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Bacteria are abundant in marine environments. They play important roles in nutrient cycling and form symbiotic interactions with eukaryotes. However, the vast majority of bacterial taxa are difficult to maintain in laboratory cultures, meaning that most microbiological research of the past century has focused on a small subset of bacteria. The advent of next generation sequencing (NGS) approaches, especially sequencing amplicons of the 16S rRNA gene, allowed researchers to detect bacteria in their natural environments, giving them the tools to conduct bacterial censuses and begin to understand how bacteria interact with their surroundings. Using 16S rRNA gene amplicon sequencing to describe the composition and structure of marine bacterial communities associated with animals residing in natural systems may provide vital indicators of health. However, if these animals reside in human-built environments, water treatment methods likely affect the composition of their bacterial communities, and conclusions drawn from researchers studying natural systems may not apply to those in a laboratory setting. Therefore, before conducting experiments on marine animals in a human built environment, it is crucial to understand how different water treatment approaches affect marine bacterial communities. If the effects of water treatment are not

taken into account and/or standardized within the field of marine bacterial ecology, then experiments may be difficult to reproduce. This dissertation begins by describing the natural variation of the microbiome of the symbiotic intertidal sea anemone *Anthopleura elegantissima*. It then progresses to examine the microbiome of human-built systems, exploring how seawater treatment shapes the bacterial communities in a survey of the Hatfield Marine Science Center's microbiome. Finally, it tests an ecological hypothesis put forth by those researching intensive aquaculture systems: that blooms of opportunistic pathogens can be suppressed by operating systems near their bacterial capacity to select against fast-growing taxa.

16S rRNA Gene Amplicon Sequencing Reveals Trends in Marine Bacterial Diversity and Taxonomic Composition in Natural and Human-built Systems

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Ian Arthur Morelan

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APPROVED:

_____

Major Professor, representing Molecular and Cellular Biology

_____

Director of the Molecular and Cellular Biology Program

_____

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Ian Arthur Morelan, Author

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CONTRIBUTIONS OF AUTHORS

Chapter 2: Dee Denver, Thomas Sharpton, and Rebecca Vega Thurber helped conceive and design the study. Christopher Gaulke, Thomas Sharpton, and Rebecca Vega Thurber contributed to data analysis. Dee Denver, Chris Gaulke, Thomas Sharpton, and Rebecca Vega Thurber provided critical revisions.

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Chapter 4: Olav Vadstein and Ingrid Bakke helped conceive and design the study. Madeleine Gundersen contributed to laboratory work and data analysis. Dee Denver provided critical revisions.

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16S rRNA Gene Amplicon Sequencing Reveals Trends in Marine Bacterial Diversity and Taxonomic Composition in Natural and Human-built Systems

Chapter 1: Introduction

Bacteria are abundant in marine environments. They have been known to play vital roles in nutrient cycling and form diverse symbioses with eukaryotes since the 19th century. However, many ecological interactions between marine bacterial taxa and their environments remain to be poorly understood. Observing bacterial behaviors in their natural environment is not scalable with current technological approaches like fluorescence *in situ* hybridization (Eilers et al., 2000). Marine microbiological research often involves the culturing of bacteria, which is not trivial: most of these taxa are difficult to isolate and culture (Giovannoni and Stingl, 2007; Kopke et al., 2005). This has created a strong research bias toward the characterization of generalist bacterial taxa that can grow on common laboratory media. This problem has long been understood, with pioneering microbial ecologist Sergei Winogradsky declaring that the natural environment harbors "a swarming mass of microscopic organisms, a variety that defies imagination", and that removing these organisms from their natural environments limits the extrapolations of findings from culture-based studies (Bertrand, 2015). Ecological interactions between bacteria and the environment, and the diversifying forces that drive those interactions, lie primarily in bacterial metabolisms and genetic material (Summers, 2002). Thus, the most useful tools available to contemporary researchers wishing to deduce relationships between bacteria and their environments are molecular and biochemical in nature. Until recently, with the development of new DNA sequencing technologies, these methods were difficult to scale. Recently, tools have been developed to feasibly conduct censuses of free-living bacteria in their natural environments, an essential next step in understanding the ecology of these elusive microbes.

Next generation sequencing and its applications in microbial ecology

The advent of Next Generation Sequencing (NGS) technology with the introduction of high-throughput pyrosequencing gave researchers a feasible, scalable method to detect unculturable bacteria in their natural environments (Margulies et al., 2005). There are currently two primary approaches to applying NGS to microbial ecology, each with its own costs and benefits. The direct shotgun approach, used to generated metagenomes or transcriptomes, involves sequencing all the DNA or RNA in a given sample. A shotgun metagenome or transcriptome can provide functional information about a microbial community because it explicitly observes genes in the environment (Sharpton, 2014). However, the generation and analysis of shotgun metagenomes is currently costly and computationally difficult, so contemporary shotgun metagenome studies are often carried out on a small scale. An alternative approach is to amplify and sequence only the 16S ribosomal RNA gene. Bacteria, archaea, and eukaryotes all encode this gene, with some regions conserved across the tree of life. Universal PCR primers can be designed to anneal to these portions of the gene and amplify the (presumably) neutrally-evolving bases in between (Lane et al., 1985). While the idea of sequencing environmental 16S rRNA sequences is not new, next generation sequencing made it possible on a scale orders of magnitudes greater (Caporaso et al., 2012). A limitation of 16S rRNA gene amplicon sequencing is that it provides little information about the function of a microbial community, making it less preferable to shotgun metagenome sequencing on a per-sample basis. Furthermore, 16S rRNA gene copy numbers vary across taxa, confounding efforts to estimate precise abundances of bacteria (Louca et al., 2018). However, 16S rRNA gene amplicon sequencing is substantially less expensive than

shotgun approaches, which allows for far more samples to be sequenced in a given study. For the purposes of this dissertation, which investigates broad patterns in the community ecology of bacteria, 16S rRNA gene amplicon sequencing was chosen as the method of choice.

Bridging the gap between bioinformatics and ecology

The goal of most 16S rRNA amplicon sequencing studies is to translate DNA sequence data into ecological information. Therefore, the analysis of this data type requires interdisciplinary approaches, and draws from bioinformatics and community ecology. Because defining a bacterial species based on 16S rRNA amplicon sequence is not possible, most microbial ecologists instead use Operational Taxonomic Units (OTUs), which are clusters of 16S rRNA sequences that are similar beyond an arbitrary threshold, usually 97% similarity (Stackebrandt and Goebel, 1994). It was recently discovered, however, that most variation in individual 16S rRNA sequences is due to chimeras introduced during PCR and sequencing errors, and when exact amplicon sequences are resolved, the use of OTUs is not necessary (Callahan et al., 2016). Algorithms likes DADA2, published during the course of this dissertation, can resolve these exact amplicon sequences by learning sequencing error patterns and correcting them; the output of these algorithms are referred to as amplicon sequence variants (ASVs) (Callahan et al., 2016). ASVs are more effective as units of study than OTUs, because while they are still somewhat arbitrary, they can be more directly compared across multiple studies. However, because OTUs have been used since the advent of high-throughput 16S rRNA sequencing, the field is experiencing a slow transition. While ASVs may be preferred to OTUs in future publications, and one chapter of this dissertation uses this approach, both

approaches will eventually be replaced by higher throughput metagenome and transcriptome sequencing approaches.

Microbial ecology as a nascent field

There is much deserved excitement in biology about the importance of microbiomes. Bacterial communities have profound impacts on human health and have caused a paradigm shift in treatments of some conditions like *Clostridium difficile* (Bakken et al., 2011). However, given the attention and hype that microbiomes have received, the field risks overselling the importance of individual studies (Sze and Schloss, 2016). The complexity and high-throughput nature of NGS data has demanded the development of new statistical approaches. While many conclusions drawn by contemporary researchers are valid, it is likely that some of most important patterns in taxonomic composition and community structure are still overlooked due to limitations of current statistical approaches (McMurdie and Holmes, 2014; Weiss et al., 2017).
16S rRNA amplicon sequencing can be used to link bacterial taxa to specific environmental conditions at scales previously not possible, and when synthesized across many studies, this approach has the potential to catalogue the imagination-defying diversity of microorganisms described by Winogradsky (Thompson et al., 2017).
There remain questions about how well bacterial communities can be represented using approaches developed by macro-organismal ecologists and *vice versa* (Barberán, 2014). Some ecological phenomena first described in macro-organismal communities also apply to bacteria. For example, succession is well established in bacterial communities, and resembles patterns seen in macro-organismal communities (Gulmann et al., 2015; Lax et al., 2017; Li et al., 2014). However, bacteria evolve in fundamentally different modes

from their eukaryotic counterparts, sharing their genes with distantly related taxa via horizontal gene transfer. Better understanding the scales at which bacterial taxa vary and disperse can offer insights into differences and commonalities between bacterial and macro-organismal communities.

Challenges and approaches to describing within-sample (i.e. alpha) diversity

OTUs are often treated as bacterial species within a study, and they are the units most commonly used to measure within-sample diversity. As with macro-organismal communities, OTU abundance distributions vary with respect to environmental variables. An OTU abundance distribution may be imagined as operational taxonomic units (OTUs) ordered by rank abundance on the x-axis with the 16S rRNA gene amplicon abundances of those OTUs on the y-axis. The shape of this distribution varies considerably among bacterial communities, and few mechanisms have been offered to explain the differences (Shade, 2017). Furthermore, different parts of the amplicon abundance distributions may respond to different types of environmental pressures, depending on whether these pressures affect dominant or rare taxa. Many current researchers use one or two mathematically unrelated metrics, possibly overlooking differences between bacterial communities (Shade, 2017).

This dissertation primarily uses Hill diversity profiles, which are not commonly used to analyze bacterial diversity, to examine OTU abundance distributions and describe how they change with various factors (Hill, 1973). Hill diversity profiles differentially weight abundances of individual taxa, corresponding with different "orders" of diversity. A Hill diversity of order 0, for example, does not weight OTU abundances and is equivalent to species richness. A Hill diversity of order infinity weights abundances so

heavily that only the most abundant OTU is considered. Between these two extremes, Hill diversities of orders 1 and 2 are easily related to alpha diversity metrics commonly used by other researchers: a Hill diversity of order 1 is equivalent to the exponential Shannon index and a Hill diversity of order 2 is equivalent to the inverse Simpson metric. A key finding of this dissertation is that different environmental factors affect the OTU abundance distribution in distinct ways. Some factors affect diversities of all orders, some affect only richness, some affect only diversities of order infinity, and so on. The results of this dissertation may help classify these environmental factors based on the way that they affect OTU abundance distributions in marine systems. As more researchers investigate how environmental factors affect OTU abundance distributions, shifts in these distributions may be explicitly linked to the specific ways environmental factors shape bacterial networks. For example, a loss in order 0 diversity when all other orders are unaffected might often correspond with an environmental factor selecting against a highly diverse taxon consisting of many low-abundance OTUs. A decrease in infinite order diversity when all other orders are unaffected may correspond with an environmental factor that selects against an abundant OTU that weakly interacts with other species. Finally, a decrease in all orders of diversity might suggest selection against an abundant keystone "hub" species or an overall decrease in niche space.

Challenges and approaches to describing between-sample differences (i.e. beta diversity)

As with measuring differences in the OTU abundance distribution of bacterial communities, abundance weighting affects conclusions about the differences in composition between bacterial communities. This dissertation uses both presence-

absence metrics (e.g. Sørensen or unweighted UniFrac), and abundance-weighted metrics (e.g. Bray-Curtis or weighted UniFrac) to explore differences in composition between samples. As with the alpha diversity metrics, patterns sometimes differ between the abundance-weighted and presence-absence metrics, highlighting the importance of using both approaches. This two-pronged approach can give a more balanced picture of what forces shape ecological communities (Anderson et al., 2011).

Calculations of beta diversity generate pairwise distances between bacterial communities; however, these values offer little interpretative value on their own, due to the high dimensionality of community matrices. In order to visualize these beta diversity matrices, it is common to use an ordination technique to reduce the dimensionality of the distance matrix and visualize the dissimilarities in two dimensions. A common technique used among microbial ecologists is Principal Coordinates Analysis (PCoA).This ordination technique generates a set of orthogonal axes that explain the variability in the dissimilarity matrix. An extension of this technique is Constrained Analysis of Principal Coordinates (CAP) ordination, whereby a redundancy analysis is performed on the output of PCoA (Legendre and Anderson, 1999). This leads to an ordination whose axes correspond with the variation explained by the constraining variable. When clustering patterns differ between presence-absence and abundance-weighted metrics, inferences can be made about beta diversity patterns that would not be possible when using only one. Tight clustering when using a presence-absence metric but not an abundance-weighted metric suggests that only low abundance taxa differ between communities. Tight clustering when using an abundance-weighted metric, but not when using a presence-absence metric, suggests that high abundance taxa differ between communities,

but low abundance taxa are shared. These allow for a nuanced understanding of bacterial beta diversity.

Bacteria associated with cnidarian holobionts

Bacteria have long been understood to play important ecological roles in marine and seawater environments. The term holobiont, which refers to an assemblage of species that forms an ecological unit, has been a particularly useful model to understand the relationship between corals and their symbiotic algae, bacteria, and viruses (Glasl et al., 2016; Vega Thurber et al., 2009). Describing the core microbiome of a cnidarian holobiont can provide insights into its health. A stable core microbiome may indicate a high degree of host selection, corresponding with a healthy animal (Grottoli et al., 2018). A few bacterial taxa are often observed in coral microbiomes, and some have been associated with coral disease. The genus *Vibrio*, for example, is common in diseased corals (Munn, 2015). Bacteria in the genus *Roseobacter* are often found in healthy coral holobionts, leading some to hypothesize that these bacteria have a protective effect (Peixoto et al., 2017). Alpha diversity of holobiont bacterial communities has previously been hypothesized as a potential indicator of health; in some cases, the richness of the coral microbiome increases under stress, and this is thought to be due to a loss of host control over microbiome composition (McDevitt-Irwin et al., 2017).

The first chapter of this dissertation describes the microbiome associated with *Anthopleura elegantissima*, a temperate intertidal sea anemone that forms symbioses with both the brown algal *Symbiodinium* and the green algal *Elliptochloris marina*. This symbiotic anemone is distributed from Baja California, Mexico, to Alaska, United States. Across most of its range, *A. elegantissima* hosts *Symbiodinium*, but in cold, low-light

conditions, it occasionally hosts *E. marina.* The relationship between cnidarian microbiomes and their symbioses with *Symbiodinium* have long been known to be important, and shifts in microbial community structure have been linked to the health of the coral holobiont. In most cases, the stability of holobionts in response to heat stress is poor—just a few degrees can lead to dysbiosis and the expulsion of algal symbionts (Brown, 1997). The symbiosis between *A. elegantissima* and *Symbiodinium* is comparatively stable; it endures double-digit swings in temperature daily between tides (Bingham et al., 2011). Chapter 2 of this dissertation examines the bacterial component of this notably durable holobiont.

Diversity of bacterial communities in marine built environments

The chemical composition of seawater surrounding cnidarian holobionts influences their microbiomes (Ziegler et al., 2016). Therefore, the effects of different water treatments on the composition of seawater bacterial communities are crucial to consider when designing experiments involving marine holobionts. However, few studies have described the microbiomes of marine built environments like aquaria and aquaculture facilities. The Shedd Aquarium recently began a collaboration with Argonne National Laboratories to document the microbiome of their artificial seawater facilities, and the Georgia Aquarium has conducted a study on their *Ocean Voyage* artificial seawater exhibit, but these are the only aquarium microbiome projects to date (Patin et al., 2018; Van Bonn et al., 2015). Chapter 3 of this dissertation examines the microbiome of the Hatfield Marine Science Center (HMSC) aquarium, and it is the first study to describe the microbiome associated with an aquarium operating on a flow-through system where natural seawater is pumped, minimally treated, and used only once. Better understanding

how seawater treatment systems shape bacterial communities is essential to provide considerations for researchers wishing to use these facilities in experiments. Chapter 4 of this dissertation seeks to explain why some water treatment approaches are more effective at suppressing blooms of opportunistic pathogens than others in aquaculture systems. It uses bioreactors as models of recirculating aquaculture systems (RAS) and flow-through systems (FTS). These two chapters address a substantial gap in the literature, providing information useful for researchers to design experiments and engineers to design aquaria and aquaculture facilities.

Conclusion

Taken as a whole, this dissertation elucidates relationships between high-level bacterial taxa and their marine environments. The guiding research questions have the potential for near-future applications concerning health diagnoses of holobionts and design of aquaculture and aquarium water treatment systems. This dissertation is characterized by close attention to trends in alpha and beta diversity of bacterial communities by the use of both abundance-weighted and presence-absence metrics. It explores both spatial and temporal scales to refine our understanding of what factors influence distributions of bacterial taxa.

Chapter 2: Microbiome variation in an intertidal sea anemone across latitudes and symbiotic states

Abstract

Many cnidarians form symbiotic relationships with brown dinoflagellate algae in the genus *Symbiodinium*. Bacteria are important to this symbiosis, with diverse functions such as providing nutrients to the symbiont and pathogen protection to the cnidarian. Disrupted bacterial communities are associated with thermally stressed cnidarians, which have a higher likelihood of expelling their symbionts, an event called bleaching. To better understand the association between thermal tolerance and bacterial community structure, we studied communities associated with an exceptionally thermal tolerant cnidarian, *Anthopleura elegantissima*. This intertidal symbiotic sea anemone is distributed from the subtropical waters of Baja California to subarctic Alaska, and experiences daily temperature fluctuations of up to 20ºC. It is also flexible in its symbioses, predominantly hosting *Symbiodinium,* but occasionally hosting the green algae *Elliptochloris marina* or existing without symbionts in an aposymbiotic state. We used 16S rRNA gene amplicon sequencing to characterize the natural variation of microbial communities associated with *Anthopleura elegantissima* in these three symbiotic states and across a latitudinal gradient. In this study, we identified a core microbiome, made up predominantly of low-abundance taxa. We found that the communities associated with *A. elegantissima* were weakly linked to latitude. Alpha diversity analyses revealed significantly higher richness values for bacterial communities associated with anemones hosting *E. marina*. Lastly the microbiomes associated with anemones in different symbiotic states were compositionally distinct. Taken together, our results suggest that the structure of bacterial

communities associated with these temperate cnidarians is tightly linked to symbiotic state and weakly linked to other biogeographic phenomena.

Introduction

Many cnidaria in class *Anthozoa*, including corals and sea anemones, form mutualistic relationships with endosymbiotic brown dinoflagellate algae in the genus *Symbiodinium.* Bacterial symbionts facilitate this relationship, providing metabolic and protective functions such as supplying nitrogen to the brown dinoflagellate algae and inhibiting growth of pathogens on cnidarians (Lema et al., 2012; Rypien et al., 2010). The community of a cnidarian, its algal endosymbionts, and all associated microbiota is often modeled as an ecological unit called a holobiont (Rohwer et al., 2002). Destabilization or stress on one holobiont component can propagate through the community and cause the system to collapse. Thermal stress, for example, can diminish the protective functions of commensal and symbiotic bacteria of scleractinian corals, allowing opportunistic pathogenic bacteria such as *Vibrio shiloi* to bloom (Frydenborg et al., 2014). However, destabilized bacterial communities do not always precede bleaching, and bleaching is not always caused by pathogens; in some cases, thermal stress can directly lead to bleaching, with destabilized bacterial communities following the bleaching event (Ainsworth and Hoegh-Guldberg, 2009). Some cnidarians, such as the intertidal sea anemone *Anthopleura elegantissima,* experience extreme thermal variation in their natural habitat with no adverse effects to their symbiosis with the brown algal *Symbiodinium* (Bingham et al., 2011). *A. elegantissima* also occasionally hosts a green algal symbiont, *Elliptochloris marina*, but this symbiosis is highly susceptible to bleaching (Dimond et al., 2013). Observing the microbiota associated with *A.*

elegantissima in these different symbiotic states may provide clues into the relationship between stability of bacterial communities and stability of cnidarian-algal symbioses.

A. elegantissima is a symbiotic intertidal sea anemone distributed from subtropical Baja California to subarctic Alaska. It exhibits broad thermal tolerance, experiencing fluctuations of up to 20ºC within minutes without obvious signs of stress (Bingham et al., 2011). It is uniquely flexible in its symbioses compared to other cnidarians, and can host the brown algae *Symbiodinium*, the green algae *E. marina*, or no symbionts at all in an aposymbiotic state. Irradiance conditions and temperature are the main predictors of symbiotic state: generally, *A. elegantissima* is found in either the brown symbiotic state (hosting *Symbiodinium*) when in the light or in the aposymbiotic state in the dark (Bates et al., 2010). Anemones in the green symbiotic state (hosting *E.marina*), on the other hand, are found in the more restrictive ecological range of low-temperature and low-but-nonzero irradiance conditions, making up <1% of anemones as far north as Vancouver, British Columbia (Bates et al., 2010). *A. elegantissima* can also be found hosting both symbionts, particularly in the Salish Sea, where east-facing slopes allow for a gradient of irradiances (Dimond et al., 2011). The paucity of anemones in the green symbiotic state is concordant with experimental evidence that the green algae are less beneficial to *A. elegantissima*; brown algal symbionts are estimated to contribute about five times as much carbon to their hosts compared to green algal symbionts (Verde and McCloskey, 1996). Furthermore, *A. elegantissima*, which are facultative sexual reproducing animals, exhibit higher rates of sexual reproduction in the green symbiotic state and higher rates of asexual reproduction in the brown symbiotic state (Bingham et al., 2014). This is in agreement with the common observation that in facultative sexual

organisms, sexual reproduction is usually associated with poor conditions (Ram and Hadany, 2016).

The robustness of *A. elegantissima*'s symbiosis with the brown algal *Symbiodinium*, as well as its flexibility to exist in different symbiotic states, make it an ideal model to investigate high-level patterns of host-symbiont-microbiome associations. In this study, we used 16S amplicon sequencing to characterize the microbial communities associated with *A. elegantissima* across a latitudinal gradient and in different symbiotic states. Our goals were to a) evaluate how host-associated microbial communities vary across a large-scale temperature and irradiance gradient (i.e. latitude) and b) compare the microbiome of *A. elegantissima* in three of its symbiotic states (brown, green, and aposymbiotic) to assess the degree to which microbial communities associate with symbiotic states.

Materials and Methods

Sample collections and preparation

We collected 119 anemones from eight sites ranging from Cape Mendocino, California, to Point of Arches, Washington, USA during August and September 2014 (Figure 1). We began collections approximately one hour before low tide. To account for possible effects of factors associated with tide (e.g. temperature, exposure time, substrate), we collected equal numbers of samples from each biological intertidal zone (low, middle, high). The low zone was defined by presence of feather boa kelp, the middle zone by mussel beds, and the high zone by barnacles (Paine, 1974). For questions concerning symbiotic state, we collected fifteen anemones from the middle tidal zone at Boiler Bay, OR, USA, in fall 2014: five brown, five green, and five aposymbiotic. Symbiont typing was performed by

careful visual inspection in the laboratory. We collected anemones using forceps and sterile nitrile gloves. A fresh pair of gloves was used for each sample, and forceps were cleaned using 70% ethanol between samples. For storage, we placed samples in Whirl-pak® sampling bags (Nasco), and froze the samples on dry ice in the field.

DNA Isolation, 16S PCR, and Sequencing

Samples were stored at -80°C prior to DNA extractions for 16S PCR. Whole anemones were homogenized using mortar and pestle. DNA was isolated from the homogenate using PowerSoil DNA Extraction kits (MOBIO, Carlsbad, CA, USA). We sequenced three kit blanks and two PCR negative blanks to assess contamination introduced during this step. The V4 region of the 16S rRNA gene was amplified using 515F-806R primers modified for Illumina indexing (Kozich et al., 2013) and AccuStart II PCR ToughMix (Gaithersburg, MD, USA). PCR was performed in triplicate with the following program: 1 x 2 minutes at 95°C, 30 x (20 seconds at 95°C, 15 seconds at 50°C, 5 minutes at 72°C), 1 x 10 minutes at 72°C. We included two PCR negatives to assess contamination introduced during this step. Samples were pooled, quantified using qPCR, and normalized before sequencing the MiSeq platform (Illumina, San Diego, CA, USA) using the single-end 150 bp chemistry.

16S Amplicon Pre-Processing

After demultiplexing and initial quality filtering (Q>20) using the Quantitative Insights in Microbial Ecology (QIIME) (version 1.9) pipeline split_libraries_fastq.py (Caporaso et al., 2010), sequences were clustered into *de novo* operational taxonomic units (OTUs) using Swarm (Mahé et al., 2014). Singleton OTUs were discarded to minimize false

OTUs (Auer et al., 2017). Chimeric sequences removed using uchime (Edgar 2011, v4.2.40) against the Silva gold chimera reference (Quast et al., 2012). Taxonomy was assigned using BLAST (Altschul et al., 1990) with an e-value cutoff of 0.001 against the Greengenes 13.8 reference, the default database for QIIME. OTUs identified as chloroplasts and mitochondria were removed from the OTU table. Additionally, OTUs were identified as contaminants and removed if their relative abundances were above 1% in the kit blanks or PCR negatives. We chose these thresholds to avoid removing OTUs that might appear in the kit blanks or PCR negatives due to Illumina sample bleeding or cross-contamination from samples.

Core microbiome of A. elegantissima

Commonly used taxonomic classifiers and databases often differ at lower taxonomic ranks (Bokulich et al. 2018), so the Order level was chosen as the unit for core taxa analyses, so our results could be compared to studies using different classifiers and databases. Because the term "core" is not well defined (Shade and Handelsman, 2012), three separate core taxa sets were identified using 95%, 75%, and 50% prevalence cutoffs. Quantiles of the relative abundances of these orders were also included to relate the abundances of these taxa to their prevalence (Appendix table 2).

Alpha diversity metrics

To account for random biases associated with rarefaction, OTU tables were rarefied 10 times and the Chao1 metric, Faith's Phylogenetic Diversity, and Shannon Index were calculated for each rarefied table. Mean values were used for statistical tests and plots. The 114-sample OTU table for latitude analyses was rarefied to a depth of 5,000. The 15-

sample OTU table for symbiosis analyses was rarefied to a minimum depth of 2,632 so that all samples could be included in the analysis. We conducted Kruskal-Wallis tests to assess differences in alpha diversity among groups and Mann-Whitney U tests to assess differences between groups.

