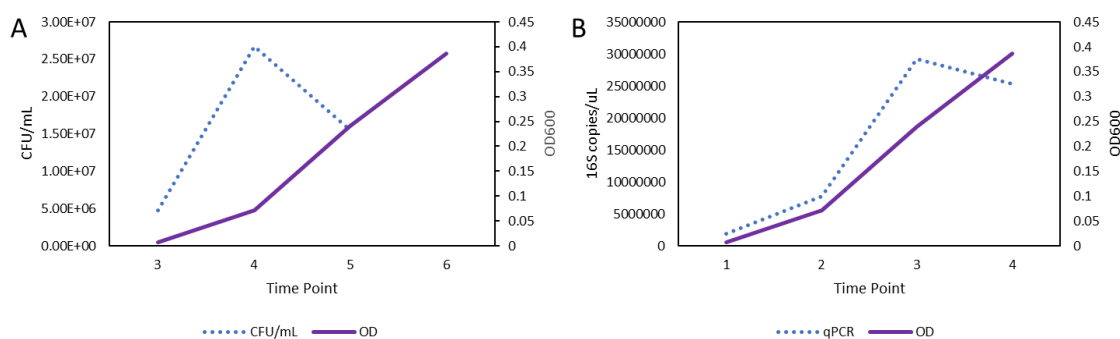

Figure 29. Comparison of measurements for culture growth from hour 3 through hour 6 (for isolate DL-MA-07). Optical density at wavelength 600 was compared with (A) plated CFU/mL and (B) 16S rRNA gene copies per uL of DNA. Samples from the same culture were used for all measurements.