Beta diversity

For beta diversity analyses, we conducted permutational multivariate analyses of variance (PERMANOVA) tests using the R function vegan::adonis (Oksanen et al., 2018) to identify associations between symbiotic state or latitude and Bray-Curtis dissimilarities. Constrained Analysis of Principal Coordinates (CAP) was performed using the R function vegan::capscale to test for linear relationships between explanatory variables and beta diversity metrics. In CAP, a distance matrix is ordinated using Principal Coordinates Analysis (PCoA), and the resulting coordinates are tested for linear associations with variables of interest (e.g. latitude or symbiotic state) using redundancy analysis, an extension of multiple linear regression (Oksanen et al., 2018). The results of redundancy analysis can then be used to constrain the PCoA ordination, resulting in an ordination with axes constrained to only explain variance explained by the variable of interest.

To test for correlations between beta diversity and latitude, we conducted Mantel tests using the R function vegan::mantel. We conducted the test for Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac metrics.

Taxonomic differences between symbiotic states

To identify taxa associated with symbiotic states, we conducted indicator taxon analyses on OTU tables rarefied to minimum depth and agglomerated at the phylum, class, and

order ranks using the indicspecies::multipatt function in R (De Cáceres and Legendre, 2009). We used the IndVal.g metric, which considers differential abundance as well as presence/absence information to detect associations between taxa and combinations of sample groups (Dufrêne and Legendre, 1997; De Cáceres et al., 2010). Prior to this analysis, we filtered OTUs with mean relative abundances below 10^{-3} and present in fewer than two samples to reduce noise from OTUs near the detection limit and increase the power of the analysis. Taxa associated with symbiotic state with an FDR q-value < 0.2 were recorded along with the indicator value, p-value, and FDR q-value (Appendix table 3). We plotted the log10 relative abundances as well as prevalences of the orders identified as indicators of symbiotic state (Figure 4).

Results

Pre-processing

The processed data contained 2,456,818 reads associated with 18,709 OTUs and had a median sample depth of 11,399 reads. We split this table into two sub-tables for analyses concerning latitude and symbiotic state. The table for analyses concerning latitude contained 114 samples with a median sample depth of 13,240 reads. The table for analyses concerning symbiotic state contained 15 samples with a median sample depth of 6,986 reads. Metadata, including site, latitude, symbiotic state, sequencing depth, and alpha diversity metrics for each sample, can be found in Appendix table 1. We removed 12 OTUs, visualized in Appendix Figure 1. These OTUs were all taxonomically assigned to genera previously identified as common kit contaminants: *Sphingomonas, Janthinobacterium, Burkholderia, Variovorax, Herbaspirillum, Methylobacterium, Aeromicrobium, Pseudomonas, Phyllobacterium,* and *Bacillus* (Salter et al., 2014). Two

OTUs we removed were unassigned at the genus level, but belonged to families *Comamonadaceae* and *Enterobacteriaceae.*

The core microbiome of A. elegantissima in the brown symbiotic state consists mostly of orders typically associated with coastal seawater

To identify microbiota that stably associate with *A. elegantissima* across the sampled geographic range, we identified core orders at 50%, 75%, and 95% prevalence thresholds (Appendix table 2). *Flavobacteriales, Rhodobacterales, Rhizobiales* and *Alteromonadales,* orders commonly associated with coastal seawater, had the highest median relative abundances of the core orders with relative abundances of 14%, 9%, 4%, and 4% respectively. All of the orders with prevalence > 95% (Table 1) were previously found to associate with symbiotic cnidarians and/or seawater (Glasl et al., 2016; McDevitt-Irwin et al., 2017; Pootakham et al., 2017).

Microbial communities associated with green anemones have higher richness, but not evenness

We analyzed alpha diversity using Chao1, Faith's PD, and Shannon Index metrics (Figure 2A-C). Chao1, an estimator of the total number of OTUs, had significantly higher median values in communities associated with green anemones compared to the pool of brown and aposymbiotic anemones (Mann-Whitney U, $p = 0.003$). This result is concordant with patterns seen in Faith's PD metric (Mann-Whitney U, $p = 0.039$), which demonstrates that this increase in richness is not simply due to the presence of a few hyper-diverse species or genera. Median Shannon Index values did not differ significantly among the symbiont types (Kruskal-Wallis, $p = 0.147$), but tended to have

higher values in symbiotic anemones compared to aposymbiotic anemones (Mann-Whitney U, p=0.055).

A rarefaction analysis of the samples aggregated by symbiotic state provides further evidence that communities associated with green anemones have higher richness than brown and aposymbiotic anemones (Figure 2D). At a rarefaction depth of 10,000, the agglomerated green-associated communities contained 1,164 observed OTUs, brown-associated communities contained 840 OTUs, and aposymbiotic-associated communities had 633 OTUs.

Symbiotic state is associated with microbial community structure

We used four beta diversity metrics and Constrained Analysis of Principal Coordinates on rarefied OTU tables to investigate the differences in community structure attributed to symbiotic state (Figure 3A-D). These metrics, which differ in their weighting of abundance and phylogenetic information, were all significantly linked to symbiotic state. Clustering of Bray-Curtis values, which are abundance-weighted, suggest that abundant species differ among the symbiotic states (PERMANOVA, $p = 0.025$, $R^2=0.196$). Clustering of Weighted UniFrac, which also takes into account phylogenetic information, further confirms this observation, while also suggesting that differences in closely-related OTUs do not account for the observed differences (PERMANOVA, $p = 0.025$, $R^2=0.262$).

We observed differences in Jaccard distances (calculated from presence/absence data), which suggests that OTU incidences, not just abundances, differ among the symbiotic states (PERMANOVA, $p = 0.019$, $R^2=0.172$). Differences in Unweighted UniFrac values (PERMANOVA, p = 0.001, 0.241), a phylogenetically informed metric also calculated

from presence/absence data, further supports this observation and suggests that these differences in frequencies are not driven by closely-related OTUs.

In summary, similar observations between abundance-weighted and presence/absence metrics suggest that the differences among these communities cannot be explained entirely by high- or low-abundance OTUs. Furthermore, the similarities in results from phylogenetically informed and phylogenetically uninformed metrics suggest that these observations are not skewed by the presence of hyper-diverse species or genera, which tend to inflate incidence-based metrics.

Symbiotic states are associated with indicator taxa

Indicator taxon analysis identified taxa associated with symbiotic state (Appendix table 3). We plotted the relative abundances and prevalence of the five orders identified as indicators to visualize patterns in the distributions of these orders (Figure 4A-E).

The order *Legionellales* was identified as an indicator of the brown symbiotic state (FDR q-value = 0.076). This order was detected in communities associated with all symbiotic states, but had higher prevalence and relative abundances in the brown symbiotic state. This order was dominated by an OTU taxonomically assigned to the *Nebulobacter yamunensis.*

Orders *Rhodocyclales*, *Spirochaetes*, and *FS117-23B-02* were identified as indicators of the "Not green" symbiotic state (i.e. associated with brown and aposymbiotic states) (FDR q-values = 0.193, 0.076, and 0.076 respectively). *Rhodocyclales* was present in every sample included in the analysis, but had lower relative abundances in green-associated communities. *Spirochaetales* had 100% prevalence in brown- and aposymbiotic- associated communities, but was only present in one green-associated

community. The order *FS117-23B-02*, in the class *Dehalococcoides*, was present in 90% of aposymbiotic- or brown- associated communities, but not detected in any of the green-associated communities.

The order *Thiohalorhabdales* was identified as an indicator of symbiosis (i.e. associated with brown- or green-associated communities) (FDR q-value = 0.076). *Thiohalorhabdales* had both lower prevalence and abundances in aposymbiotic-associated communities than in symbiotic communities.

Alpha diversity of A. elegantissima-associated communities is not linked to latitude

We did not detect an association between latitude and Shannon index, Chao1, or Faith's Phylogenetic Diversity values using a Kruskal-Wallis test or Spearman correlation (Figure 5A-C). A rarefaction analysis of the samples aggregated by site similarly did not reveal any trends between latitude and number of observed OTUs (Figure 5D). A median of 3148 OTUs were observed per site.

Latitude is weakly linked to beta diversity

We used four beta diversity metrics for CAP ordinations and Mantel tests (Materials and Methods) to evaluate trends in beta diversity over a latitudinal gradient. As with analyses concerning symbiotic state, results were robust to metric choice, so only Bray-Curtis results are reported in our CAP analyses (Figure 6). This ordination was only constrained by site, so both CAP axes describe variation explained by site. Tidal zone was found to have a small but significant association with Bray-Curtis dissimilarities (PERMANOVA, $p = 0.026$, $R^2=0.025$). We analyzed latitude both as a quantitative and categorical variable to evaluate linear relationships between latitude and Bray-Curtis dissimilarities as well as

potential non-linear differences among sites. When treated as a quantitative variable, latitude was found to be very weakly linked to Bray-Curtis dissimilarities (PERMANOVA, $p = 0.001$, $R^2 = 0.022$). As a qualitative factor, latitude explained more variance in Bray-Curtis dissimilarities, suggesting that the relationship between site location and beta diversity is not linear (PERMANOVA, $p = 0.001$, $R^2 = 0.126$). Finally, we conducted Mantel tests to test for correlations between geographic distances and beta diversity distances. We found significant positive correlations for Bray-Curtis ($r=0.10$, $p=0.01$), Jaccard ($r=0.10$, $p=0.01$), and Unweighted UniFrac ($r=0.16$, $p=0.001$) distances. Weighted UniFrac distances did not correlate with geographic distances ($r=0.03$, $p=0.23$).

Taxonomic composition is relatively stable across latitudes

We also examined the relationship between abundant phyla (defined as >1% abundance) and latitude (Figure 7). *Proteobacteria* and *Bacteroidetes* were the dominant phyla, making up 56% and 25% of anemone-associated microbiomes respectively. We did not detect any relationship between abundances of these phyla and latitude (Spearman, FDR q-value > 0.2). Of the less abundant phyla, 3 correlated with latitude: *Tenericutes* (Spearman rho = 0.39, FDR q-value =0.002), *Acidobacteria* (Spearman rho = 0.31, FDR q-value = 0.005), and *Firmicutes* (Spearman rho = 0.22, FDR q-value = 0.086). Spearman correlation analyses were then used to test for monotonically increasing or decreasing abundances of orders present in at least 50% of samples across the sampled range. We identified eleven orders with a significant monotonic relationship with latitude (FDR q-value < 0.2). We plotted the relative abundances and prevalences of the four orders with the highest relative abundances because correlations from low abundance and

sparse features are more likely to be spurious (Figure 8). *Mycoplasmatales* (FDR q-value = 2.7 x 10^{-4}, rho = 0.39) exhibited a positive trend in both prevalence and relative abundance. *Legionellales* (FDR q-value = $9.6x10^{-4}$, rho = 0.31) displayed a unimodal distribution in both prevalence and relative abundances, peaking at the 45^{th}-47^{th} parallels. Abundances of *Alteromonadales* (FDR q-value= $4.9x10^{-8}$, rho = -0.48) did not display a clear visual trend despite having the strongest relationship with latitude of the orders included in this analysis. Order *Bacillales* (FDR q-value = 1.2 x 10^{-1}, rho = 0.22) had lower prevalence in lower latitudes, but differences in abundances were not visually apparent.

Discussion

Indicator taxon analyses reveal relationships between specific taxa and symbiotic state

We identified taxa associated with different symbiotic states. The order *Legionellales,* dominated by an OTU assigned to the genus *Nebulobacter,* was found to be associated with the brown symbiotic state. Bacteria in the genus *Nebulobacter* are typically symbionts of ciliates (Boscaro et al., 2012). This OTU also significantly correlated with latitude, appearing to exhibit a unimodal distribution across latitudes, with abundances and prevalence peaking between the 45^{th} and 47^{th} parallels.

Spirochaetales, Rhodocyclales, and an order in class *Dehalococcoides* (*FS117-23b-02*) were negatively associated with the green symbiotic state. Communities associated with the green symbiotic state displayed higher richness and comparable evenness to the other states, so on average, taxa are expected have lower relative abundances due to the compositional nature of rarefied data (Jackson, 1997). With this this caveat, the complete absence of the order in *Dehalococcoides* in green-associated communities is striking,

given that this order is present in abundances of almost 10% in some brown- and aposymbiotic- associated communities. The absence of this order in green-associated communities may well be symbiont-related, but without an observation in a controlled setting, this possibility is difficult to evaluate.

The order *Thiohalorhabdales* was found to be an indicator of symbiosis (i.e. associated with the brown or green symbiotic state). This order is made up of obligate sulfur-oxidizing bacteria, and has been found in high abundances on temperate corals (Sharp et al., 2017). Additionally, sulfur cycling has been previously identified as a discriminator between bacterial communities associated with symbiotic and aposymbiotic sea anemones (Röthig et al., 2016; Hadaidi et al., 2017).

The microbiome of A. elegantissima is stable across a latitudinal gradient of >1000km

The most abundant orders in the core microbiome of *A. elegantissima—Flavobacterales, Rhodobacterales, Rhizobiales* and *Alteromonadales*-- are commonly found in coastal seawater (Bryson et al., 2017; López-Pérez et al., 2016), so we hypothesize that seawater surrounding the sea anemones influences the composition of their microbiomes.

We did not find any strong relationships between latitude and alpha diversity of communities associated with *A. elegantissima.* While richness is linked to latitude at a global scale (Ladau et al., 2013), no trend was found between richness and latitude in microbial communities of intertidal sand in California (Boehm et al., 2014). Our beta diversity analyses reveal an association between beta diversity and latitude. However, we observe that this association is very weak, suggesting that intra-site factors, such as wave action, may play an important role in shaping the microbial communities associated with these anemones. We did not find a significant relationship between Weighted UniFrac

distances and geographic distances; this suggests a microbiome whose most abundant members do not vary with latitude. A significant correlation between Unweighted UniFrac distances and geographic distances suggests a low-abundance component of these microbiomes that does vary with latitude. Among the orders with abundances that significantly correlate with latitude, we did not observe clear monotonic trends, so we suspect that differences between sites are better explained by local ecological conditions. Indeed, habitat and substrate have previously been found to correlate with bacterial community structure in symbiotic corals (Roder et al., 2015).While we found a weak relationship between latitude and community structure, it may be fruitful to examine effects of habitat, land use and wave conditions on anemone-associated communities in future efforts; latitude, land use classification, wave conditions have previously been found to influence intertidal sand communities in California (Boehm et al., 2014). Finally, developmental stage has recently been found to be tightly coupled to microbial community structure in a symbiotic sea anemone (Mortzfeld et al., 2016), so this factor should be considered in future studies.

Elliptochloris-associated communities exhibit higher richness values

We found a significant association between symbiotic state and richness measures of anemone-associated microbial communities. Communities associated with anemones in the green symbiotic state had higher richness values than anemones in the brown or aposymbiotic states. We hypothesize that this is corresponding to a stressed state, because higher richness often associates with stress in coral-associated communities; this is thought to be due to a diminishing of microbiome regulation when hosts are stressed (McDevitt-Irwin et al., 2017). This is further supported when previous observations that

anemones in the green symbiotic state favor sexual reproduction over asexual reproduction typical of the brown symbiotic state (Bingham et al., 2014), are considered alongside the strong correlation between stress and sexual reproduction in facultative sexual organisms (Ram and Hadany, 2016). Finally, *Anthopleura elegantissima* only stably associate with *Elliptochloris* in a narrow range of temperatures and irradiances, suggesting that the symbiosis with *Elliptochloris* is less favorable (Bates et al., 2010). We found a robust association between symbiotic state and beta diversity with anemones in the green, brown, and aposymbiotic states having distinct community compositions. We propose that symbiont presence and type has a minor role in the selection of *A. elegantissima*- associated microbial communities or that microbial communities have a role in selection of algal symbionts. However, because we collected the samples from natural populations, we must consider the possibility that unexplored environmental factors are responsible for the differences in community composition that we observed. Furthermore, due to a relatively few number of samples collected in each symbiotic state (n=5), these results must be interpreted with caution.

Conclusion

We identified a core microbiome of *A. elegantissima* across a >1000-kilometer range. Beta diversity analyses reveal differences in the structure of bacterial communities across this range, but these differences may be better explained by between-site factors other than latitude. We detected associations between symbiotic state and the structure of microbial communities associated with *A. elegantissima*. Despite the significance of these associations, it is difficult to rule out confounding effects of habitat differences among the anemones in these symbiotic states. We suggest that a common garden experiment

whereby anemones in different symbiotic states acclimate to identical conditions, then are bleached, would provide more certainty that the observed differences can be attributed to symbiotic state, and could be more readily compared to existing literature on cnidarian bleaching.

Table 1. Orders core to the microbiome A. elegantissima at over 95% prevalence.

Phylum	**Class**	**Order**	**Median relative abundance**
Bacteroidetes	Flavobacteriia	Flavobacteriales	0.1409
Proteobacteria	Alphaproteobacteria	Rhodobacterales	0.0862
Proteobacteria	Alphaproteobacteria	Rhizobiales	0.0435
Proteobacteria	Gammaproteobacteria	Alteromonadales	0.0432
Proteobacteria	Gammaproteobacteria	Thiotrichales	0.027
Bacteroidetes	[Saprospirae]	[Saprospirales]	0.0251
Proteobacteria	Betaproteobacteria	Burkholderiales	0.0195
Proteobacteria	Gammaproteobacteria	Vibrionales	0.0154
Proteobacteria	Alphaproteobacteria	Sphingomonadales	0.0173
Actinobacteria	Acidimicrobiia	Acidimicrobiales	0.0166
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	0.017
Proteobacteria	Gammaproteobacteria	Pseudomonadales	0.0084
Actinobacteria	Actinobacteria	Actinomycetales	0.008
Proteobacteria	Betaproteobacteria	Rhodocyclales	0.0029
Proteobacteria	Gammaproteobacteria	Xanthomonadales	0.0022

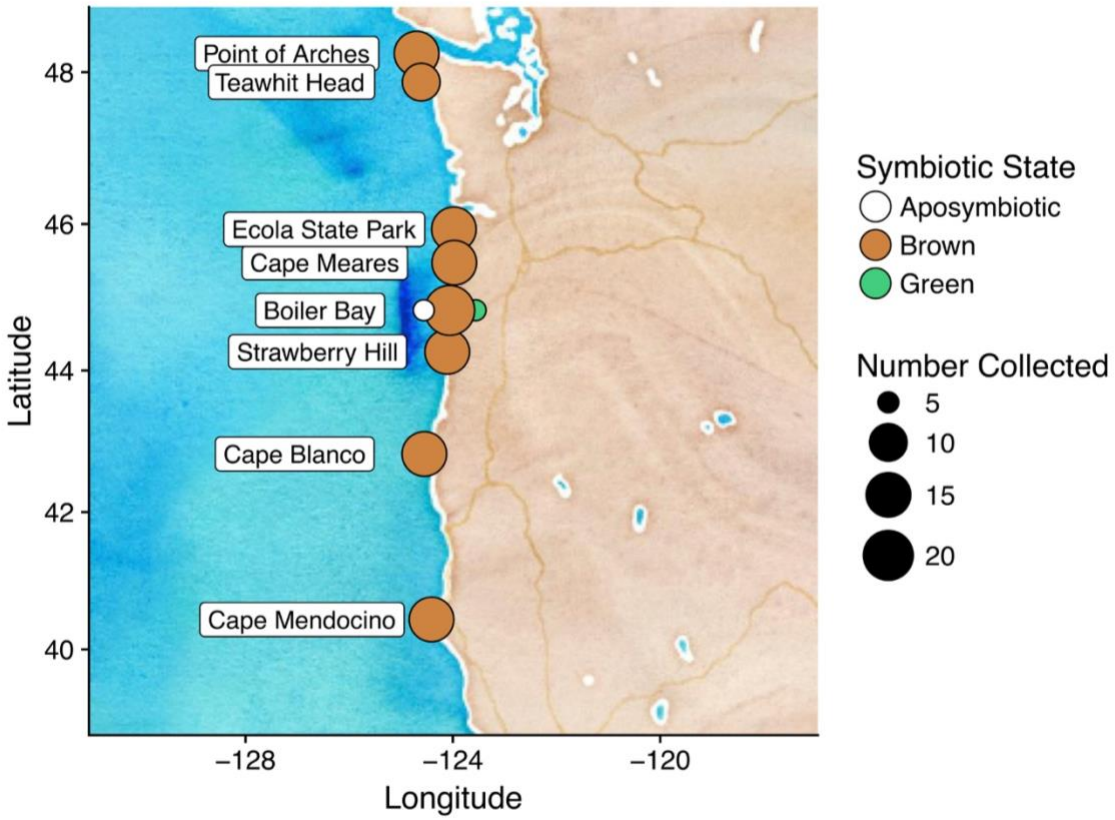


Figure 1. We collected A. elegantissima from eight sites in the Pacific rocky intertidal over a latitudinal range of eight degrees. We returned to Boiler Bay to collect samples for analyses concerning symbiotic state.

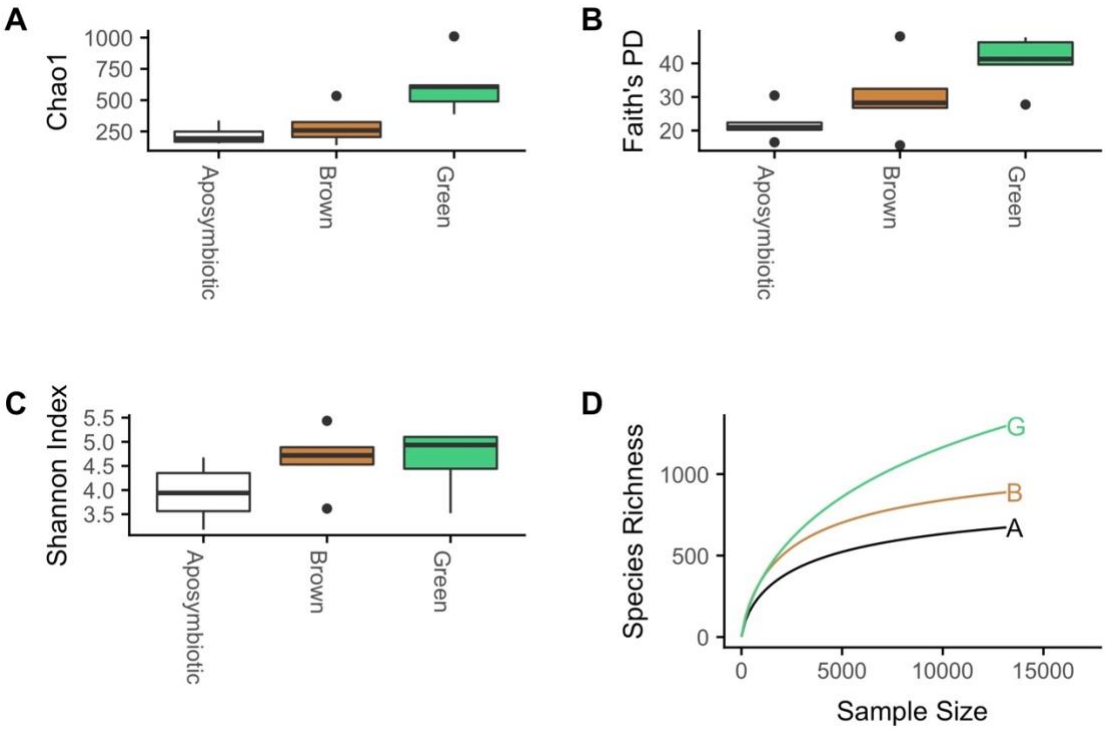


Figure 2: Microbial communities associated with the green symbiotic state have significantly higher median (A) Chao1 (Mann-Whitney U, p = 0.003) and (B) Faith's PD values (Mann-Whitney U, p= 0.039). There is some evidence that communities associated with symbiotic anemones have higher median (C) Shannon index values than those of aposymbiotic anemones (Mann-Whitney U, p = 0.055). (D) Rarefaction curves of aggregated samples (n=5 for each symbiotic state) provide estimates of the total number of OTUs observed in communities associated with each symbiotic state.

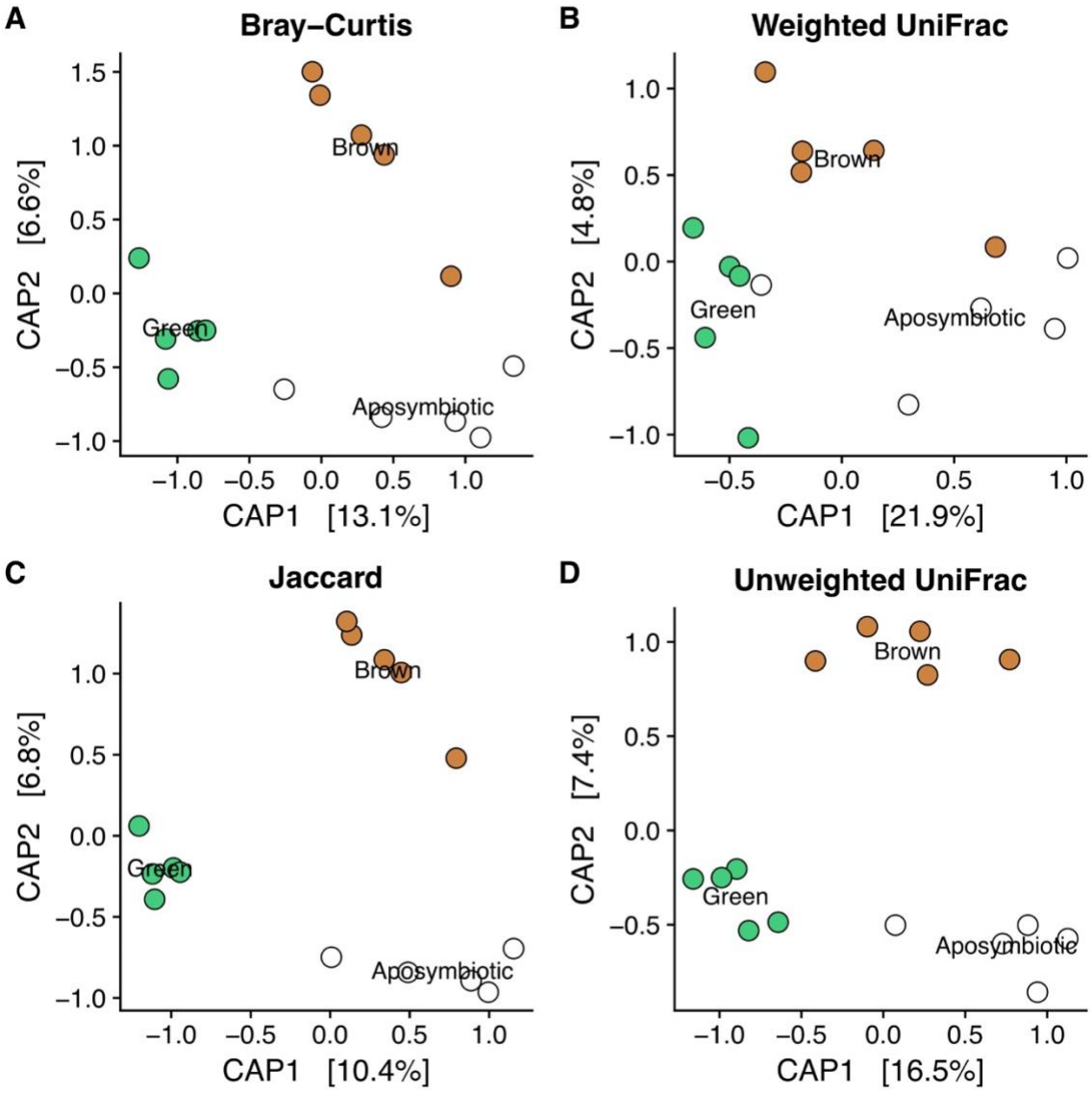


Figure 3: CAP ordination plots of (A) Bray-Curtis, (B) Weighted UniFrac, (C) Jaccard, and (D) Unweighted UniFrac beta diversity metrics reveal structure in microbial communities associated with A. elegantissima in different symbiotic states. Clustering by symbiotic state was robust to the metric used.

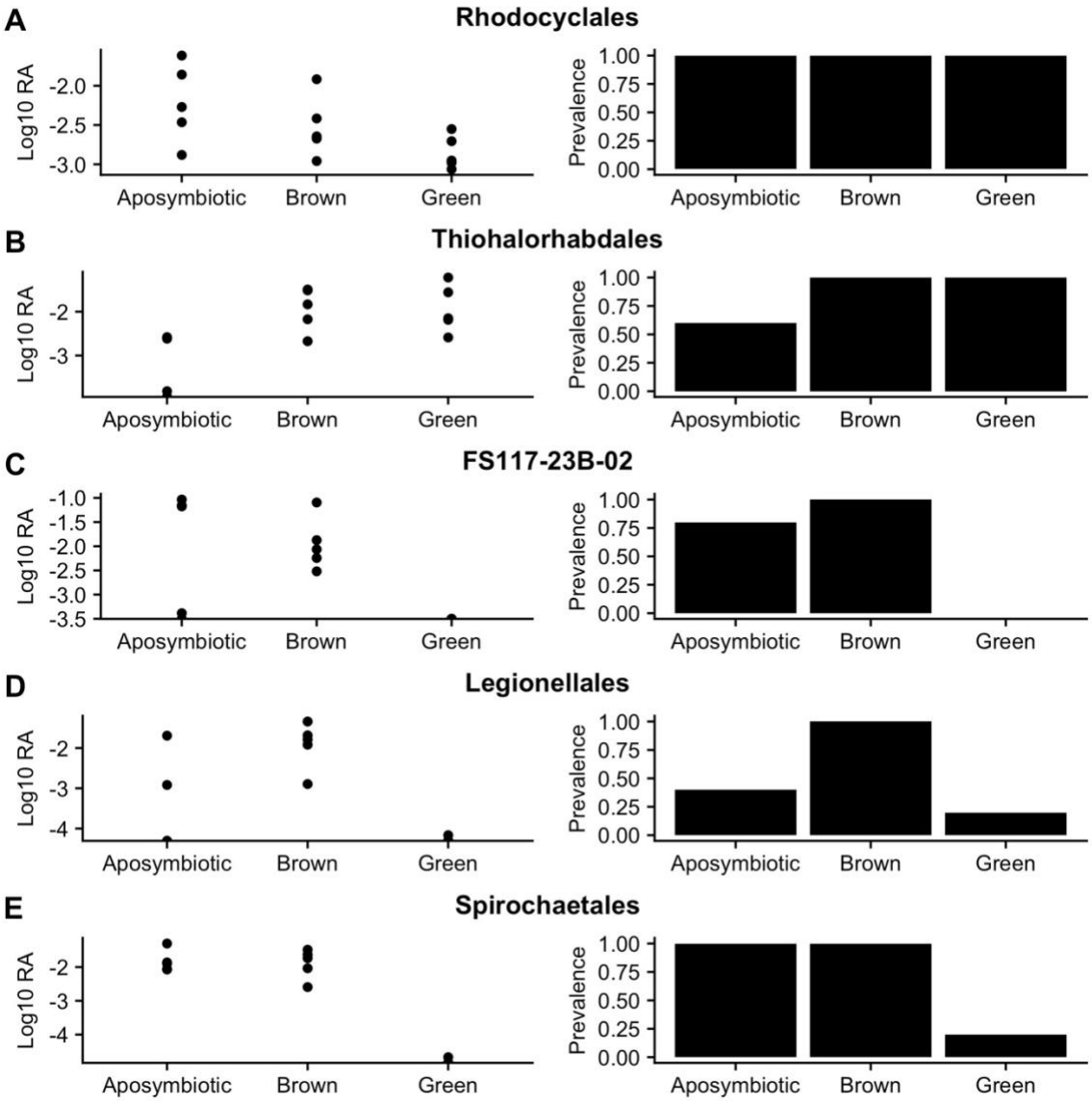


Figure 4: Log10 relative abundances (left) and prevalence (right) reveal abundance and presence/absence patterns of orders identified as indicators of symbiotic state (FDR q-value < 0.2): (A) Rhodocyclales (B) Thiohalorhabdales, (C) FS117-23B-02, (D) Legionellales and (E) Spirochaetales.

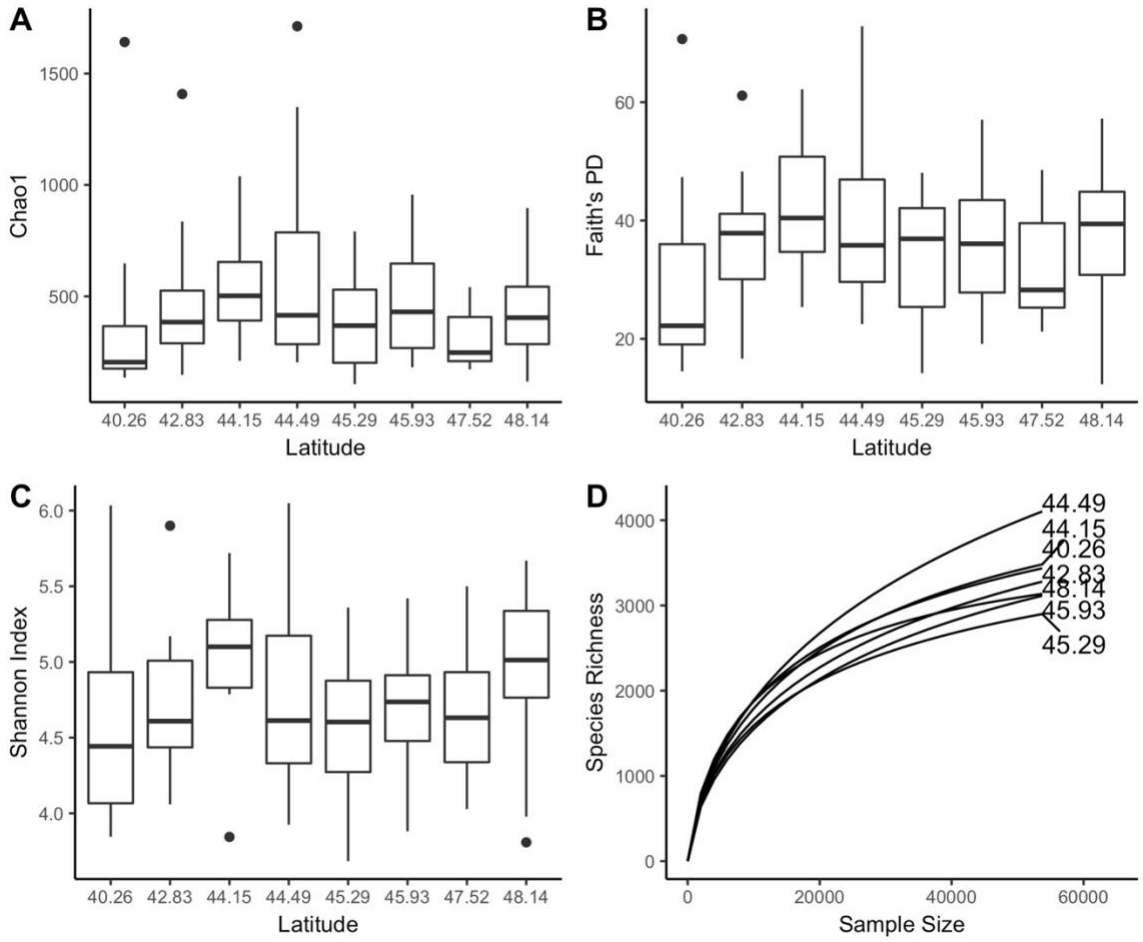


Figure 5: We did not find an association between latitude and (A) Chao1, (B) Faith's PD, or (C) Shannon Index values using a Spearman correlation or Kruskal-Wallis test. (D) A rarefaction curve of samples aggregated by site (n=14 or 15) provides estimates of the total number of OTUs observed at each site. At a depth of 50,000 reads, a median of 3,204 OTUs were observed.

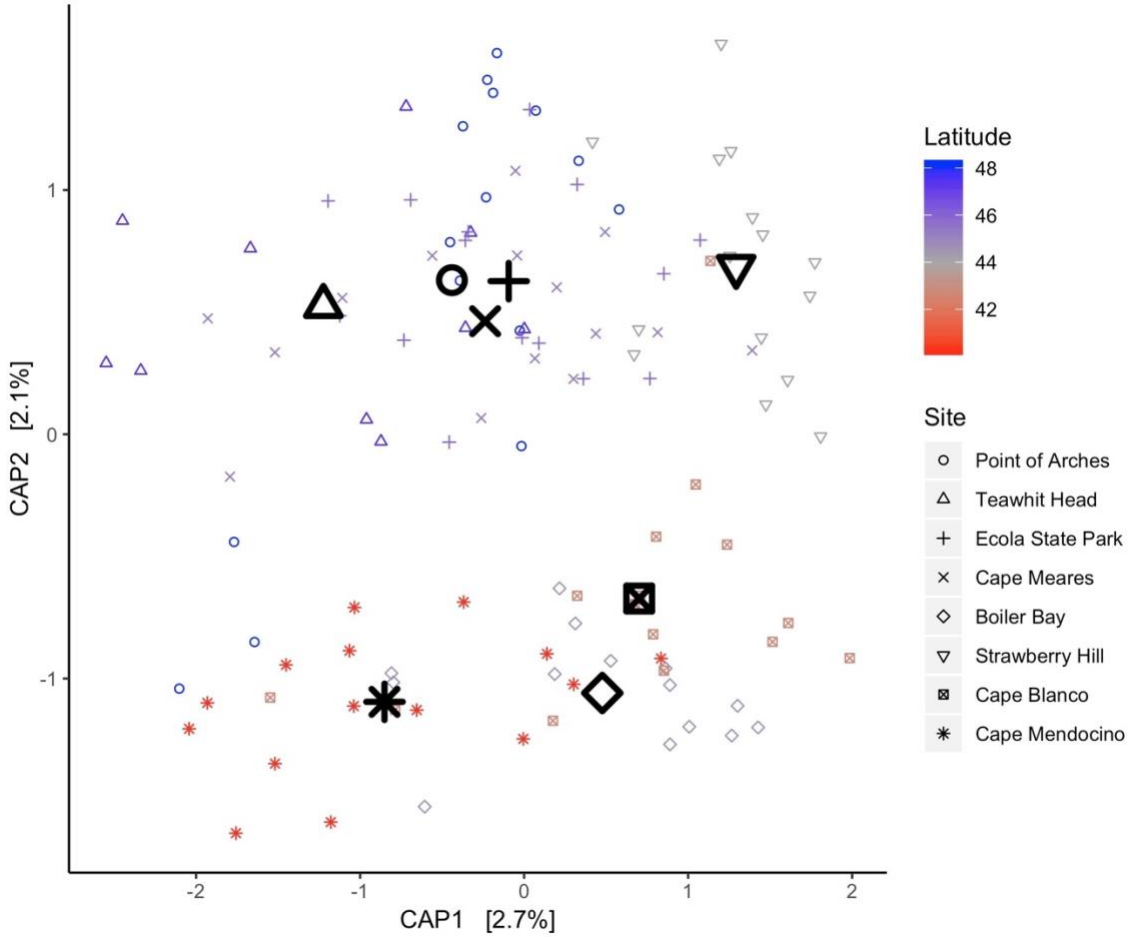


Figure 6: A CAP ordination on Bray-Curtis dissimilarities between A. elegantissima communities across latitudes reveals some structure. Latitude explains a small part of the variance across the range when treated as a quantitative variable ($p=0.002$, $R^2= 0.022$). Site labels were placed at the centroids of samples from each location.

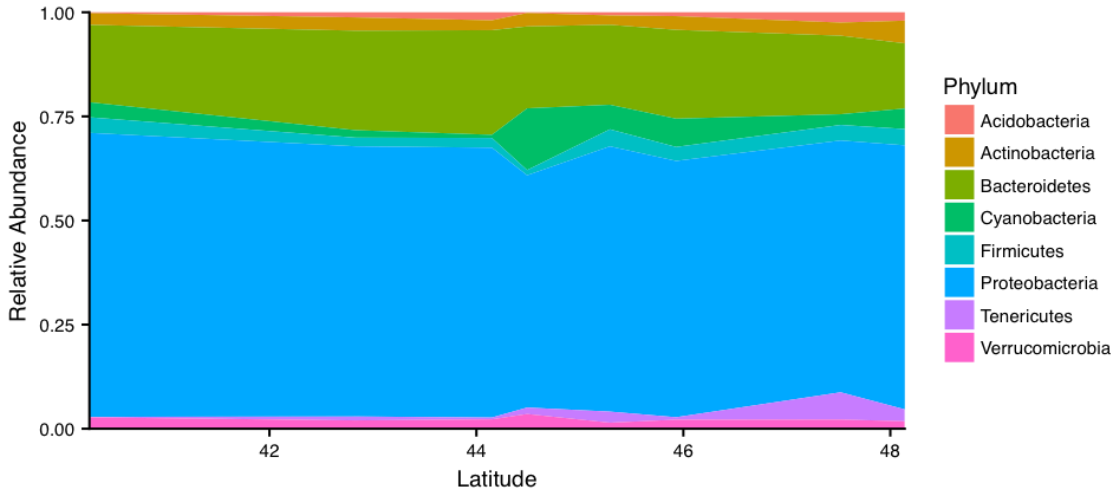


Figure 7: Taxonomic composition of communities associated with A. elegantissima is dominated by Proteobacteria and Bacteroidetes across latitudes.

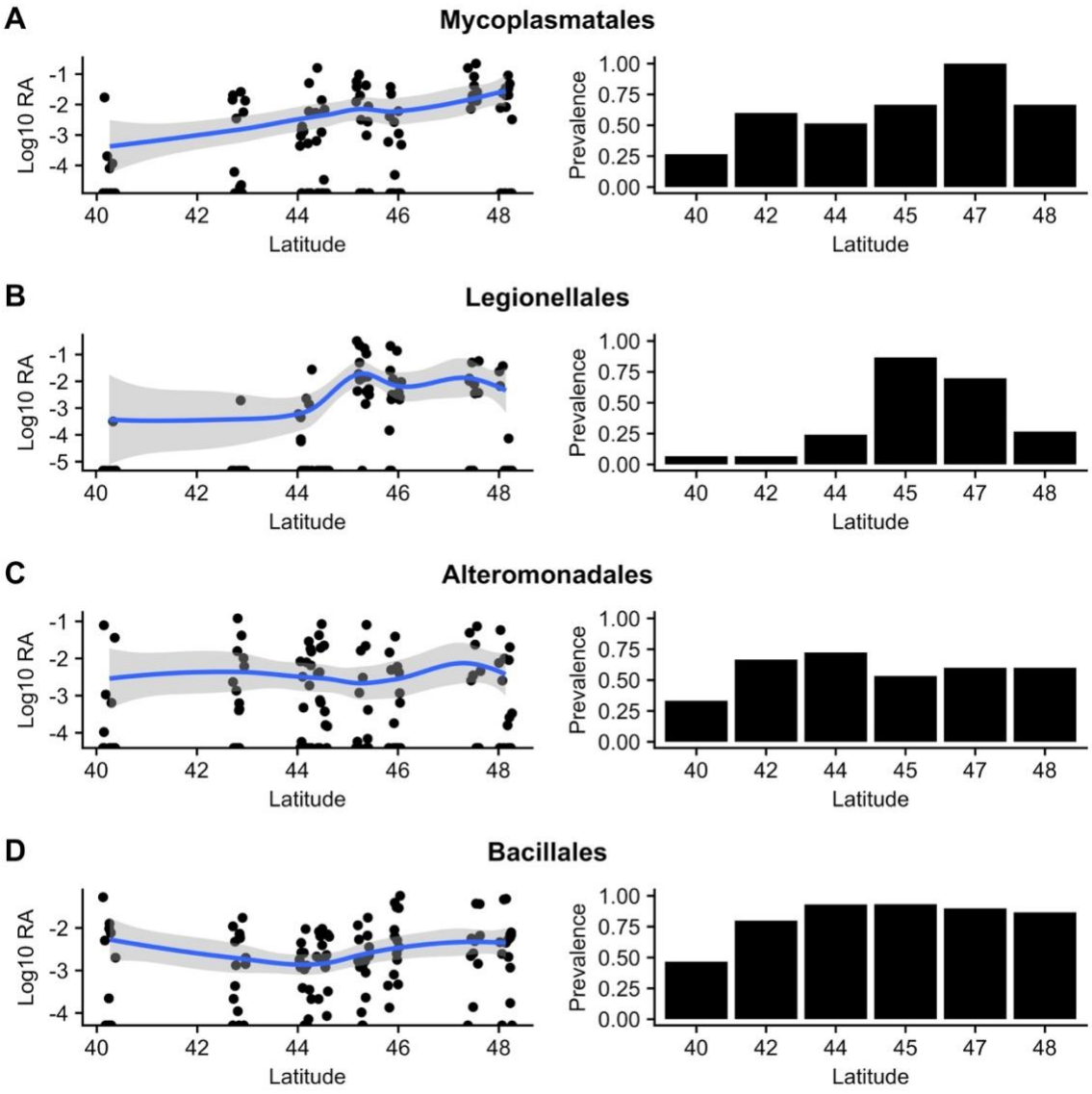


Figure 8: Log10 relative abundances (left) and prevalence (right) of the four most abundant core orders (prevalence > 50%) reveal trends in abundance and presence/absence across eight degrees of latitude: (A) Mycoplasmatales, (B) Legionellales, (C) Alteromonadales and (D) Bacillales.

Chapter 3: Snapshot of a coastal aquarium's microbiome reveals considerable bacterial diversity within and between tanks and water treatments

Abstract

Bacteria are abundant in aquaria that operate on both flow-through and recirculating systems, and when properly managed, can provide beneficial functions to display organisms. However, when mismanaged, blooms of opportunistic pathogens can be harmful and costly. Despite the importance of bacteria to the functioning of seawater aquaria, little is known about the bacterial composition of these marine built environment microbiomes. This study aims to describe the composition and structure of seawater and biofilm bacterial communities inhabiting the water treatment facilities and visitor center tanks at the Hatfield Marine Science Center (HMSC) in Newport, Oregon, United States. It follows raw seawater through the treatment process used to supply the visitor center aquarium: from the Yaquina Bay estuary, after settling out particles in a reservoir, and after rapid sand filtration. We identify substantial changes in community structure due to settling, but surprisingly find that rapid sand filtration minimally affects the composition of bacterial communities. We also examine the effects of recirculation and heating on the seawater and biofilm bacteria composition of a few tanks in the aquarium. We find that recirculation and heating both select for a small subset of bacteria, some of which were not detected in the Yaquina Bay estuary. Finally, we describe the dermal tissue microbiomes of two sea stars found at the visitor center: *Dermasterias imbricata* and *Pisaster ochraceus.* The findings of this study provide a high-level microbiome map of a coastal aquarium that predominantly operates on a flow-through system.

Introduction

Bacteria are abundant in marine environments. These marine bacteria perform important functions in global biogeochemical cycling (Kolber, 2001), food webs (Azam et al., 1983), and animal health (McFall-Ngai et al., 2013; Reshef et al., 2006). Bacteria play similar roles in saltwater aquaria and can be selected for desirable properties. For example, aquarists have long harnessed the properties of *Nitrosomonas* and *Nitrobacter* to metabolize toxic nitrogenous waste produced by fish (Burrell et al., 2001). Furthermore, some water treatment strategies select for non-pathogenic bacteria, improving the health of animals (Cabello, 2006). Despite the known importance of these microbial communities in aquarium management, little is known about their composition until recently, when the Shedd Aquarium Microbiome project began an effort to investigate the composition and function of microbial communities in an artificial seawater aquarium (Van Bonn et al., 2015; Kearns et al., 2017). This effort is an important start but has limited implications because many coastal aquaria do not use artificial seawater; for example, the Monterey Bay Aquarium is equipped with a flow-through system that pumps minimally treated seawater through display tanks at a rate of about 7,500 liters per minute (Christiansen and Davis, 2001).

Here we provide a first glimpse into the composition of seawater, tank biofilm, and animal-associated bacterial communities in a coastal aquarium that primarily relies on a flow-through system. We explore the effects of water treatment and recirculation on the composition of microbial communities. We also examine differences in microbial composition between planktonic and biofilm communities. To conduct a census of the bacterial communities, we used universal 16S PCR primers combined with high-throughput sequencing. We aimed to conduct a broad, snapshot survey of these systems;

while this study offers a glimpse into the bacterial composition inhabiting these systems, we emphasize that more research is needed to understand the dynamics and functions of these bacterial communities.

Materials and Methods

Facilities

We conducted our study at the Hatfield Marine Science Center (HMSC) aquarium in Newport, Oregon. The HMSC seawater system draws seawater from the surface of Yaquina Bay estuary at a rate of 7,500 liters per minute. To maintain high salinity, water is pumped only during the 90 minutes preceding and following high tides. The seawater is pumped into a 3-million-liter reservoir and rests to settle out organic and inorganic matter. From here, it passes through a 50-micron sand filtration unit and is delivered to the rest of the system. We monitored temperatures of the Yaquina Bay estuary and reservoir as well as salinity of Yaquina Bay for the duration of our collection.

Sample collection, DNA isolation, and 16S library preparation

In our survey, we collected water and biofilm samples from aquarium tanks and treatment facilities at the Hatfield Marine Science Center (HMSC) aquarium over three consecutive days in July 2017. Tank descriptions can be found in Table 1. We collected biofilms using sterile swabs and stored them in sterile phosphate buffered saline solution. For water samples, we filtered one liter of seawater through gamma irradiated 0.2-micron filters (Pall, USA) using a peristaltic pump. We ran one liter of deionized water through the peristaltic tubing between water samples. Samples were kept on ice during the

collection and stored at -80C at the end of each day. We sequenced a total of six blanks: three swabs and three filters.

We sent swab tips and filters to the Center for Genome Research and Biocomputing at Oregon State University for DNA isolation and PCR. They conducted automated DNA isolation on the KingFisher Platform using Mag-Bind® Stool DNA 96 kits (Omega BioTek, USA). They prepared 16S amplicon libraries according to the two-step PCR protocol described by Illumina (16S Metagenomic Sequencing Library Preparation, 2013). They used primers targeting the V3-V4 region (forward:5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3'; reverse:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3') (Klindworth et al., 2013).

Libraries were sequenced using a MiSeq v2 Nano 2x250 bp kit (Illumina, USA).

Sequence preprocessing

We used the DADA2 pipeline to infer amplicon sequence variants (ASVs) and assign taxonomy (Callahan et al., 2016). Amplicon sequencing variants are exact 16S rRNA gene sequence variants that can be used in analyses similar to operational taxonomic units but benefit from finer resolution and better reproducibility of analyses (Callahan et al., 2017). Starting with raw sequences, we trimmed 10 nucleotides on the left, and truncated forward reads to a length of 245 bases and reverse reads to a length of 240 bases. We allowed a maximum estimated error (maxEE) per read of two in quality filtering. We used one million forward and reverse reads to learn Illumina sequencing error patterns, which were applied to correct errors in the ASV inference step of the

DADA2 pipeline. We removed chimeras using the command removeBimeraDenovo in DADA2. We utilized the Silva Project's database (version 128) as a reference for taxonomic assignment (Quast et al., 2012). To build a phylogenetic tree of the ASVs, we used the R package DECIPHER (Wright, 2017) . We used the R package decontam (Davis et al., 2017) to identify and filter probable contaminating sequences; if an ASV was more prevalent in negative controls than in samples, we considered it to be a contaminant and removed it from our dataset. We removed samples with fewer than 100 reads from the data prior to analysis.

Alpha diversity analyses

We conducted alpha diversity analyses using the R package vegan (Oksanen et al., 2018). To characterize within-sample (alpha) diversity, we calculated Hill diversities (H_a) using the equation:

$$H_a = \left(\sum_{i=1}^{S} p_i^a \right)^{1/(1-a)}$$

Where *p* is the proportional abundance of the *i*th species, *S* is the number of species present, and *a* is a scaling factor. Hill diversities have favorable properties for characterizing alpha diversity. Notably, when $a = 0$, $H_a = S$ (i.e. species richness). As *a* increases, abundant species contribute more to the value. H_a can also be easily related to popular alpha diversity metrics; when $a = 1$, the limit of H_a is equivalent to the exponential Shannon index. When $a = 2$, H_a is equivalent to the inverse Simpson index. Finally, when a = infinity, H_a is equal to the reciprocal of the abundance of the most dominant ASV. We calculated Hill diversities on ASV tables randomly subsampled to a

depth of 2000 reads. This led to underestimates of species richness (Hill diversities when a=0), but eliminated biases due to library depth variance.

Beta diversity analyses

To analyze compositional differences between treatments, we first calculated pairwise Bray-Curtis distances between samples. We then performed a permutational MANOVA using the R function vegan::adonis to evaluate the significance of treatments. We then used indicator taxon analysis using the R function indicspecies::multipatt on the top 100 most abundant ASVs to identify ASVs associated with our variables of interest, with an FDR cutoff of 0.1. For biofilm-seawater comparisons, where there were only three samples per treatment, we tested the top 50 ASVs and relaxed the FDR cutoff to 0.2. Due to small sample sizes, we present the results as exploratory rather than hypothesis-testing. To further explore compositional differences, we filtered our data to only include ASVs as indicators of treatments or treatment groups and plotted the relative abundances of these ASVs to visualize the differences in abundances of these ASVs among samples.

Results

Pre-processing

After quality filtering and chimera filtering, our ASV table had a total of 823,184 reads with a median sample depth of 8,278 reads. DADA2 identified 25,010 unique ASVs, and decontam identified 249 of these ASVs that had higher frequencies in the six kit blanks than in the other samples. After removal of the contaminating ASVs, our ASV table had a total of 702,701 reads with a median sample depth of 5,994 reads. Samples had a mean richness of 306 observed ASVs, with a standard deviation of 206.

Settling in a reservoir alters bacterial community diversity and composition, but subsequent sand filtration does not

We compared the bacterial community compositions of seawater samples collected from four locations over the course of three days: a) The seawater system's intake point ("Dock"), b) seawater after settling in the reservoir but pre-sand-filtration ("Pre_sandfilter"), c) seawater after rapid sand filtration ("Post_sandfilter"), and d) seawater collected from the pipe delivering water to most of the Hatfield Marine Science Center visitor center aquarium ("Aquarium_mainline"). We found no significant differences in richness values among these treatments (Figure 9A, Kruskal-Wallis $p = 0.19$). We found Hill diversities of order 1 (Figure 9B) and order 2 (Figure 9C) to be suggestively different (Kruskal-Wallis $p=0.09$ and $p=0.09$), with water samples from the estuary appearing to have higher diversity than seawater collected downstream. Finally, we found Hill diversities of order infinity to be significantly different (Figure 9D) (Kruskal-Wallis, $p=0.02$), with samples from the estuary having higher diversity than those found in the aquarium system.

We used Principal Coordinates Analysis to visualize beta diversities measured using the Bray-Curtis metric (Figure 10). Samples of raw seawater collected from the Yaquina Bay estuary exhibited significantly different composition compared to samples collected from within the seawater system (PERMANOVA, $p = 0.01$). Indicator taxon analyses identified 24 ASVs that significantly predict a single or combination of treatment steps (FDR q-value <0.1). 22 of these 24 ASVs were associated with the raw seawater, further implicating settling as the most important step in shaping community composition in this system (Figure 11A). The most abundant of these ASVs were taxonomically assigned to the genera *Planktomarina* and *Cellulophaga*. One ASV in the

genus *Sulfitobacter* was identified as an indicator of water within the HMSC seawater system (Figure 11B), and one ASV unassigned at the genus level but assigned to family *Flavobacteriaceae* was identified as an indicator of both raw and aquarium mainline seawater (Figure 11C).

Biofilms exhibit higher diversities and distinct composition compared to their surrounding seawater

Because biofilms were collected from different surface types in different tanks (Table 2), we conducted analyses separately for each tank. Regardless of biofilm type (sea star dermal tissue or tank surface), we observed greater diversity in biofilms compared to seawater in the same tank. This was true for every tank and Hill diversities of all orders (Figure 12).

Beta diversity analyses revealed that biofilms from different tanks exhibited less dispersion than water samples in different tanks (betadisper, $p = 0.02$) (Figure 13). Interestingly, the median distance to the centroid of biofilm beta diversities was higher than the corresponding value for seawater beta diversities (Appendix figure 2). In other words, this analysis determined that biofilms were more loosely clustered than the water samples, despite substantial differences between recirculating, heated and flowthrough seawater communities. It should be noted that this tight clustering of seawater samples is likely driven by high similarity among flowthrough tanks. The seawater from these tanks cannot be treated as independent, because they come from the same source, so this result should be interpreted with caution. Indicator taxon analysis revealed ASVs associated with biofilms in each tank except the coral holding tank; we plotted the abundances of these ASVs colored by genus (Figure 14A-C). Each biofilm sample differed from its

surrounding water in a different way. In the biofilms collected from the surface of the rockfish tank, we identified ASVs in eight genera that differed from the flow-through seawater (Figure 14A). In particular, ASVs in *Rubritalea*, *Litorlinea*, and *Nitrospira* were abundant in tank biofilms, but were absent in seawater samples.

Biofilms collected from the dermal tissue of *Dermasterias imbricata* sea stars also differed from flow-through seawater; *Bdellovibrio*, *Sphingomonas*, *Ilumatobacter* and *Rubritalea* were all found to be indicators of *D. imbricata* dermal tissue (Figure 14B). Of the eight ASVs identified as indicators of dermal tissue, four were alphaproteobacteria that not taxonomically classified past the level of class. Because these ASVs made up a substantial abundance of the bacteria associated with *D. imbricata* dermal tissue (Figure 14B), we queried the NCBI nucleotide repository using the ASV sequences of these unclassified bacteria. The top three hits in the repository, matching 95% of the four query sequences, were bacteria associated with the sea star *Paterella sp.* in New Zealand (NCBI accession numbers: FJ393607.1, FJ393578.1, FJ393577.1).

Finally, samples collected from the dermal tissue of *Pisaster ochraceus* differed from the recirculating seawater in their tank; we identified 12 genera (Figure 14C). ASVs assigned to *Stenotrophomonas*, *Sphingomonas*, and *Salinispira*, as well as an ASV in order *Spirochaetales* (but unclassified at the genus and family level), were the most abundant indicators of *P. ochraceus* dermal tissue compared to recirculating seawater. We queried the NCBI nucleotide repository using the sequence from the poorly classified *Spirochaetales* ASV. The top hit of the query was an 88% match to a coral-associated bacterium (NCBI accession number: GQ413925.1).

We next analyzed ASVs characteristic of seawater. In the rockfish and touch tank flow-through seawater, the most abundant ASVs associated with seawater were assigned to genera *Amylibacter* (family *Rhodobacteraceae*) and *Tenacibaculum* (family *Flavobacteriaceae*) (Figure 15A-B). ASVs associated with cold recirculating seawater were distinct from those associated with flow-through seawater (Figure 15C). The most abundant of these ASVs were in genera *Pseudofulvibacter, Colwellia, Aestuariibacter,* and one unclassified ASV was in family *Alteromonadaceae*. The top hit of this ASV sequence in the NCBI nucleotide repository was a 100% match to *Glaciecola sp.* isolated from an estuary (NCBI accession number: HQ875494.1).

Biofilms collected from different surfaces exhibit variable richness and community composition

We found significant differences among richness values (Kruskal-Wallis p=0.01) in biofilm samples collected from the surface of the coral holding tank, *P. ochraceus* dermal tissue (Recirculating), the surface of the rockfish tank, and *D. imbricata* (Touch) dermal tissue (Figure 16A). Differences in Hill diversities of orders 1 and 2 exhibited a similar trend (Figure 16B-C) (Kruskal-Wallis p-values = 0.06 and 0.06). We did not find any relationship between biofilm type and diversity of order infinity (Kruskal-Wallis, p=0.63) (Figure 16D). Indicator taxon analyses revealed ASVs associated with each biofilm type. The most abundant ASVs associated with *D. imbricata* tissue were in genus *Bdellovibiro* and two ASVs in class alphaproteobacteria (Figure 17A). An uncultured bacterium found in a marine sediment sample was the top hit for these ASV sequences, and exhibited 91% similarity to these sequences (NCBI accession number: KX173227.1). The most abundant ASVs associated with *P. ochraceus* were assigned to genera *Sphingomonas* and

Stenotrophomonas (Figure 17B). The most abundant ASVs associated with the rockfish tank biofilm were assigned to genera *Rubritalea, Nitrosospira,* and *Lutibacter* (Figure 18A). The most abundant ASVs associated with the coral holding tank were assigned to genus *Portibacter* and order *Chromatiales* (Figure 18B). The sequence from the ASVs in order *Chromatiales* exhibited 98% similarity to the sequence of an uncultured gammaproteobacterium collected from a hydrothermal vent (NCBI accesssion number: AB611486.1).

Recirculation and heating lead to fewer dominant taxa and distinct seawater bacterial communities

We next compared the Hill diversities between seawater samples collected from the coral holding (heated recirculating), coldwater recirculating, rockfish flowthrough, and touch flowthrough tanks. We found no significant differences in richness (Kruskal-Wallis, $p = 0.49$) (Figure 19A), but for diversities of order 1, 2, and infinity (Figure 19B-D), flowthrough tanks exhibited higher diversities (Kruskal-Wallis, $p=0.03,0.01$, and 0.01 respectively). ASVs associated with diverse genera were associated with flowthrough seawater (Figure 20A). The most abundant of these were in genera *Amylibacter* and *Tenacibaculum*, which were identified in the previous section as indicators of flowthrough seawater compared to sea star dermal tissue and tank surface biofilms. In the coral holding tank water, for which we found no associated taxa compared to biofilms collected from the tank in the previous section, we found that the most abundant ASVs were unassigned at the genus level (Figure 20B). The most abundant of these were assigned to orders *Alteromonadales, SAR11, Rhodobacterales, Chromatiales,* and an alphaproteobacteria unassigned at the order level. The closest match (100%) to this ASV

sequence in NCBI's nucleotide repository came from a bacterium isolated from marine sediment (NCBI accession number: KX177464.1). The ASVs associated with coldwater recirculating seawater (Figure 20C) followed a similar pattern to the flowthrough water ASVs: the most abundant ASVs associated with recirculating tank seawater were assigned to genera *Pseudofulvibacter, Colwellia,* and *Aestuariibacter.*

Discussion

Settling alters seawater microbiome diversity and composition

We found that settling in a reservoir leads to a shift in bacterial community diversity and composition. We found no differences in richness, but seawater in Yaquina Bay displayed greater values of diversity of higher orders compared to seawater in the aquarium's system. Greater values of high-order diversities suggest that the seawater has more dominant taxa than water in the reservoir. These results highlight the importance of using diversity profiles to explore alpha diversity rather than attempting to summarize a taxa distribution with a single metric (Kang et al., 2016).

Indicator taxon analyses identified diverse ASVs found in the seawater in Yaquina Bay but not in the pre-sandfilter seawater. The ASV with the sharpest drop in relative abundance was assigned to genus *Cellulophaga*; members of this genus are commonly associated with algae or mud (Johansen et al., 1999). We hypothesize that *Cellulophaga* bacteria were attached to particles or algae and settled out. The genus *Planktomarina* also exhibited a drop in relative abundance during settling. While its ecology is still poorly understood, *Planktomarina* is a member of the *Roseobacter* clade affiliated (RCA) cluster, and is an abundant bacterioplankton in temperate ocean water (Giebel et al., 2011).

We found the genus *Sulfitobacter*, a sulfite-oxidizing member of the RCA cluster, to be an indicator of the seawater in the aquarium's system. Like *Planktomarina*, *Sulfitobacter* is ubiquitous in seawater, but it has also been isolated from seagrass and sea stars (Ivanova, 2004), and it has been demonstrated to form biofilms (Bruhn et al., 2007). We hypothesize that *Sulfitobacter* form biofilms in the reservoir and their presence in the rest of the system result from the shedding of these biofilms. Finally, an ASV in family *Flavobacteraceae* was identified as an indicator of Yaquina Bay seawater and aquarium mainline water, but was not detected in pre- or post-sandfilter water. However, its relative abundances ranged from 0.1% to 0.5%-- it is possible that it was present in pre- and post-sandfilter but below our limit of detection.

Biofilms from different tanks and surface types exhibit distinct compositions

We found that biofilms had greater alpha diversity than the seawater surrounding them, regardless of whether the biofilm was collected from a tank surface or sea star. We hypothesize that this is due to species sorting during the succession of bacterial communities over the course of biofilm formation (Gulmann et al., 2015). Two ASVs abundant in *D. imbricata* dermal tissues were not similar to sequences in the NCBI nucleotide repository (maximum similarity of 91%), suggesting that these bacteria may belong to a previously undescribed genus. We speculate that these bacteria are symbionts of *D. imbricata*, given their high abundance on their dermal tissue and lack of presence elsewhere. Abundant ASVs associated with *P. ochraceus* dermal tissues were assigned to genera *Sphingomonas* and *Stenotrophomonas.* Both of these genera are generalists and can be opportunistic pathogens of humans (Ryan and Adley, 2010; Brooke, 2012). The

presence of these potential opportunistic pathogens may be of interest to those researching sea star wasting disease.

ASVs associated with rockfish tank biofilms were assigned to genera *Rubritalea, Lutibacter* and *Nitrosospira. Rubritalea,* in the phylum *Verrucomicrobia* are commonly found associated with marine animals (Freitas et al., 2012) and have previously been found in the guts of health fish (Li et al., 2016). *Lutibacter* are typically found in marine sediments (Choi et al., 2013). *Nitrosospira* are ammonia-oxidizing bacteria, a desirable property in aquaria (Burrell et al., 2001). Finally, the most abundant ASVs associated with the coral holding tank were assigned to genus *Portibacter* (family *Saprospiraceae*) and order *Chromatiales. Saprospiraceae* are thought to be involved in the breakdown of complex carbon sources and are abundant in seawater and activated sludge (McIlroy and Nielsen, 2014). The closest hit in the NCBI repository to the *Chromatiales* ASV was to a bacterium collected from a hydrothermal vent. It is not clear whether the presence of coral or the heating of the water selected for these taxa.

Recirculation leads to dominance of a few bacterial taxa that are typically present in low abundances in seawater

Comparing Hill diversities, we found that richness did not differ substantially between system type (flowthrough, coldwater recirculating, heated recirculating). However, Hill diversities of flowthrough seawater were about four times those of both coldwater and heated recirculating tanks. This means that flowthrough water has more ASVs with meaningful abundances than recirculating tanks. Indeed, the heated recirculating seawater was dominated by a few uncharacterized ASVs. Interestingly, the most dominant of these were poorly categorized, but belonged to orders *Alteromonadales, SAR11,*

Rhodobacterales, and *Chromatiales,* which are not uncommon in seawater. We thought it noteworthy that *SAR11* was abundant in the coral holding tank because it is notoriously difficult to culture (Rappé et al., 2002), and its abundances were reduced during settling. We hypothesize that the warm water selected for *SAR11* and the other poorly classified ASVs. The most dominant ASVs in the coldwater recirculating tank, assigned to genera *Pseudofulvibacter, Colwellia,* and *Aestuariibacter,* were not found in meaningful abundances in flowthrough tank water. *Pseudofulvibacter* has been isolated from numerous marine sources, including tidal flats (Kyoung Kwon et al., 2016), so we believe it likely exists in low abundances in the source seawater, but an unknown factor related to coldwater recirculation selected for its presence. *Colwellia* is typically associated with polar and deep seawater, but has also been isolated from sea stars (Choi et al., 2010), and was found on the *P. ocraceus* in the recirculating tank. Therefore, we hypothesize that the presence of *Colwellia* in the water was due to the influence of the *P. ocraceus* dermal tissue microbiome.

Conclusion

Here we provide a first glimpse into the microbiome of a coastal aquarium. We find substantial differences in composition between tanks, and a significant change in bacterial composition between seawater in the system and Yaquina Bay. We emphasize that this study is preliminary and does not have sufficient independence between samples or sample sizes to deduce generalizable trends. However, given the diversity discovered in this system, we propose the coastal aquarium as a promising research avenue for microbial ecology.

Table 2: Sample collection details

Location/Tank	Water	Biofilm	Biofilm type	Details
Dock	X	0	NA	Collected from Yaquina Bay estuary
Pre_sandfilter	X	0	NA	Collected from reservoir line to sandfilter
Post_sandfilter	X	0	NA	Collected from outlet of sandfilter
Aquarium_mainline	X	0	NA	Collected from HSMC aquarium
Rockfish	X	X	Tank surface	
Recirculating	X	X	*P. ocraceus* dermal tissue	
Touch	X	X	*D. imbricata* dermal tissue	Collected from HMSC aquarium touch tank
Coral holding	X	X	Tank surface	

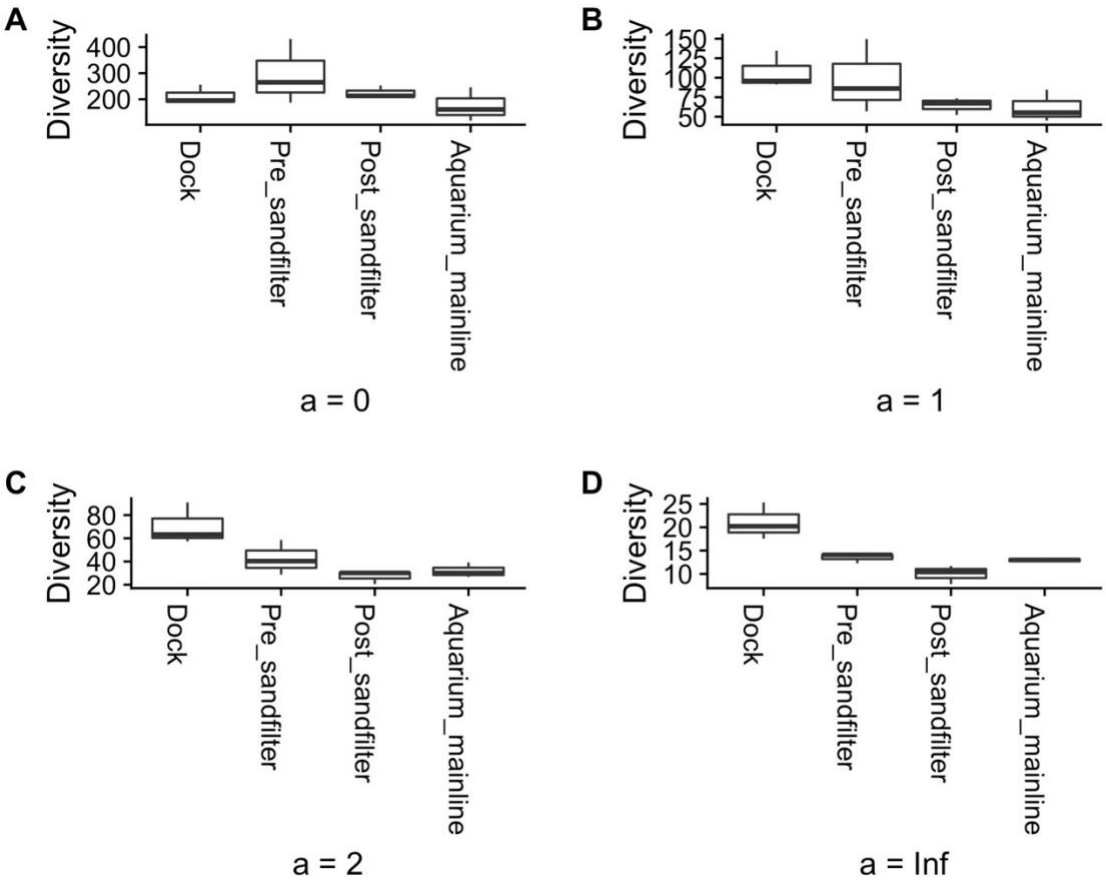


Figure 9: Hill diversities of A) order 0, B) order 1, C) order 2, and D) order infinity reveal differences in bacterial diversity between Yaquina Bay (Dock) and the seawater system at Hatfield Marine Science Center.

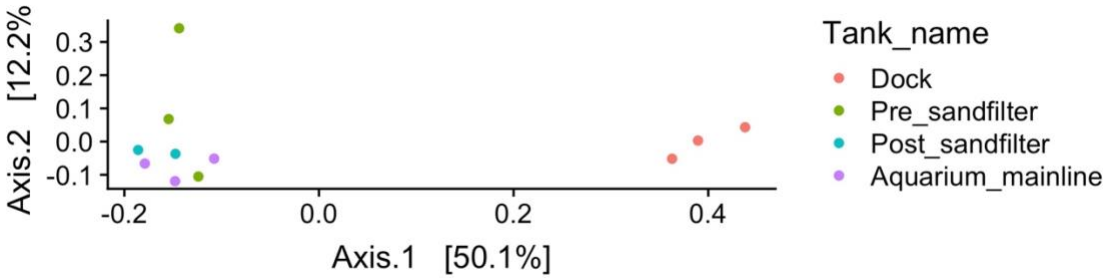


Figure 10: A PCoA ordination of Bray-Curtis dissimilarities reveals that seawater collected from Yaquina Bay (Dock) has a significantly different bacterial community composition compared to sample collected from the seawater system (PERMANOVA, p=0.01).

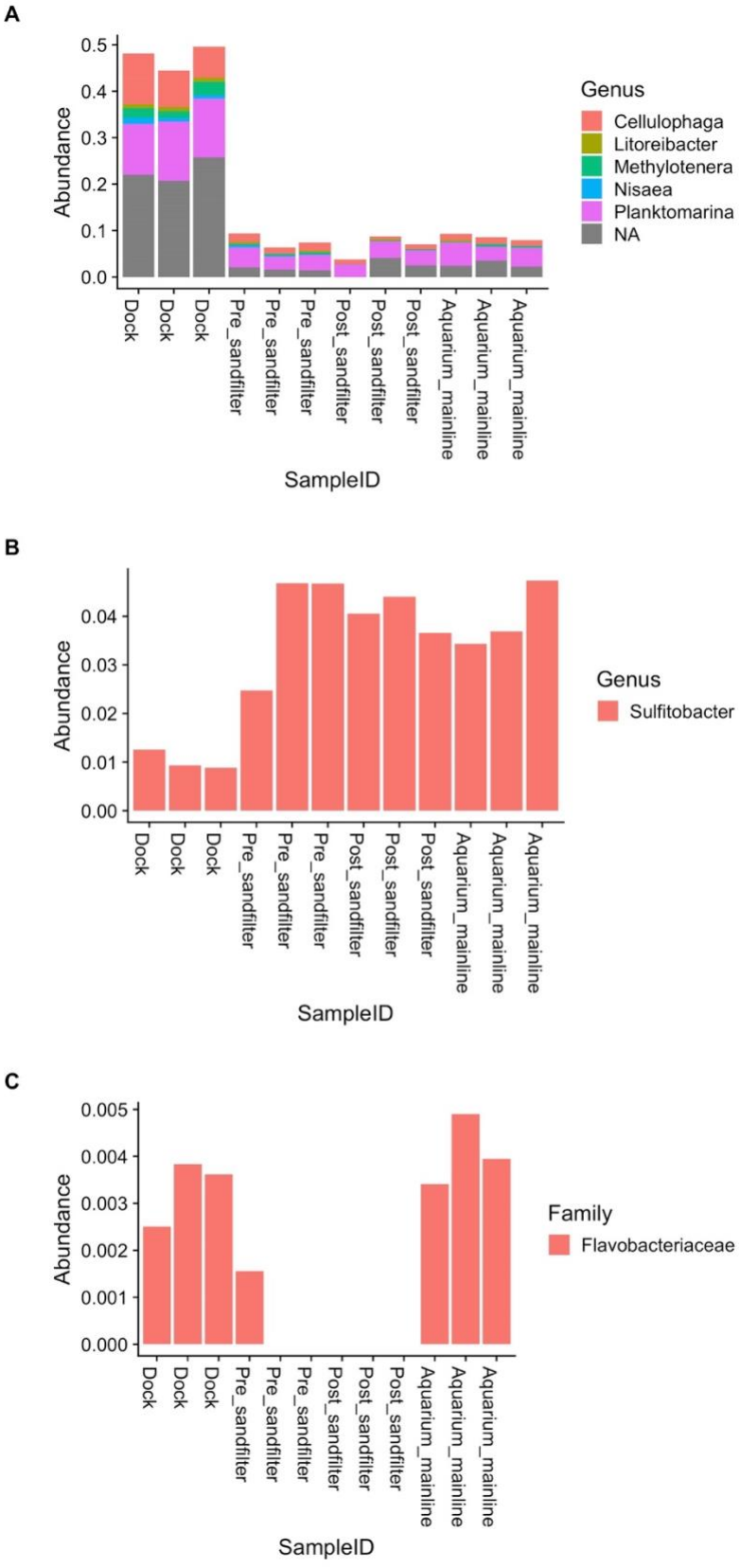

A
Abundance
0.5
0.4
0.3
0.2
0.1
0.0
Dock
Dock
Dock
Pre_sandfilter
Pre_sandfilter
Pre_sandfilter
Post_sandfilter
Post_sandfilter
Post_sandfilter
Aquarium_mainline
Aquarium_mainline
Aquarium_mainline
SampleID
Genus
Cellulophaga
Litoreibacter
Methylotenera
Nisaea
Planktomarina
NA
B
Abundance
0.04
0.03
0.02
0.01
0.00
Dock
Dock
Dock
Pre_sandfilter
Pre_sandfilter
Pre_sandfilter
Post_sandfilter
Post_sandfilter
Post_sandfilter
Aquarium_mainline
Aquarium_mainline
Aquarium_mainline
SampleID
Genus
Sulfitobacter
C
Abundance
0.005
0.004
0.003
0.002
0.001
0.000
Dock
Dock
Dock
Pre_sandfilter
Pre_sandfilter
Pre_sandfilter
Post_sandfilter
Post_sandfilter
Post_sandfilter
Aquarium_mainline
Aquarium_mainline
Aquarium_mainline
SampleID
Family
Flavobacteriaceae

Figure 11: Relative abundances of ASVs associated with A) Yaquina Bay (Dock), B) the HMSC seawater system, and C) Yaquina Bay and the aquarium mainline.

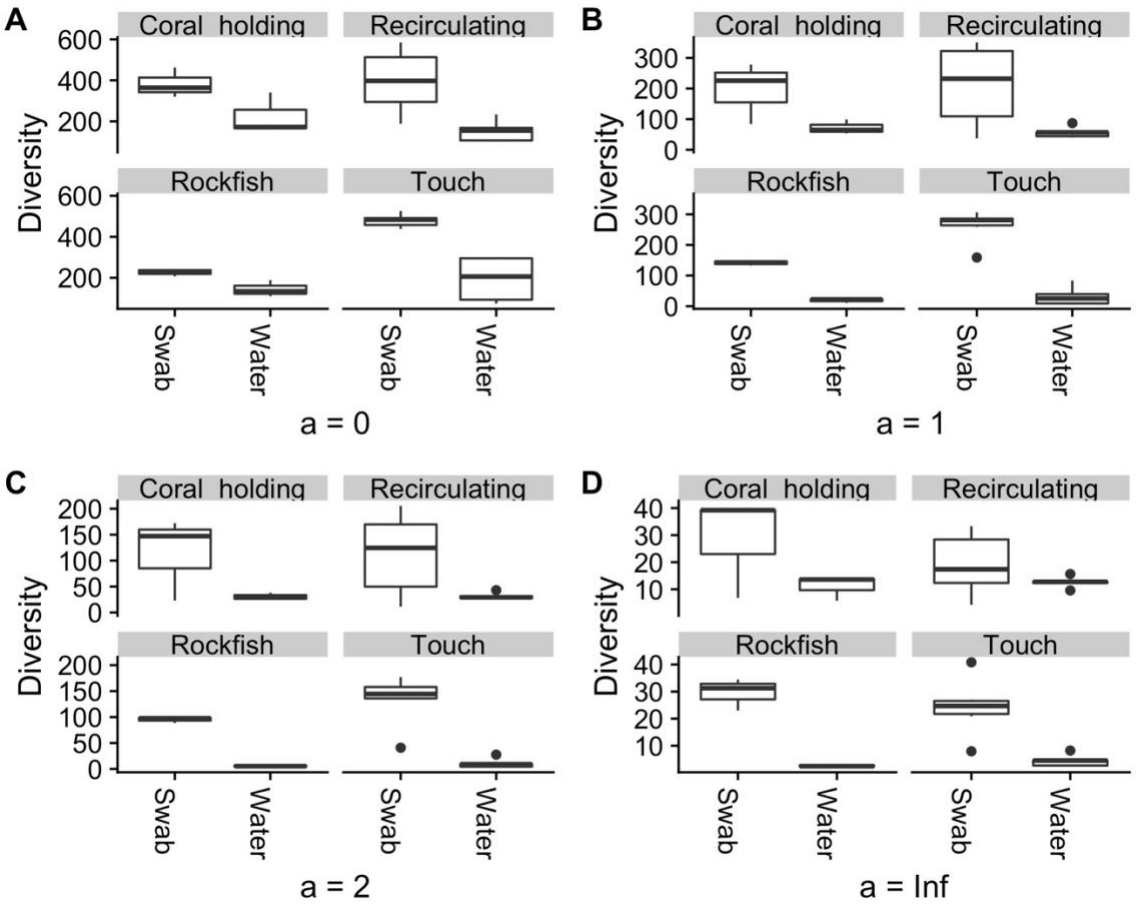


Figure 12: Hill diversity profiles of A) order 0, B) order 1, C) order 2, and D) order infinity reveal that biofilms, regardless of source, exhibit higher diversity than their surrounding seawater.

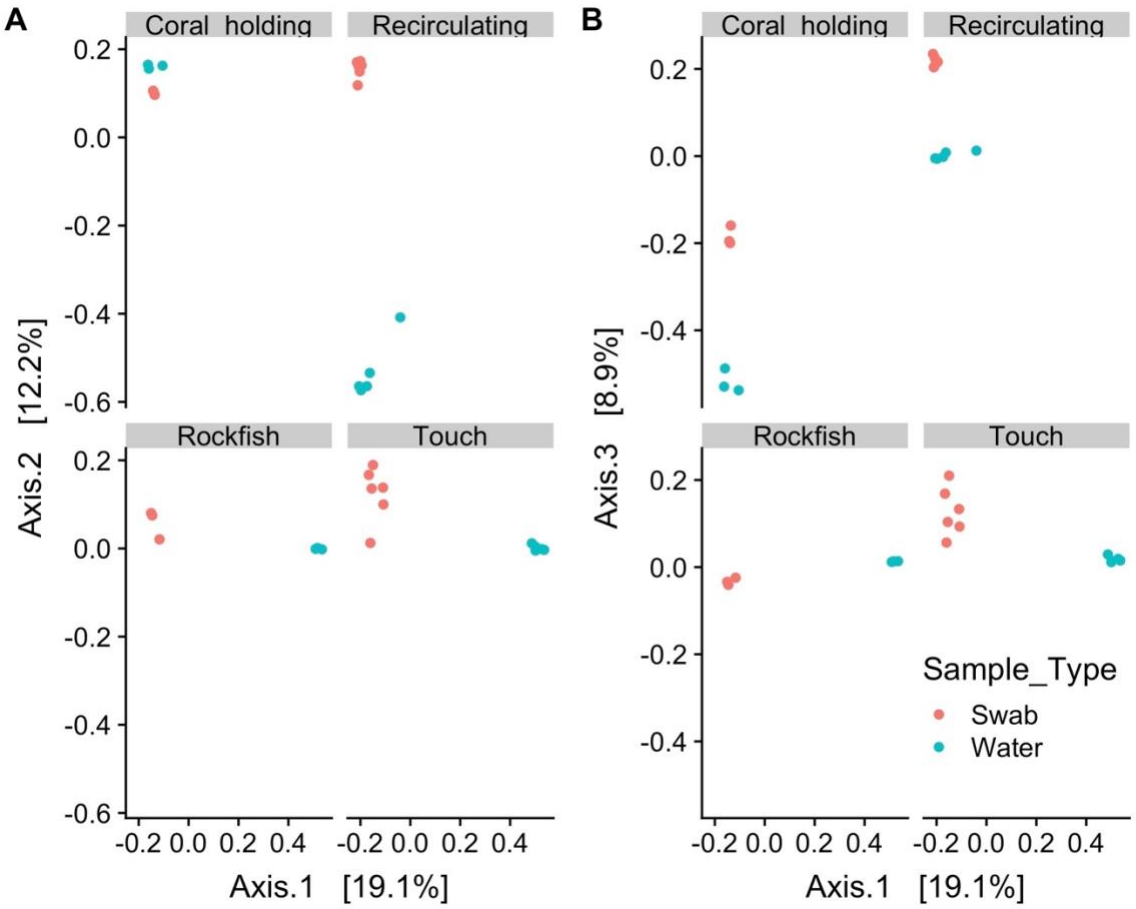


Figure 13: A PCoA ordination of Bray-Curtis dissimilarities demonstrates that differences between water system types (flowthrough, coldwater recirculating, and heated recirculating) are greater than differences between biofilms.

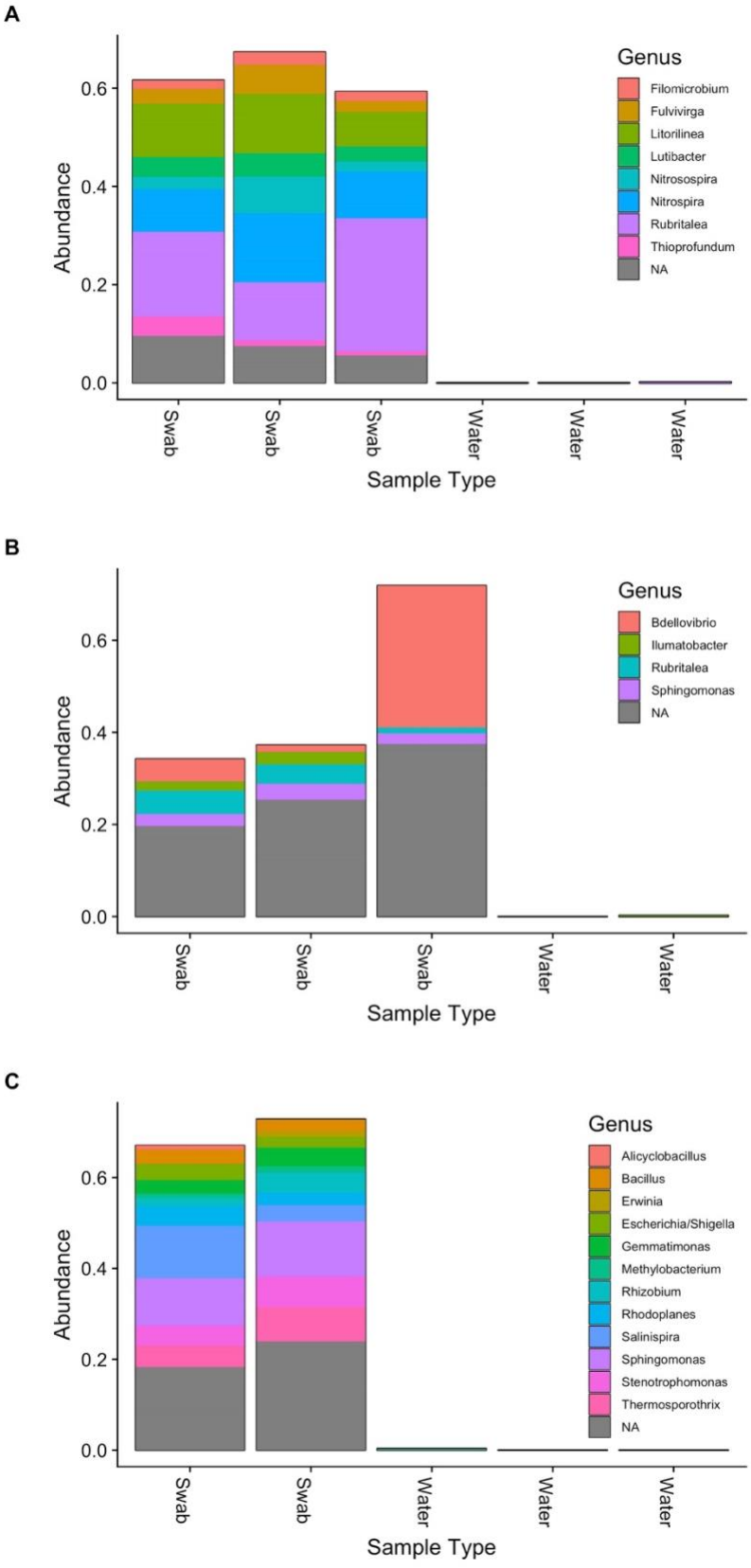
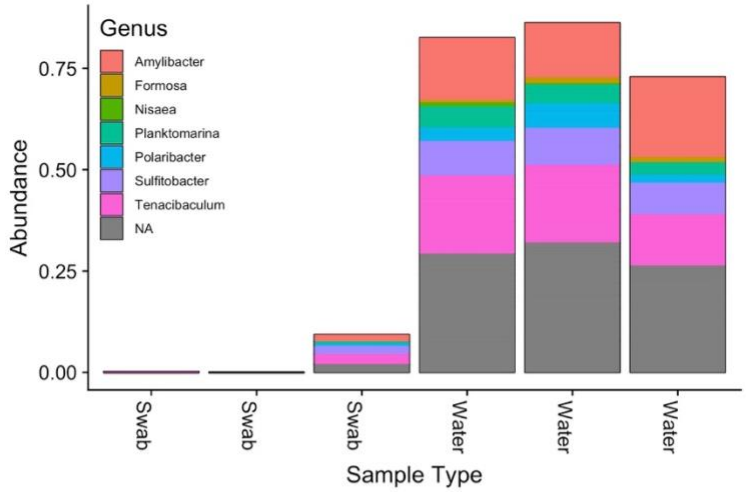
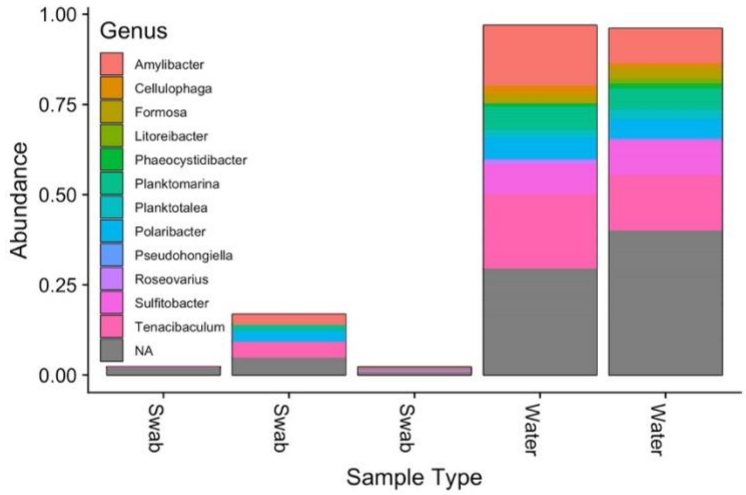

A
Abundance
0.0
0.2
0.4
0.6
Swab
Swab
Swab
Water
Water
Water
Sample Type
Genus
Filomicrobium
Fulvivirga
Litorilinea
Lutibacter
Nitrosospira
Nitrospira
Rubritalea
Thioprofundum
NA
B
Abundance
0.0
0.2
0.4
0.6
Swab
Swab
Swab
Water
Water
Sample Type
Genus
Bdellovibrio
Ilumatobacter
Rubritalea
Sphingomonas
NA
C
Abundance
0.0
0.2
0.4
0.6
Swab
Swab
Water
Water
Water
Sample Type
Genus
Alicyclobacillus
Bacillus
Erwinia
Escherichia/Shigella
Gemmatimonas
Methylobacterium
Rhizobium
Rhodoplanes
Salinispira
Sphingomonas
Stenotrophomonas
Thermosporothrix
NA

Figure 14: Relative abundances of ASVs associated with A) rockfish tank surface biofilms, B) *D. imbricata* dermal tissue biofilms, and C) *P. ochraceus* dermal tissue biofilms compared to their surrounding water.

A


Genus
Amylibacter
Formosa
Nisaea
Planktomarina
Polaribacter
Sulfitobacter
Tenacibaculum
NA
Abundance
0.00
0.25
0.50
0.75
Swab
Swab
Swab
Water
Water
Water
Sample Type

B


Genus
Amylibacter
Cellulophaga
Formosa
Litoreibacter
Phaeocystidibacter
Planktomarina
Planktotalea
Polaribacter
Pseudohongiella
Roseovarius
Sulfitobacter
Tenacibaculum
NA
Abundance
0.00
0.25
0.50
0.75
1.00
Swab
Swab
Swab
Water
Water
Sample Type

C

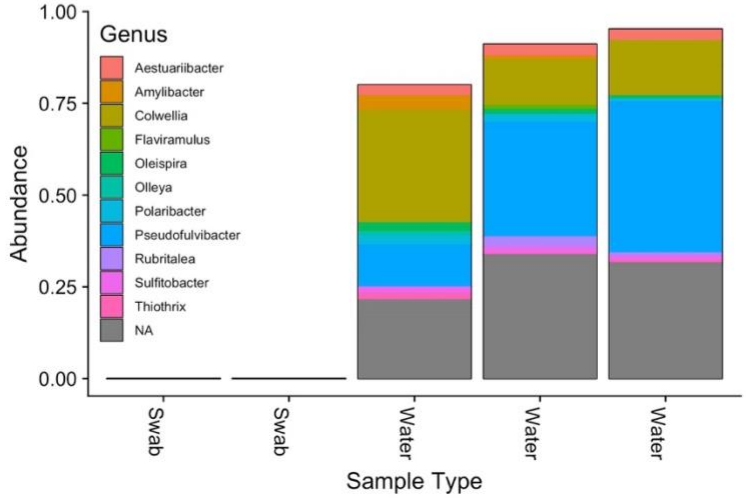

Genus
Aestuariibacter
Amylibacter
Colwellia
Flaviramulus
Oleispira
Olleya
Polaribacter
Pseudofulvibacter
Rubritalea
Sulfitobacter
Thiothrix
NA
Abundance
0.00
0.25
0.50
0.75
1.00
Swab
Swab
Water
Water
Water
Sample Type

Figure 15: Relative abundances of ASVs associated with A) rockfish tank flowthrough water, B) touch tank flowthrough water, and C) coldwater recirculating water compared to their corresponding biofilms.

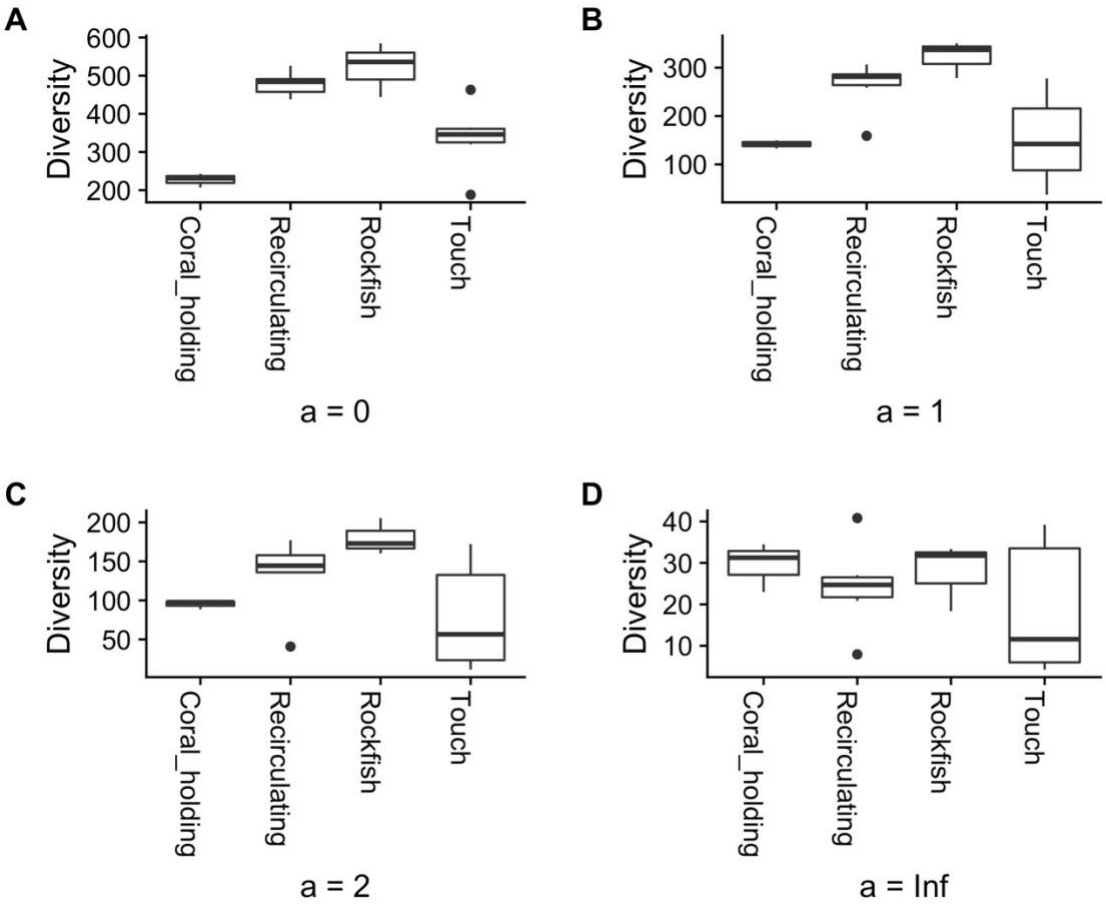


Figure 16: Hill diversity profiles of A) order 0, B) order 1, C) order 2, and D) order infinity reveal that biofilms of different types exhibit different richnesses, but have similar Hill diversities of order infinity.

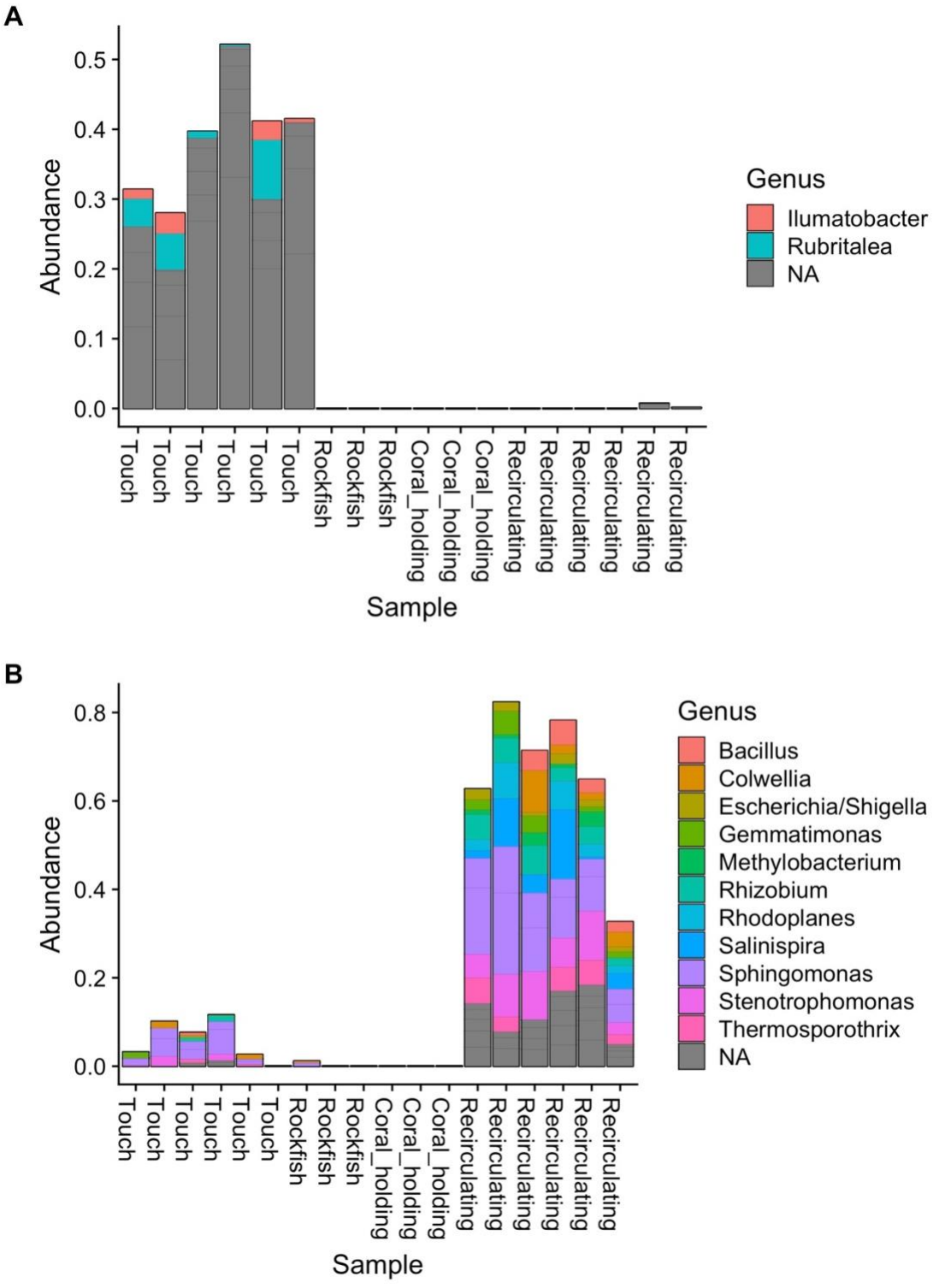


Figure 17: Relative abundances of ASVs associated with A) *D. imbricata* dermal tissue and B) *P. ochraceus* dermal tissue.

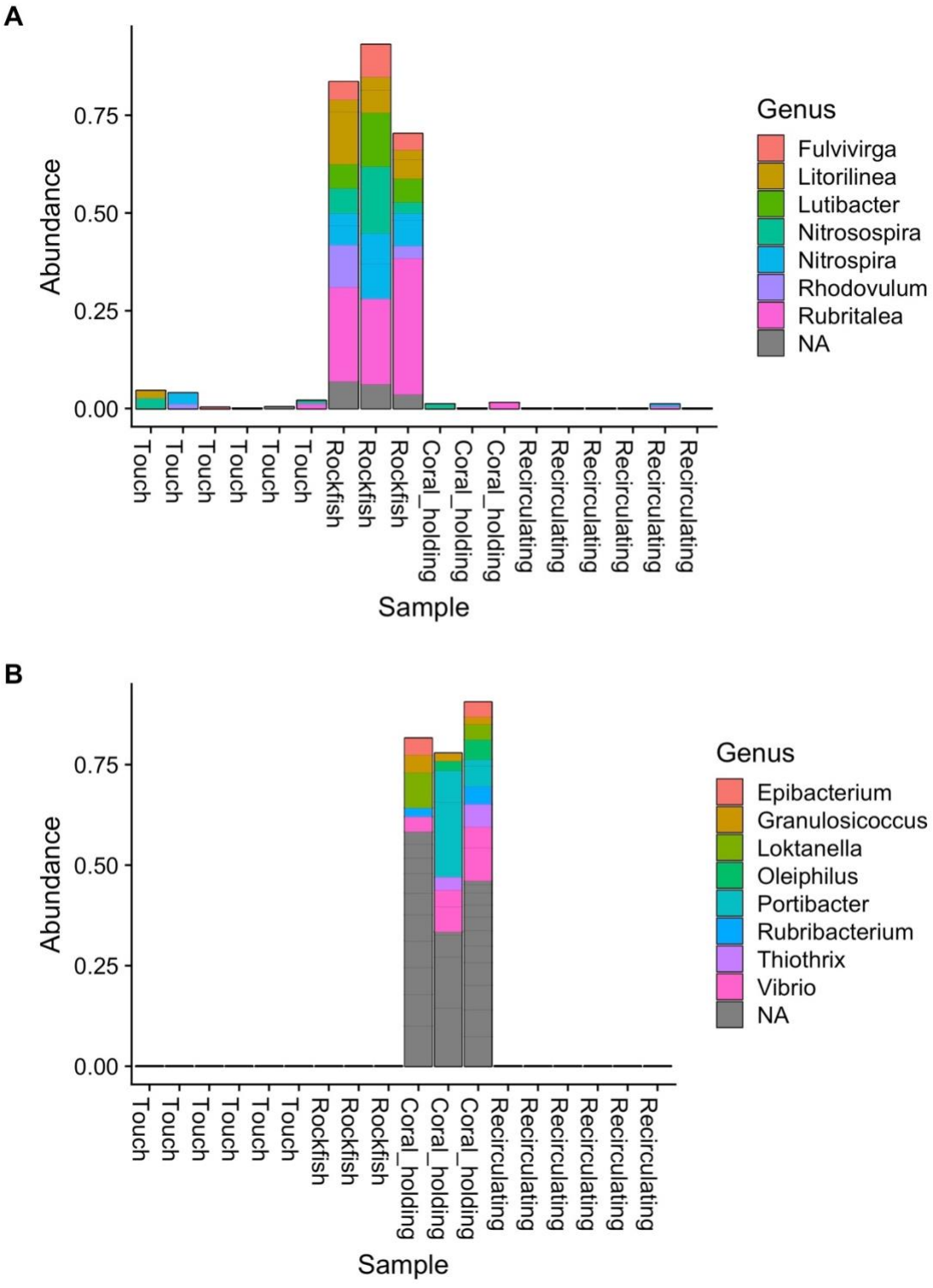


Figure 18: Relative abundances of ASVs associated with A) rockfish tank surface biofilms and B) coral holding tank surface biofilms.

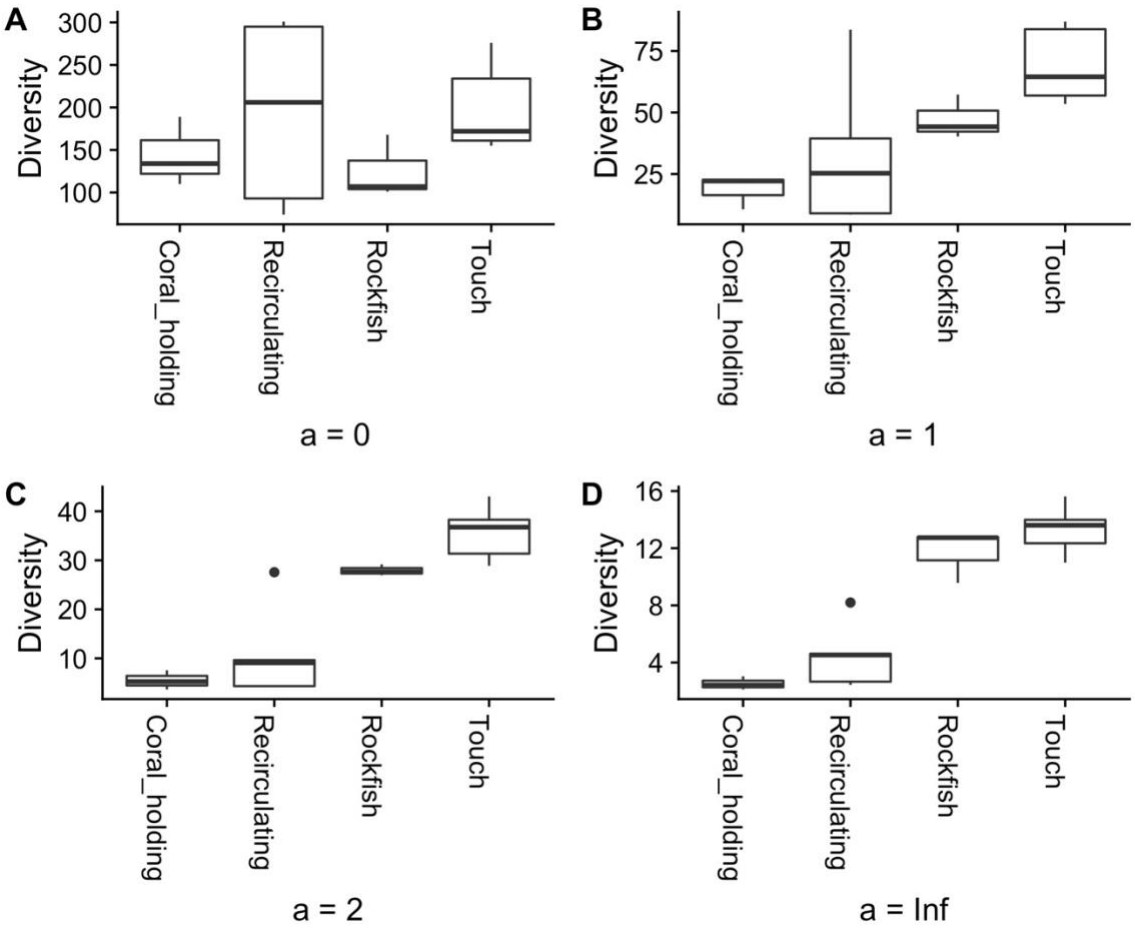


Figure 19: Hill diversity profiles of A) order 0, B) order 1, C) order 2, and D) order infinity reveal that seawater from different tanks exhibit similar (and variable) richness values, but differ at higher orders of diversity.

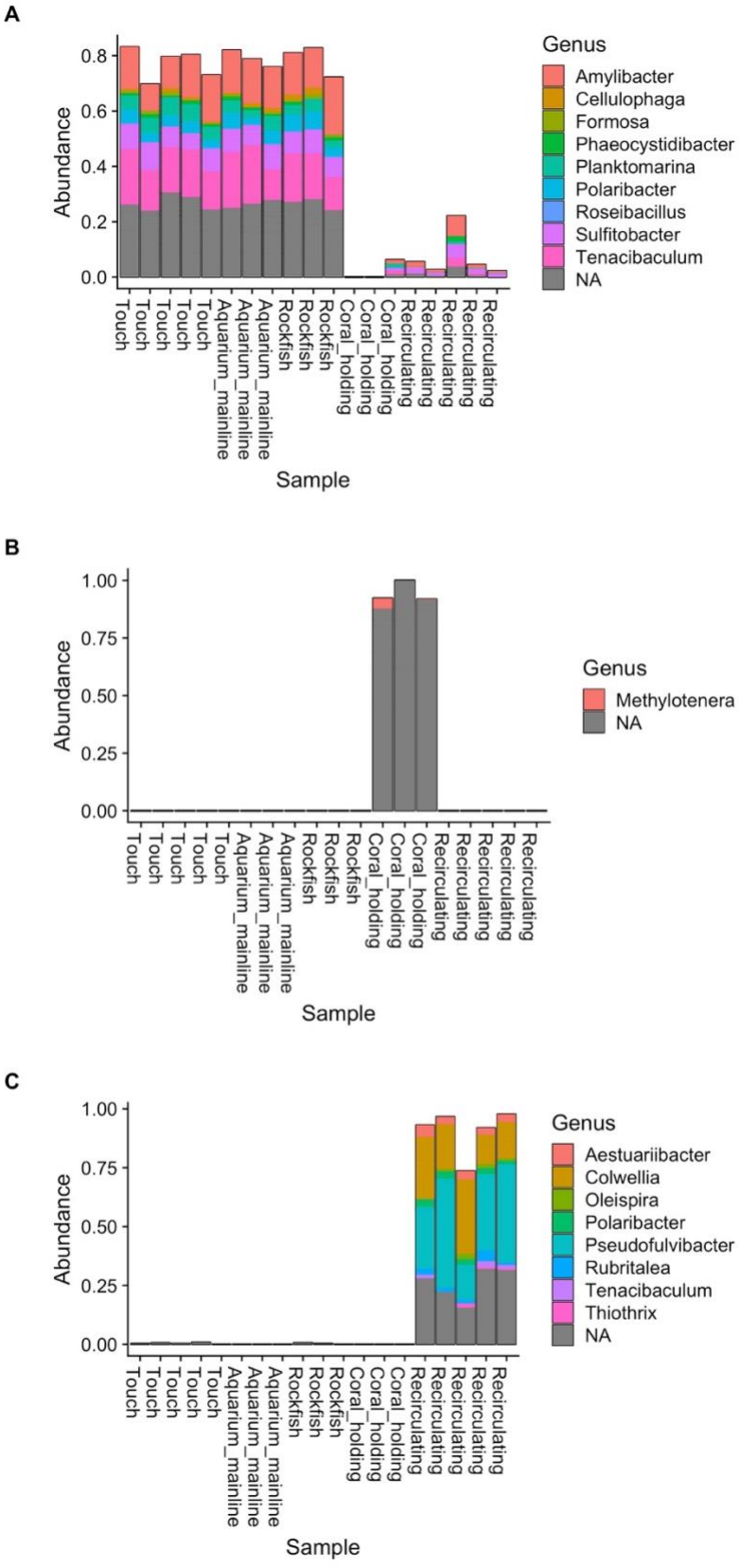

A
Abundance
0.0
0.2
0.4
0.6
0.8
Touch
Touch
Touch
Touch
Touch
Aquarium_mainline
Aquarium_mainline
Aquarium_mainline
Rockfish
Rockfish
Rockfish
Coral_holding
Coral_holding
Coral_holding
Recirculating
Recirculating
Recirculating
Recirculating
Recirculating
Sample
Genus
Amylibacter
Cellulophaga
Formosa
Phaeocystidibacter
Planktomarina
Polaribacter
Roseibacillus
Sulfitobacter
Tenacibaculum
NA
B
Abundance
0.00
0.25
0.50
0.75
1.00
Touch
Touch
Touch
Touch
Touch
Aquarium_mainline
Aquarium_mainline
Aquarium_mainline
Rockfish
Rockfish
Rockfish
Coral_holding
Coral_holding
Coral_holding
Recirculating
Recirculating
Recirculating
Recirculating
Recirculating
Sample
Genus
Methylotenera
NA
C
Abundance
0.00
0.25
0.50
0.75
1.00
Touch
Touch
Touch
Touch
Touch
Aquarium_mainline
Aquarium_mainline
Aquarium_mainline
Rockfish
Rockfish
Rockfish
Coral_holding
Coral_holding
Coral_holding
Recirculating
Recirculating
Recirculating
Recirculating
Recirculating
Sample
Genus
Aestuariibacter
Colwellia
Oleispira
Polaribacter
Pseudofulvibacter
Rubritalea
Tenacibaculum
Thiothrix
NA

Figure 20: Relative abundances of ASVs associated with A) Touch tank water, B) rockfish tank water, C) coral holding tank water, and D) recirculating tank water compared to other water types.

Chapter 4: Bacterial community dynamics of model aquaculture systems reveal that K-selection has an inhibitory effect on Vibrio growth

Abstract

Fish provide nutrition for a substantial fraction of the world's growing population. Production of wild-caught fish has been flat since the 1980s, so the aquaculture industry must respond to increasing demand. Recirculating aquaculture system (RAS) design is the most promising approach to minimize the resource demands and environmental impact of fish farming. Due it its intensive nature, however, RAS faces many challenges before it is widely implemented. Fish raised in high densities are susceptible to blooms of opportunistic pathogens. Water treatment strategies sufficient for microbial management in traditional flow-through systems (FTS), like ozonation and UV irradiation, can have the counterintuitive effect of exacerbating these blooms instead of suppressing them. Some have proposed that these disinfection techniques are not effective in the microbial management of RAS because they drive microbial communities below their ecological carrying capacity, selecting for fast-growing bacteria known as r-strategists, which are often opportunistic pathogens. These researchers reason that selecting for bacteria that are competitive at their ecological carrying capacity, known as K-strategists, might suppress blooms of opportunistic pathogens. This study uses seawater-inoculated bioreactor models of recirculating aquaculture systems to test the hypothesis that K-selection can inhibit blooms of opportunistic pathogens. We used 16S rRNA gene amplicon sequencing to track the composition of bacterial communities in r- and K-selected reactors over a 28-day-experiment. We found that r-selected treatments were dominated by the gammaproteobacterial genus *Vibrio*, which contains many opportunistic fish pathogens, while K-selected treatments were dominated by the alphaproteobacterial

genus *Roseovarius*, which contains no known fish pathogens. Our findings provide further support for K-selection as a water management strategy for RAS.

Introduction

Aquaculture is a rapidly growing global industry, with its contribution to total food supply increasing as wild-caught fish production has remained flat since the 1980s (Anderson et al., 2018). As the industry grows, its environmental impact rises, and demand for new approaches to water quality management increases (Edwards, 2015). Many current aquaculture facilities utilize a flow-through system (FTS) design. This "single-pass" approach requires a substantial amount of energy and resources to treat (and often heat) incoming water; furthermore, FTS produce diffuse effluent, making environmental impact difficult to mitigate (Martins et al., 2010). Recirculating aquaculture system (RAS) design has been proposed as a sustainable alternative to flow-through systems (Piedrahita, 2003). This approach dramatically reduces resource demands and the volume of effluent, producing concentrated nitrogen-rich sludge that can be used as fertilizer (Brod et al., 2017). However, RAS has a higher upfront cost and has only been implemented with limited success; this is primarily due to the challenges associated with managing water quality in a system with a high density of fish (Badiola et al., 2012) .

One substantial challenge to the RAS approach is that nutrients available to opportunistic pathogenic bacteria are orders of magnitude higher in RAS than in FTS; this is due to a combination of fish excrement and excess feed in the water (van Rijn, 2013). If water quality is not carefully managed, the intensive nature of RAS production can create ideal growth conditions for larval fish pathogens in the genera *Vibrio*, *Aeromonas* and

Photobacterium (King et al., 2004). The intuitive approach to prevent blooms of these pathogens might be to treat water with UV irradiation, ozonation, or antibiotics (Cabello, 2006; Sharrer and Summerfelt, 2007). However, this water management approach is resource-demanding, and recent ecologically-informed research suggests that reducing bacterial counts does not result in better outcomes and in many cases has negative effects on fish health (Defoirdt et al., 2011; Rurangwa and Verdegem, 2015). Here, we experimentally test one ecologically informed management approach that seeks to explain this phenomenon and reduce the abundances of harmful bacteria in RAS by selecting for bacteria that will outcompete them (De Schryver and Vadstein, 2014).

In classical ecology, r- and K-selection are used as heuristics to describe two distinct life history strategies of organisms. K- selected organisms evolved to live in ecosystems functioning near their carrying capacity: these organisms, known as K-strategists, are typically classified as specialists and are highly competitive in resource-poor environments (Pianka, 1970). Organisms that are r-selected have adapted to live in recently disrupted environments with high levels of available nutrients: these organisms are typically classified as generalists and have adapted to maximize growth rates at the expense of being competitive in nutrient-poor environments (Pianka, 1970). The r- and K- life history heuristics also apply to bacteria (Vadstein et al., 2018b). Many opportunistic pathogens (e.g. *Vibrio*) have high maximal growth rates, and have previously been described as r-strategists (Weinbauer et al., 2006). Furthermore, beneficial bacteria that most efficiently convert harmful ammonia found in fish excrement to relatively innocuous nitrate, are slow-growing specialists, making them K-strategists (Puyol et al., 2013; van der Star et al., 2008). Taken together, these

observations have led researchers to propose that operating aquaculture systems near their bacterial carrying capacity may have the property of reducing abundances of opportunistic pathogens and increasing abundances of beneficial bacteria (Skjermo et al., 1997). According to this hypothesis, common disinfection practices (which reduce total bacterial counts) drive bacterial communities below their carrying capacities and favor opportunistic pathogens (Skjermo et al., 1997). Recent research has suggested that maintaining a high microbial load may encourage succession of bacterial communities, enriching for K-selected bacteria and inhibiting the growth of opportunistic bacteria (Vadstein et al., 2018a). K-selection as a water management strategy has been linked to better outcomes such as larval survival, growth rates, size, and appetite (Attramadal et al., 2012; Salvesen et al., 1999). While this research is on firm theoretical and empirical ground, it thus far has not explicitly probed for high-resolution taxonomic differences in composition between r- and K-selected bacterial communities in a fully controlled laboratory experiment.

In this study, we use laboratory-scale bioreactors as models of aquaculture systems to test the hypothesis that opportunistic pathogens are favored in r-selected environments and are outcompeted in environments operating near their carrying capacity. We also explore the possible confounding effect of carrying capacity on the outcomes of r- or K-selection. We use 16S rRNA gene amplicon sequencing to describe the composition of bacterial communities and flow cytometry to measure RNA content per cell and estimate cell counts. We find evidence in support of the hypothesis that r-selection favors opportunistic fish pathogens, with the observation that *Vibrio* dominate r-selected

environments, but are outcompeted by *Roseovarius* when nutrient levels are kept constant.

Materials and Methods

Experimental design

To test effects of selection regime (r/K) and nutrient concentration (High/Low) on bacterial community structure, we implemented a 2x2 experimental design with three technical replicate laboratory-scale bioreactors for each treatment, making for 12 total reactors. We used Duran 500 mL borosilicate reactors. All reactors were aerated with air pumped through a 0.2-micron filter and stirred using stir bars. Cultures were transferred to autoclaved reactors every eight days to ensure that communities primarily reflect bacterioplankton rather than shedding or detachment of biofilms. We kept reactors in a 15°C water bath to simulate typical aquaculture conditions for rearing of larval Atlantic cod. We used M65 media (equal parts peptone, tryptone, and yeast extract, dissolved in 0.2-micron filtered seawater) with a concentration of 5 mg/L in the "High" nutrient level reactors and 1 mg / L in the "Low" nutrient level reactors.

To encourage maximal growth rate selection (r-selection), we used fed-batch reactors. We used 5 mL of seawater, collected at a depth of 70m from the Trondheim Fjord, to inoculate 245 mL of media in the reactors. Then, every two days, we used 5 mL of this culture to inoculate 245 mL of fresh media to ensure that carrying capacity was not reached. To observe selection at carrying capacity (K-selection), we used continuous reactors. We used 250 mL of seawater to start these cultures and used a low-flow peristaltic pump to add 175 microliters of media per minute. To ensure constant volume

in the continuous reactors, an outflow tube was placed at the 250mL mark; positive air pressure was sufficient to pump excess culture into a waste container.

Flow cytometry

We collected flow cytometry samples every other day during the course of the experiment. To ascertain cell counts and measure RNA content per cell, we used the SYBR Green II dye (Thermo Fisher, S7564) to stain RNA in our cultures. We incubated samples for a minimum of 15 minutes. We used the BD Accuri C6 flow cytometer to measure forward scatter (FSC) and fluorescence (FL-1) of the stained cells. We ran samples for 2.5 minutes each at a rate of ~35uL/minute ("medium" fluidic setting). Between samples, the fluid collector was washed and samples were agitated. 0.2-micron filtered MilliQ water was run for 3 minutes after every fourth sample. We considered FSC values of above 10^5 to be aggregates, and FL-1 values below 10^4 to be noise, and filtered those events from all samples. We report sample cell counts in events per microliter and mean RNA content per cell using mean FL-1 values.

To test for effects of r- and K-selection on events per microliter and mean FL-1 values, we performed a linear mixed effects analysis. We used the R package lme4 (Bates et al., 2015) to generate models that account for non-independence by-day and by-reactor. We compared the null and full models using a likelihood ratio test, and report chi-square and p-values.

Nutrient pulsing

To determine whether our treatments resulted in different maximal growth rates among our treatments (i.e. confirm r-selection), we added concentrated M65 medium to 25mL

samples of culture to increase the M65 concentration to 25 g/L. We measured cell counts (Events per microliter) and mean RNA content per cell (mean FL-1) a) before pulsing, b) three hours after the nutrient pulse, and c) eight hours after the nutrient pulse, expecting that cultures under r-selection would both increase transcriptional activity to a greater extent and divide faster than cultures under K-selection. We conducted nutrient pulses on the inoculum (Day 0), on Day 12, and on Day 28. In 22 of 26 biological samples, mean FL-1 values increased between the start of the pulse and the third hour as expected. Four anomalous samples, the last samples to be analyzed on Day 28, exhibited distinct trends compared to their biological replicates, and showed decreases in mean FL-1 values between the beginning of the pulse and the third hour. The decrease in mean FL-1 values did not correspond with selection regime or nutrient level. We reason that ambient light photobleached the SYBR Green II dye while other samples were being measured, so we removed these samples from our analyses.

DNA isolation, PCR, and sequencing

We collected samples by pumping 50 mL of culture through a 0.2-micron syringe filter (DynaGard, product number: DG2M-110). We then used DNeasy PowerSoil Kits (Qiagen, product number: 12888-100) to extract microbial genomic DNA from the filters. We amplified a 467-bp sequence spanning the V3-V4 hypervariable regions of the 16S rRNA gene using primers Ill338F (sequence: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN CCT ACG GGW GGC AGC AG-3') and Ill805R (sequence: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN GAC TAC NVG GGT ATC TAA KCC-3'). We used Phusion Hot Start II DNA polymerase (Thermo Fisher, product number: F549L) and the following thermal cycler

settings: 1x (60 seconds @ 98°C), 28 x (15 seconds @ 98°C, 20 seconds @ 55°C, 20 seconds @ 72°C), 1 x (300 seconds @ 4°C), 1 x (∞ @ 10°C) to amplify the target sequence. We assessed PCR success by running agarose gels; if no band was present, we attempted amplification again. We used Illumina Nextera XT library preparation kits (Illumina, product number FC-131-2001) and sequenced samples on two paired-end 250bp lanes of the MiSeq platform. We used the USEARCH pipeline to quality filter, cluster reads into operational taxonomic units (OTUs) of 97% similarity, and filter chimeric reads (Edgar, 2010).

Diversity and composition

To measure alpha (within-sample) diversity, we calculated Hill diversity profiles (Hill, 1973). These profiles use a scaling factor and have the useful property of easily relating to other measures of alpha diversity. For example, a Hill diversity of order zero (i.e. the scaling factor is equal to zero) is equivalent to OTU richness. A Hill diversity of order one is equivalent to the exponential Shannon index, and a Hill diversity of order two is equivalent to the inverse Simpson index. Finally, a Hill diversity of order infinity is equivalent to the reciprocal of the relative abundance of the most abundant OTU. We randomly subsampled to a depth of 10,000 reads to estimate alpha and beta diversity values.

We used Sørensen (presence-absence) and Bray-Curtis (abundance weighted) metrics to quantify between-sample (beta) diversities. We conducted both unconstrained (Principal Coordinates Analysis) and constrained (Constrained Analysis of Principal Coordinates)

ordinations on the beta diversity matrices to evaluate the effects of r- and K- selection on between-sample diversity values.

Results

Pre-processing

After quality filtering and chimera removal, the OTU table had a total of 14,658,585 reads, with a median of 48,688 reads per sample. 2,893 total OTUs were observed; of these, 44 were identified as contaminants using the decontam package. After contaminant removal, the OTU table had 14,553,550 reads, with a median of 48,683 reads per sample. We observed a mean richness of 122 OTUs per sample (standard error: 5.8).

Culturing in M65 rapidly reduced alpha diversity and restructured bacterial communities

We observed a sharp decrease in alpha diversity in our cultures compared to the seawater inoculum collected from Trondheim Fjord at a depth of 70 meters (Figure 21). Seawater inoculum samples had an average richness of 457 OTUs per sample, and after 24 hours, the reactors had an average 188 richness OTUs per sample. By the fourth day of the experiment, we observed an average of 68 richness OTUs per sample. At this point, alpha diversity had stabilized; the average richness after the fourth day of the experiment was 66 OTUs. Taxonomic composition also changed substantially, as shown by a Principal Coordinates Analysis on Bray-Curtis distances (Figure 22) and a stacked bar plot of the top ten most abundant orders (Figure 23). K- and r- selected reactors were more similar to each other than to the inoculum, characterized by greater abundances of Orders *Vibrionales* and *Rhodobacterales* compared to the inoculum. A substantial fraction of reads in the two seawater inoculum samples were unclassified; 20.8% and 20.5% were

unclassified past Domain *Bacteria*, 19.9% and 20.1% were unclassified past Class *Gammaproteobacteria*, and another 12.2% and 11.2% were unclassified past Class *Alphaproteobacteria.* In the reactors, however, an average of 0.9% of reads (standard error: 0.2%) were unclassifiable past the level of Domain *Bacteria*, 1.7% of reads (standard error: 0.3%) were unclassifiable past Class *Gammaproteobacteria*, and 0.5% of reads (standard error: 0.1%) were unclassifiable past Class *Alphaproteobacteria.*

Selection regime corresponded with distinct cell counts and baseline RNA content per cell

We found that r-selected reactors had higher mean FL-1 values over the course of the experiment (Figure 24A). We also found that K-selected reactors contained higher cell counts over the course of the experiment, though K-selected reactors with high nutrient concentration appeared to be declining toward the end of the experiment (Figure 24B). We conducted a linear mixed effects analysis, accounting for by-reactor and by-day variability. K-selected reactor communities had significantly higher cell counts ($\chi2(1)=12.09$, $p=0.003$) and lower mean FL-1 values ($\chi2(1)=12.80$, $p=0.0003$) than r-selected reactor communities. Average and standard deviations of cell counts and mean FL-1 values can be found in Table 3.

Selection regime affected response to nutrient pulsing

We observed a clear difference between r- and K- selected bacterial communities in the rate and magnitude of mean RNA content per cell increase after a nutrient pulse (Figure 25). Bacteria in the seawater inoculum did not respond to a nutrient pulse, maintaining low values for the duration of the assay. On Days 12 and 28, bacterial communities in r-selected reactors both had higher baseline rates and increased in mean RNA content per

cell faster than communities in K-selected reactors. RNA content per cell peaked for r-selected treatments within a few hours, while RNA content per cell increased linearly for k-selected communities over the eight hours of the nutrient pulse.

Patterns in cell counts (events per microliter), were nutrient-level-dependent and inconsistent between days (Figure 25B). On Day 12, K-selected reactors with high nutrient levels experienced higher baseline level cell counts as well as higher growth rates than r-selected reactors. K-selected reactors with low nutrient levels did not experience substantial growth rates, with nearly horizontal growth curves. Irrespective of nutrient level, r-selected reactors experience similar growth rates. On Day 28, baseline cell counts were largely independent of selection and nutrient level, though r-selected reactors with high nutrient levels appeared to have lower counts than the other treatments. However, we observed differences with respect to selection regime in the difference in cell counts between the third and eighth hour of the nutrient pulse. K-selected reactors experience little growth between these time points, while r-selected reactors increase substantially.

Reactor communities and taxonomic composition varied on two dimensions: selection regime and time

The unconstrained ordination on Sørensen beta diversity values revealed a pattern in presence-absence between the early phase of the experiment and the later phase; PCoA Axis 2 corresponds with the rapid drop in diversity in the beginning of the experiment, while PCoA Axis 1 corresponds with a time gradient over the course of the experiment (Figure 26A). The unconstrained ordination on Bray-Curtis values displays a pattern over time on PCoA Axis 1, but also separates samples based on selection regime on PCoA

Axis 2 (Figure 26B). Ordinations constrained by selection regime reveal similar trends and additionally separate the Sørensen beta diversities based on selection type (Figure 27A-B).

Roseovarius dominated K-selected reactors, while Vibrionales dominated r-selected reactors

We used an area plot to visualize differences in bacterial community composition between K- and r-selected reactors (Figure 28). Throughout the experiment, K-selected reactors were dominated by the alphaproteobacterial genus *Roseovarius,* while r-selected reactors were dominated by the gammaproteobacterial genus *Vibrio*. *Vibrio* were present in meaningful abundances in K-selected reactors for the first five days, but their abundances declined as the abundances of *Roseovarius* increased. The gammaproteobacterial genus *Colwellia* was found in reactors of both selection regimes, as were unclassified alphaproteobacteria.

Discussion

Early community dynamics reveal selection for r-strategists and flushing of particle-associated taxa

During the first few days of our experiment, we observed a precipitous drop in alpha diversity in both selection regimes. The bacteria lost during this time period were difficult to classify; over half of them were not classified past the taxonomic level of class. The fact that the majority of these seawater bacteria were unclassified highlights the difficulty of characterizing and culturing marine bacteria (Giovannoni and Stingl, 2007). Many marine bacteria are particle-associated (Duret et al., 2018).; these bacteria would be represented in the inoculum, but would likely be flushed out as fresh media replaced the

inoculum. This occurred faster in the r-selected treatments, where only 5 mL of inoculum was used, than in the K-selected treatments, which began the experiment with a full volume (250mL) of inoculum. We reason that the taxa remaining after the fourth day of the experiment (when community composition stabilized) were free-living bacterioplankton that could utilize the M65 medium as a substrate. We believe that these early-phase dominant taxa were primarily r-strategists, given the dominance of gammaproteobacteria, which have previously been described as fast-growing opportunistic taxa (Weinbauer et al., 2006). Order *Alteromonadales* was more dominant in the early-phase K-selected reactors, while *Vibrionales* was more dominant in the early-phase r-selected reactors. This suggests that both of these taxa are r-strategists, thriving in nutrient rich environments, but *Alteromonadales* may utilize nutrients more efficiently, allowing them to compete with specialists that persisted longer in the K-selected reactors.

Classification of bacteria as r- or K-strategists is not trivial

In this study, we observed two assemblages; one that was sensitive to our r- or K-selection regimes, and another that was not. This suggests that the maximal growth rate / resource utilization tradeoff is not sufficient to explain the evolution of most bacteria; indeed, tradeoffs in bacterial evolution are often overlooked (Ferenci, 2016). As the failures of the r- and K- selection paradigm to explain life histories of animals and plants gave way to a more advanced understanding of life history evolution (Rose and Mueller, 1993), we suggest that our failure to classify most bacteria as r- or K-strategists may serve to open this area to discussion. With the caveat that we explored a narrow parameter space— free-living, aerobic bacteria collected from 70m in the Trondheim Fjord that could be cultured in M65 medium—we only found two taxa competing on the

maximal growth rate / resource utilization axis: *Vibrio* and *Roseovarius*. Most taxa were unaffected by selection regime, and reactor communities appeared to undergo succession without respect to the growth rate and resource competition pressures applied to them.

Roseovarius competes with Vibrio for nutrients

Perhaps the most striking finding of this study was the relative success of the *Vibrio* in r-selected treatments and its complete replacement by the *Roseovarius* in K-selected treatments. This suggests that *Vibrio* and *Roseovarius* were in direct competition, and that *Roseovarius* can better utilize nutrients at lower concentrations. We expected that an opportunistic pathogen might thrive in r-selected treatments, but would be outcompeted by many specialist taxa in K-selected treatments. However, it appears that bacterial niche space may contain more dimensions than we thought, allowing *Roseovarius* to be both a K-strategist and a generalist. *Roseovarius* species have been isolated from many sources (Park et al., 2018). *Roseovarius* have not been associated with any fish disease, but the species *Roseovarius crassostreae* is suspected as the causative agent of Juvenile Oyster Disease (Boettcher, 2005). Therefore, if *R. crassostreae* competes with *Vibrio* in aquaculture systems, then the benefits of K-selection may not extend to oyster farming.

RNA content after nutrient pulsing is a better indicator of water quality than cell counts

Current microbiological approaches to aquaculture water management often involve simple cell counts or labor-intensive culture-based techniques to measure growth rates of bacteria; these techniques provide a limited and biased view into the bacterial content of these systems (Fjellheim et al., 2012). We propose flow cytometry, a technique that is already used extensively in the water industry, as a powerful alternative to culture-based

techniques (Gillespie et al., 2014). From our nutrient pulsing experiments, we find that RNA content per cell is a good indicator of r- or K-selection, which in turn is a good predictor of some bacterial abundances. On the other hand, cell counts are dependent on nutrient concentration, which is a poor predictor of bacterial community structure.

Table 3: Summary statistics of flow cytometry data

	Average events per microliter	Standard deviation events per microliter	Average mean FL-1	Standard deviation mean FL-1
R-High	16,327.78	4,662.163	82,877.77	16,891.13
R-Low	14,539.5	8,148.52	79,117.76	7,511.454
K-High	35,896.67	12,910.83	64,497.15	9,455.278
K-Low	22,299.52	11,965.22	66,727.83	11,731.16

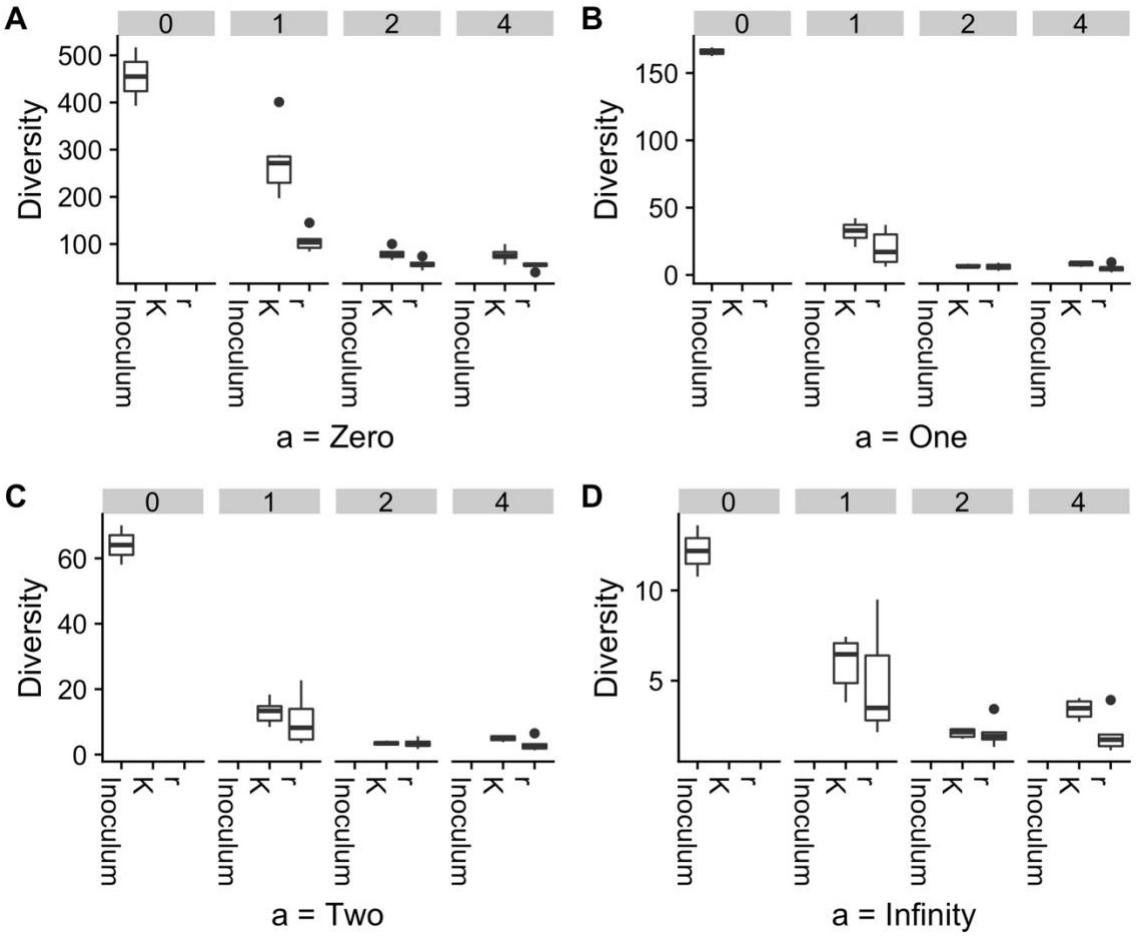


Figure 21: Hill diversities of A) order 0, B) order 1, C) order 2, and D) order infinity reveal a drop in alpha diversity during the first four days of the experiment. This decrease happened faster in r-selected treatments.

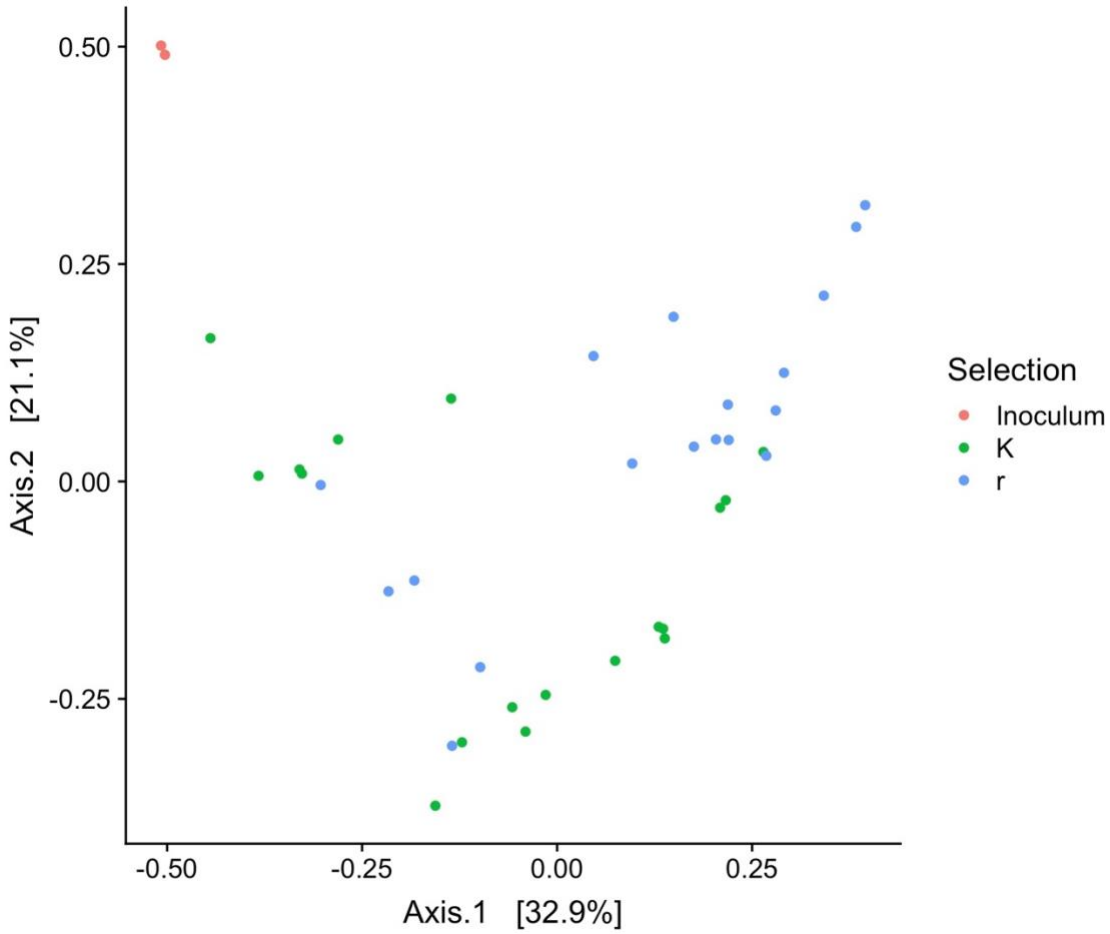


Figure 22: An unconstrained PCoA ordination on Bray-Curtis distances reveals that r- and K-selected reactors are more similar to each other than to the seawater inoculum.

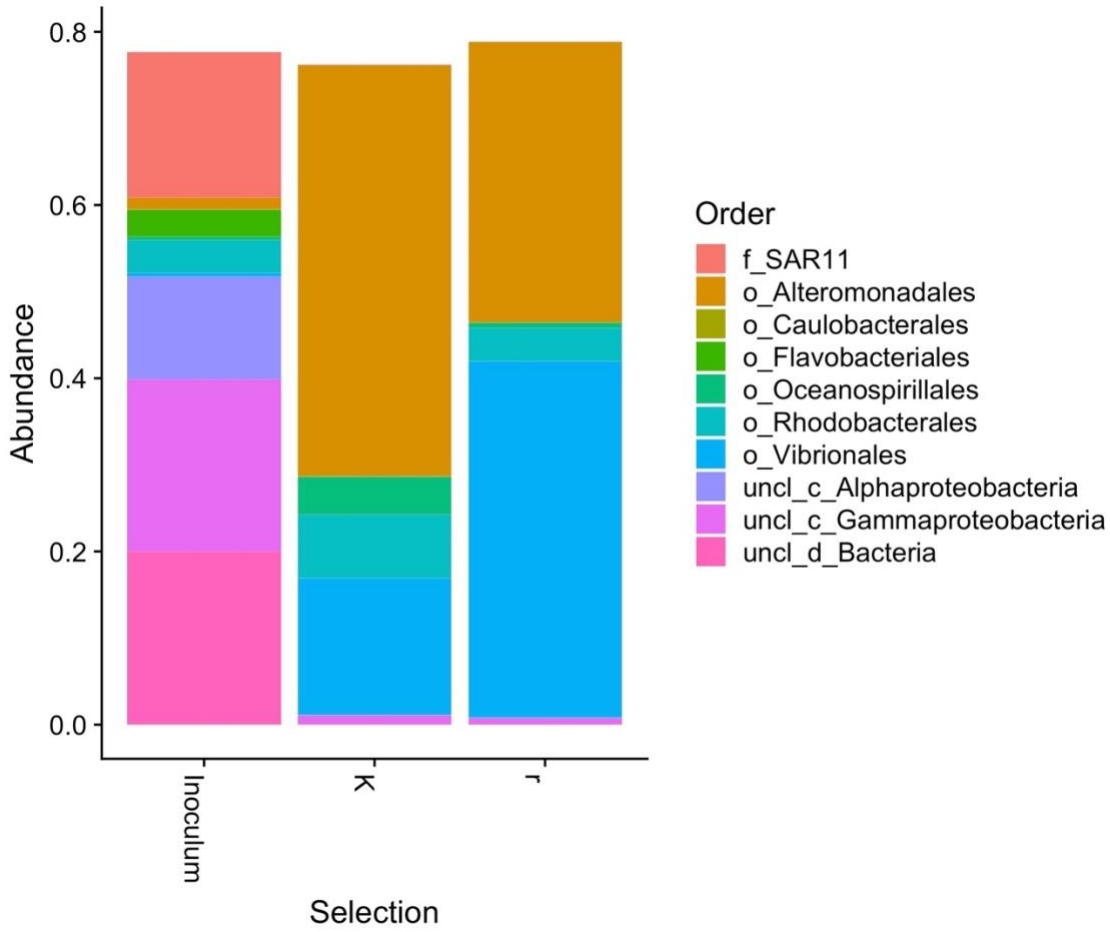


Figure 23: Stacked barplots describe the composition of bacterial communities during the first four days of the experiment. Seawater inoculum is primarily composed of unclassified OTUs, while r- and K-selected reactors are dominated by the gammaproteobacterial orders *Vibrio* and *Alteromonadales.*

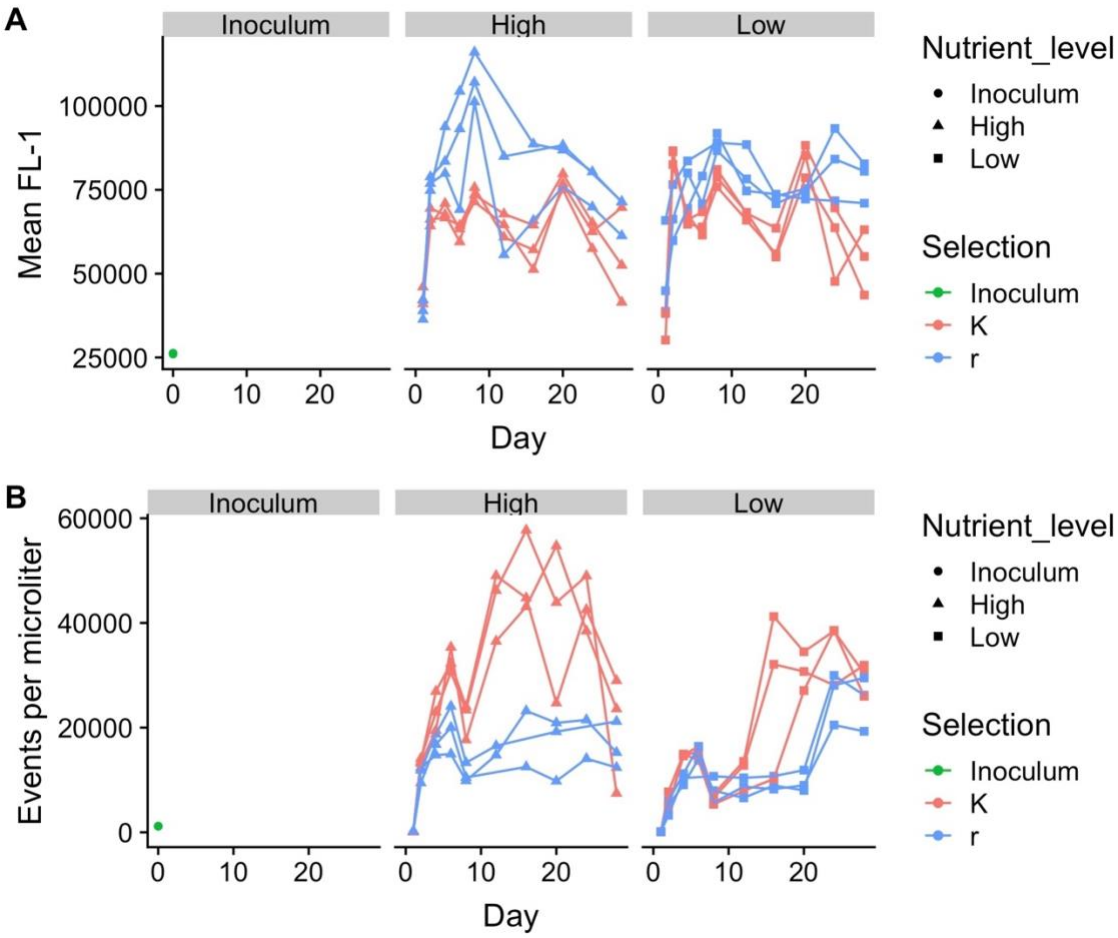


Figure 24: Baseline (A) mean FL-1 and (B) events per microliter over the course of the experiment show distinct patterns with respect to selection regime and nutrient level.

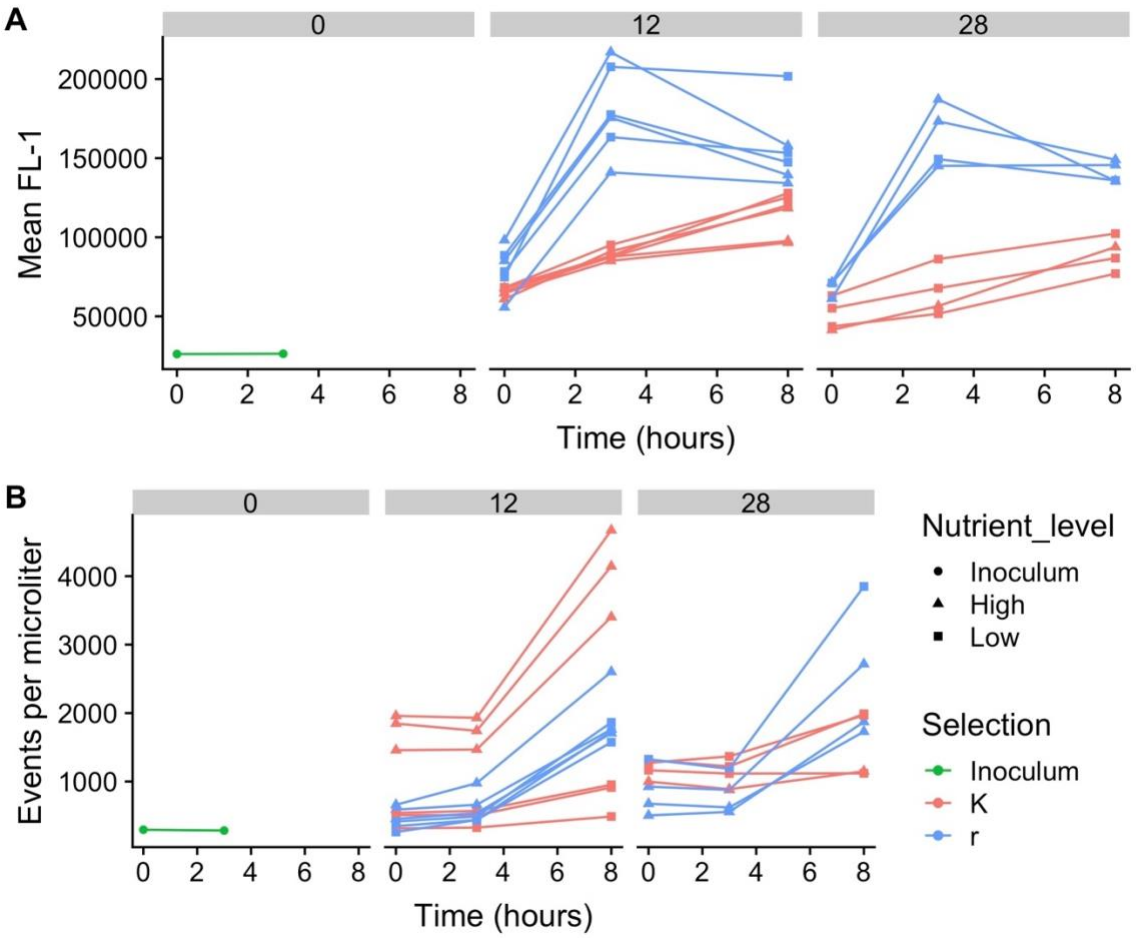


Figure 25: Bacterial community (A) mean FL-1 and (B) events per microliter before, after 3 hours, and after 8 hours of a nutrient pulse reveal that r-selected communities are faster to produce RNA and reproduce in resource-rich conditions compared to K-selected communities.

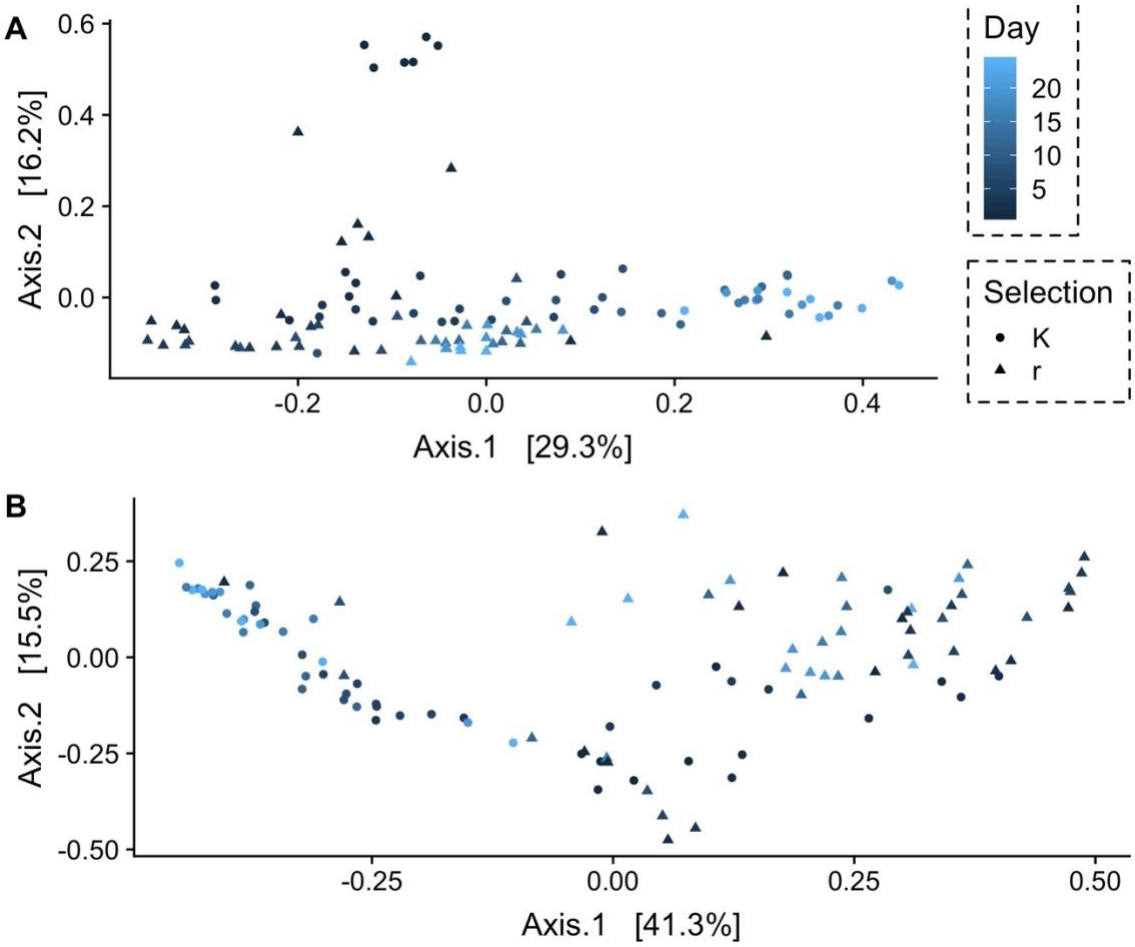


Figure 26: Unconstrained PCoA ordinations on (A) Sørensen and (B) Bray-Curtis distances visualize trends in beta diversity over time and by selection regime.

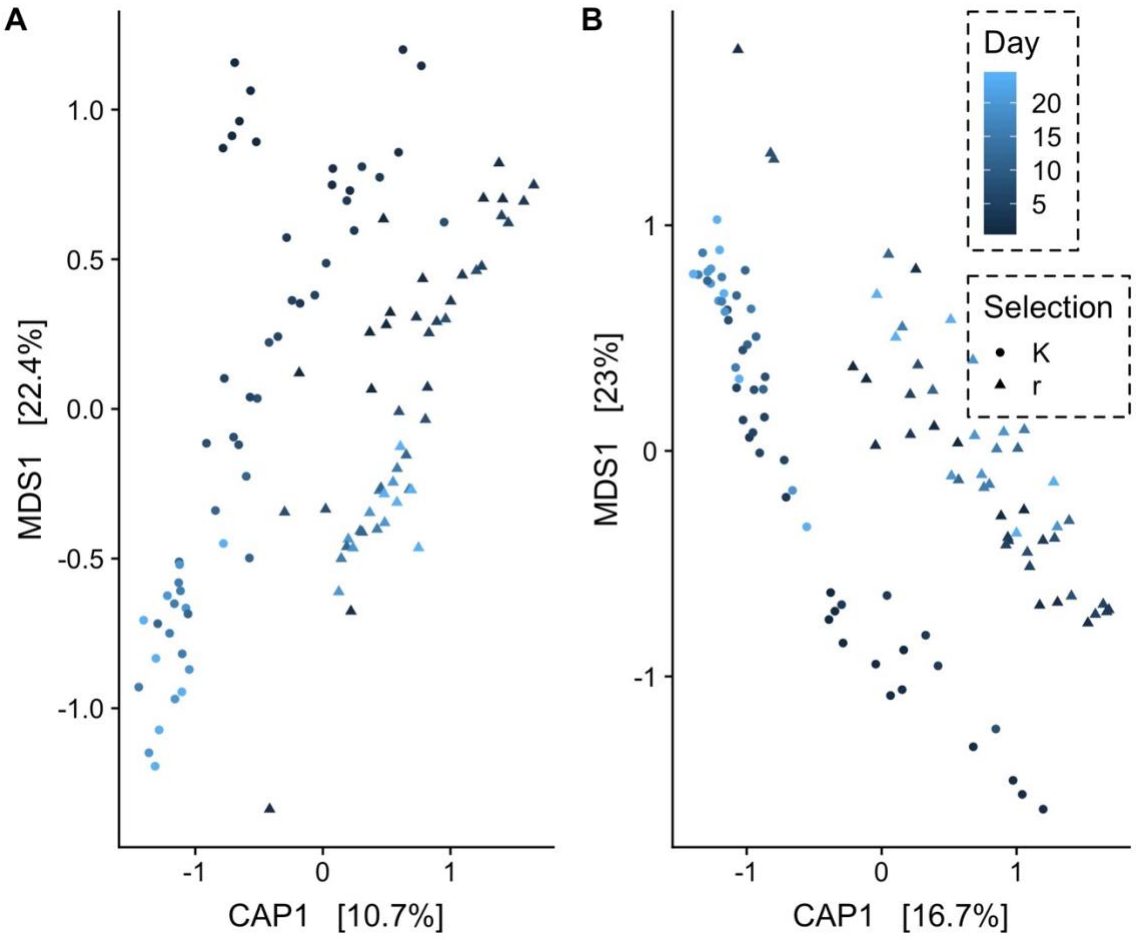


Figure 27: Constrained Analysis of Principal Coordinate ordinations on (A) Sørensen and (B) Bray-Curtis distances suggests that there are two assemblages in these bacterial communities; one that is sensitive to selection regime and one that is not.

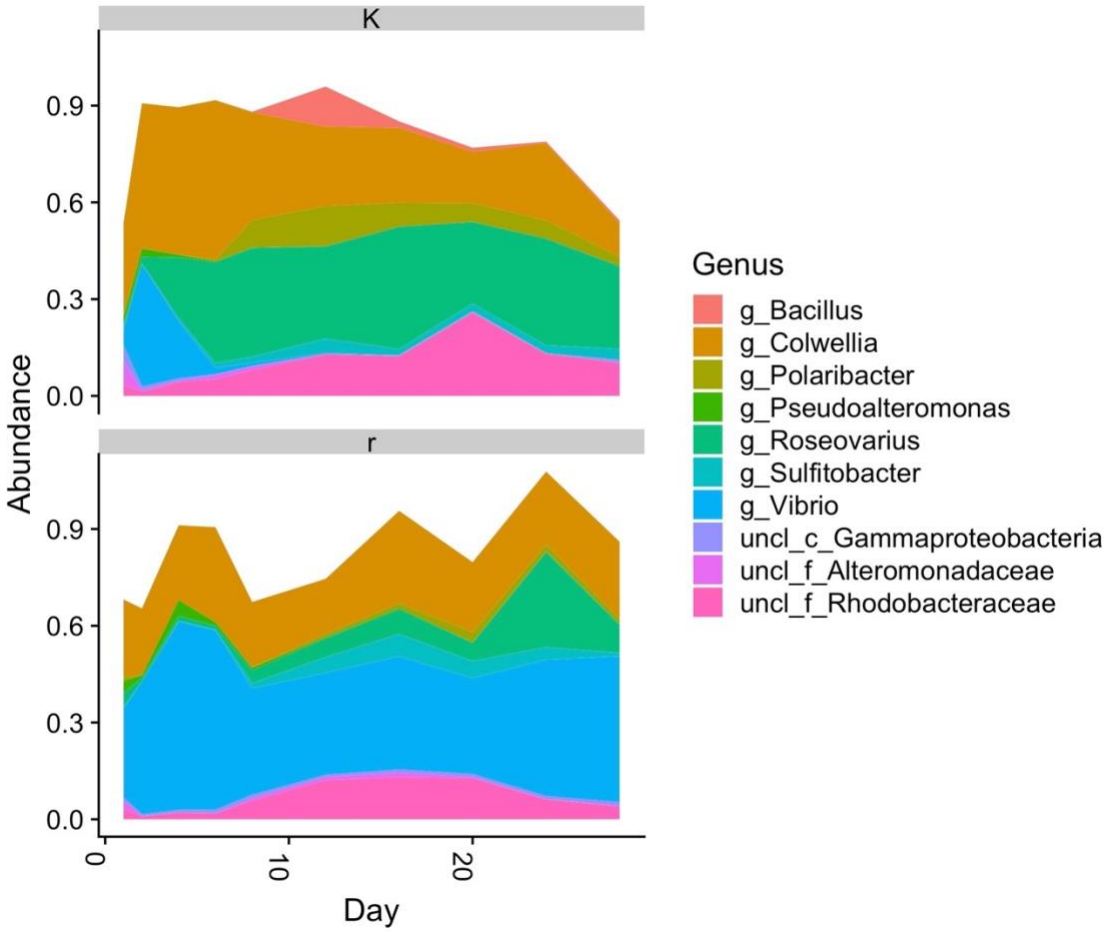


Figure 28: A stacked area plot describes the composition of r- and K- selected reactors throughout the course of the experiment. The composition of the reactors is similar during the first few days of the experiment, but *Vibrio* are outcompeted by *Roseovarius* in K-selected reactors by the tenth day of the experiment.

Chapter 5: Conclusion

This dissertation described the composition of a few previously unexplored marine bacterial communities and the factors that shape these communities. The research program progressed from marine natural systems to human-built environments. Chapter 2 described the microbiome of the temperate intertidal sea anemone *Anthopleura elegantissima* across a substantial portion of its natural range. Chapter 3 began the transition from natural systems to human-built systems, describing the effects of water treatment on seawater and biofilms. Chapter 4 focused on the completely artificial environment of seawater-inoculated bioreactors to simulate flow-through and recirculating aquaculture systems. Common themes arose over the course of the research program. First, alpha diversity values were sensitive to subtle differences in the marine environment, and different factors affected OTU abundance distributions in distinct ways. Furthermore, some taxa followed distinct patterns in both natural and human-built systems. This final chapter will summarize and synthesize the findings from the previous data chapters and propose ecological hypotheses about taxa that span natural and artificial marine systems. It will also suggest directions for future research.

Alpha diversity corresponds with environmental factors

Some of the most salient patterns observed in this dissertation concern differences in alpha diversity corresponding with environmental variables. Chapter 2 described a change in alpha diversity corresponding with the symbiotic state of the intertidal sea anemone *A. elegantissima.* Anemones in symbiosis with the green algal *Elliptochloris marina* corresponded with higher bacterial community richness compared to the anemones associated with the brown algal *Symbiodinium* or anemones in the

aposymbiotic state. However, a different pattern was observed when the Shannon index, which weights species abundances, was considered: symbiotic anemones had suggestively higher Shannon index values than aposymbiotic anemones.

The changes in alpha diversity observed in Chapter 3 and 4 both reaffirmed the commonly observed phenomenon that most seawater bacteria require specific culturing conditions. In Chapter 3 it was observed that water collected from the Yaquina Bay had substantially higher alpha diversity of all orders compared to the seawater system at the Hatfield Marine Science Center (HMSC). Similar patterns were evident in Chapter 4, when the alpha diversity of the seawater inoculum collected from Trondheim Fjord plummeted after 24 hours of inoculation of M65 cultures. These studies open the door for future research concerning the diversity trends and dynamics associated with human-built seawater systems. The results of this dissertation demonstrate that different scales of alpha diversity should always be considered when comparing bacterial communities (Kang et al., 2016). If the above analyses had only considered richness or the Shannon index, for example, they would have missed important trends. By using multiple indices or Hill diversity profiles, OTU abundance distributions were considered more thoroughly.

Defining and sampling marine bacterial communities

Because bacterial communities exist at scales that are difficult to observe, sampling a bacterial community is not trivial. The microbiomes of cnidarian holobionts have been shown to be distinct in different anatomical areas (Pollock et al., 2018). Therefore, the microbiomes samples collected from whole-organism extractions in Chapter 2 may consist of multiple non-interacting communities. The conclusions drawn from this research are likely still valid, but a more nuanced sampling approach informed by a more

solid base of evidence may have allowed for subtler trends to be found in the data. Furthermore, it is likely that the seawater collected from Yaquina Bay in Chapter 3 consisted of a combination of particle-associated and free-living bacteria, and the seawater from the flow-through system was likely made up of biofilm-shedding cells and free-living bacteria. In Chapter 4, it is possible that some of the taxa observed were associated with incompletely-dissolved media. Given the limitations of the sampling approaches used in this dissertation, one cannot easily divide samples into communities. Currently, most studies define a bacterial community *ad hoc* as whatever-was-sequenced. Future studies will likely cite these studies as foundational-but-primitive: as researchers begin to map out the microbial world in higher resolution, more refined sampling procedures will be developed. Therefore, in this rapidly developing phase of the field, the findings of this dissertation provide information that may help future researchers parse out individual communities and improve upon its sampling approaches.

Cosmopolitan taxa follow distinct patterns in marine environments

Some taxa were present in all studies in this dissertation. At the phylum level, *Proteobacteria* and *Bacteroidetes* were common to both natural and artificial marine systems and were detected in all three chapters. In most cases, phylum *Bacteroidetes* was dominated by the diverse order *Flavobacteriales*. Some species in this order have been demonstrated to be pathogens in aquaculture systems, with salmonids being particularly vulnerable to infection (Nematollahi et al., 2003). Despite some bacteria in *Flavobacteriales* being pathogenic, most evidence suggests that the majority of taxa in this order are free-living and have evolved to degrade complex carbon molecules (Kirchman, 2002).

The phylum *Proteobacteria* was also found in all three marine systems that this dissertation describes. Within this phylum, different classes—primarily *Alphaproteobacteria* and *Gammaproteobacteria*— are variably represented in different studies and treatments. In Chapter 2, for example, *Proteobacteria* associated with sea anemones was dominated by the order *Rhodobacteriales*. Order *Rhizobiales* was also associated with sea anemones, though this order was not found in any human-built systems. Members of *Rhizobiales*, which are typically associated with nitrogen fixation, may be vital components of the *A. elegantissima* holobiont, contributing nitrogen to photosynthetic symbionts of the anemones (Lema et al., 2012). Both Chapter 3 and 4 describe a separation between *Alphaproteobacteria*- and *Gammaproteobacteria*-dominated communities. In Chapter 3, OTUs in the genus *Amylibacter,* in the alphaproteobacterial order *Rhodobacteriales,* were among the most abundant in the flow-through seawater system. In the recirculating seawater system, however, two genera in the gammaproteobacterial order *Alteromonadales* dominated the seawater communities. *Rhodobacteriales* were also involved in salient trends in Chapter 4, likely competing with OTUs in gammaproteobacterial order *Vibrionales* for nutrients. Water treatments favoring a competitive environment, like K-selection or the low-water-retention time flow-through system (which resembles oligotrophic conditions of the open ocean), were dominated by alphaproteobacterial taxa. Treatments favoring generalists, like r-selected treatments, favored gammaproteobacterial taxa. This pattern supports previously described relationships between these taxa and suggests that they have evolved different ecological preferences in the marine systems examined in this dissertation (Weinbauer et al., 2006).

Below the taxonomic level of class, results support further specialization within proteobacterial orders. For example, genera *Sulfitobacter* and *Amylibacter*, both in order *Alphaproteobacteria*, stably coexisted in flow-through seawater systems. Similarly, the genera *Colwellia* and *Aestuariibacter*, both in the gammaproteobacterial order *Alteromonadales*, coexisted in recirculating water systems.

Better understanding how compositional patterns of bacterial communities break down at different taxonomic levels may help elucidate major evolutionary changes that correspond with ecological preferences. Future research might explore mechanisms that explain the ecological differences between these taxa. The results of this dissertation highlight the importance of describing compositional patterns of bacterial communities at multiple taxonomic levels.

Bacterial taxa in some seawater systems are underrepresented in databases

In Chapters 3 and 4 of this dissertation, there were stark contrasts between some treatments that led to high or low classification rates in different treatments. For example, 80% of the 16S rRNA gene amplicons in seawater used to inoculate bioreactors in Chapter 4 were unclassified past the taxonomic level of class. In Chapter 3, similar levels of OTUs associated with the sea star *D. imbricata* were unclassified past the class level. Interestingly, OTUs associated with the other sea star species we observed, *P. ochraceus*, were classifiable to the genus level. We used different taxonomic assignment methods, databases, OTU clustering algorithms, and 16S rRNA amplicon primers in the two chapters, but the within-study patterns are striking. Some of these difficult-to-classify 16S rRNA gene amplicon sequences showed sub-90% matches to any sequence in the NCBI nucleotide repository. These results suggest that microbial ecologists using

amplicon sequencing should consider these differential classification rates as a potentially confounding factor. For example, if using an agglomeration method that discards unclassified OTUs, like the default method in the Phyloseq package, researchers may make the false conclusion that better-classified samples display higher rates of diversity, when the opposite may in fact be true.

Factors shaping host-associated microbiomes in marine systems remain elusive

Chapter 2 of this dissertation aimed to describe the bacterial component of a temperate sea anemone's holobiont. The microbiome of this sea anemone was relatively stable across latitudes, but varied with respect to symbiotic state. This study was conducted in a natural system, which complicates analyses. Intra-site factors were difficult to account for, and the only factor considered was the tidal height; we assumed that this variable that encapsulates aerial exposure time, community composition of surrounding macro-organisms, and mean temperature would be the strongest predictor of bacterial community composition. However, both tidal height and latitude minimally affected bacterial community structure. Therefore, we likely overlooked an important factor shaping the bacterial communities of these sea anemones.

Bacteria of marine built environments vary considerably

Previous research concerning the microbiomes of aquaria have not examined flow-through systems or small-scale recirculating systems. The most notable contemporary studies, examining a Shedd aquarium exhibit after a 90% water change and a longitudinal study of the *Ocean Voyager* exhibit at the Georgia Aquarium, instead describe the microbiomes of large-scale artificial seawater exhibits. The Shedd Aquarium study only

characterized the microbiome of the tank at the family level despite a poor classification rate at that level, so it is difficult to compare their results to those of this dissertation (Van Bonn et al., 2015). However, they found the betaproteobacterial *Oxalobacter* to be the most abundant family, which the common DNA extraction kit contaminants *Herbaspirillum* and *Janthinobacterium* are classified under (Van Bonn et al., 2015). This study did not include any steps to remove contaminants, like a DNA extraction kit blank, so without further confirmation, the curious dominance of this betaproteobacterial family, which is typically rare in artificial and natural marine environments, cannot be considered conclusive. The study examining the *Ocean Voyager* exhibit found that taxa in *Alphaproteobacteria* dominated the pelagic fraction of the exhibit's seawater over the 14 months that the researchers collected data (Patin et al., 2018). Notably, they found that within this order, two taxa competed for dominance of the communities: *Kordiimonas* and *Phaeomarinomonas*. Similarly to the marine studies included in this dissertation, the pelagic marine communities in the *Ocean Voyager* exhibit were dominated by *Alphaproteobacteria* and *Gammproteobacteria*, with a persistent fraction consisting of *Flavobacteriales*. Elements of this pattern were reflected in a study of recirculating and flow-through aquaculture systems; however, this study also found the epsilonproteobacterial genus *Arcobacter* to dominate flow-through systems (Vestrum et al., 2018).

Chapters 3 and 4 demonstrate that the marine built environment harbors a diverse array of bacterial taxa distinct from natural systems. In particular, *Colwellia* was detected in both present recirculating seawater tanks in an aquarium and bioreactors. This genus, made up of barophiles and psychrophiles, has been previously identified as an inhabitant of

recirculating aquaculture systems (Deming et al., 1988; Methe et al., 2005; Ruan et al., 2015). In this dissertation's studies, members of this genus were underrepresented in Yaquina Bay and Trondheim Fjord seawater, but present in meaningful abundances in the built environment. Taken together, this suggests that some *Colwellia* are selected for in human-built seawater systems.

Diversity as a community lens

While alpha and beta diversities describe fundamental statistical properties of bacterial communities, they do not describe phenomena in the physical world. Bacterial communities exist as ecological networks, and diversity metrics happen to be useful ways of describing those networks. While this dissertation supports the use of abundance-weighted and presence-absence alpha and beta diversity metrics, future studies will likely be able to distinguish microbial communities based on other statistical properties, and analyses will begin, not end, with diversity measurements (Shade, 2017). Co-occurrence networks, which are currently the most feasible models available to contemporary researchers, have been demonstrated to poorly predict species interactions (Barner et al., 2018). However, co-occurrence networks are useful in some contexts; indeed, they are already commonly used (Barberán et al., 2012; Chow et al., 2014). That said, network models employing explicit connections between bacteria, such as physical distance or horizontal gene transfer rates, will likely be more informative than co-occurrence networks. Currently, these models are not currently feasible to build, so for the near future, diversity measures will likely be the descriptors of choice for microbial community networks.

The fleeting relevance of 16S rRNA amplicon sequencing

While the technique central to this dissertation has already contributed to a veritable revolution in the field of microbial ecology, its relevance to the field is already diminishing. As discussed in Chapter 1, choosing to conduct a 16S rRNA amplicon study versus a shotgun metagenomic study is primarily a question of economics. If sequencing and computing prices continue to follow even an approximation of Moore's law, the cost-benefit equation will eventually shift in favor of the more information-rich approach of shotgun metagenome sequencing.

So what will be the historic place of the 16S rRNA amplicon sequencing studies conducted in this dissertation? They will be seen as rough approximations of the taxonomic composition of the communities described. Future researchers might adjust 16S rRNA gene amplicon abundances in their mind as they read the manuscripts, compensating for differential copy numbers of the 16S rRNA amplicons in different taxa. Armed with a better understanding of how 16S rRNA copy numbers vary across taxa, however, the number of copies of the 16S rRNA gene may simply be seen as an alternative abundance metric, in the way that biomass is an alternative abundance metric to counts in macro-organismal ecosystems. Indeed, 16S rRNA copy number has been shown to correlate with a bacterium's ability to rapidly grow after an influx of nutrients (Klappenbach et al., 2000). As better methods to correct 16S rRNA copy number variances and taxonomically classify sequences are introduced, some researchers may wish to reanalyze raw sequence data. For example, the Sequence Read Archive, the National Center for Biotechnology Information's (NCBI) primary sequence repository, already has features in beta that can summarize taxonomic composition of samples. In the

coming years, more rigorous analyses will be possible. 16S rRNA gene amplicon studies will likely be considered as useful to future researchers in examining how beta diversities vary between the variables examined; while this approach is flawed in its estimation of bacterial abundances, conclusions about beta diversity will still be valid because the bias is systemic and should affect all samples in the same way.

Perspectives on marine microbial diversity

The field of microbial ecology is often framed as asking two primary questions: "who is there?" (composition), and "what are they doing?" (function). The research in this dissertation lies entirely in the former category, but as the field of microbial ecology progresses, more researchers will be able combine -omics technologies to answer both of the above questions about a given microbial community. Metaproteomics, while still limited in its throughput, can explicitly measure the function of a microbial community (Bryson et al., 2017; Maron et al., 2007). Furthermore, as the generation and analysis of shotgun metagenomes continues to become more feasible, researchers will gain a clearer picture of how viruses interact and shape marine bacterial communities (Rohwer and Vega Thurber, 2009). Relationships between microbial eukaryotes as predators, nutrient sources, and habitats; and bacteria will be better understood. The progression of the field relies on the combined efforts of technological, analytical, and observational developments: cheaper sequencing and computing, better statistical approaches, and more complete databases will lead to a renaissance in this nascent field of marine microbial community ecology.

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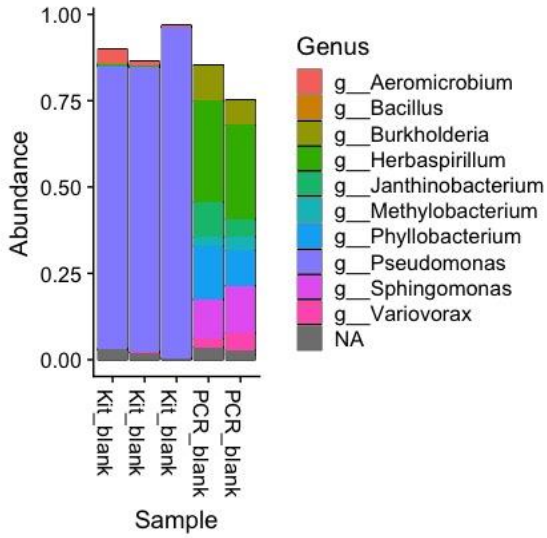
APPENDICES

Appendix A: Supplementary material for Chapter 2



Appendix Figure 1: Composition of OTUs removed from OTU table.

Appendix table 1: Sampling and alpha diversity data

Sample ID	Site	Latitude	Symbiotic State	Sequencing depth	Chao1	Shannon	Faith's PD
CMeHA	Cme	40.26	Brown	16721	152.55	3.44	19.094
CMeHB	Cme	40.26	Brown	9497	183	4.29	21.802
CMeHC	Cme	40.26	Brown	8476	154.6	4.64	20.056
CMeHD	Cme	40.26	Brown	49885	509.16	5.18	31.685
CMeHE	Cme	40.26	Brown	13949	245.85	4.85	25.942
CMeLA	Cme	40.26	Brown	2899	71.25	3.63	12.224
CMeLB	Cme	40.26	Brown	12532	137.77	4.27	14.686
CMeLC	Cme	40.26	Brown	18438	101	3.45	14.185
CMeLD	Cme	40.26	Brown	3795	114.33	3.7	14.825
CMeLE	Cme	40.26	Brown	39357	343.6	4.93	27.383
CMeMA	Cme	40.26	Brown	8628	272.67	4.36	23.062
CMeMB	Cme	40.26	Brown	4939	99.11	4.03	13.177
CMeMC	Cme	40.26	Brown	9578	104.6	4.06	12.664
CMeMD	Cme	40.26	Brown	3157	66.5	3.7	9.263
CBHA	CB	42.83	Brown	11904	182.55	4.14	20.620
CBHB	CB	42.83	Brown	29113	350.35	4.22	27.584
CBHC	CB	42.83	Brown	32530	330.64	4.5	25.404
CBHD	CB	42.83	Brown	14847	213.1	3.76	22.275

Appendix table 1: Sampling and alpha diversity data (Continued)

CBLA	CB	42.83	Brown	27264	299	4.16	25.434
CBHE	CB	42.83	Brown	6030	212.71	4.49	23.061
CBLB	CB	42.83	Brown	43680	222	4.21	24.313
CBLD	CB	42.83	Brown	65170	555.01	5.42	37.212
CBLE	CB	42.83	Brown	9833	305	4.84	27.660
CBMA	CB	42.83	Brown	12769	362.8	4.62	27.675
CBMB	CB	42.83	Brown	4038	149.11	4.25	18.252
CBMC	CB	42.83	Brown	50909	434.56	4.52	30.803
CBMD	CB	42.83	Brown	7327	78.6	3.87	11.943
CBME	CB	42.83	Brown	11299	161.1	4.08	19.351
SHHA	SH	44.15	Brown	15124	269.67	4.65	26.355
SHHB	SH	44.15	Brown	7621	269.14	4.72	26.425
SHHC	SH	44.15	Brown	9384	317.78	4.79	27.371
SHHD	SH	44.15	Brown	13267	278.02	4.57	27.415
SHHE	SH	44.15	Brown	11322	355.89	5.11	31.272
SHLA	SH	44.15	Brown	69999	311.92	3.56	21.316
SHLB	SH	44.15	Brown	12258	230.04	4.47	23.356
SHLC	SH	44.15	Brown	25018	417.57	5.25	33.390
SHLD	SH	44.15	Brown	14599	230.5	4.53	23.741
SHLE	SH	44.15	Brown	35799	377.78	4.82	33.064

Appendix table 1: Sampling and alpha diversity data (Continued)

SHMA	SH	44.15	Brown	43760	439.36	4.6	32.938
SHMB	SH	44.15	Brown	15791	222.32	4.49	22.567
SHMC	SH	44.15	Brown	7574	186	4.43	20.731
SHMD	SH	44.15	Brown	9357	274.02	4.44	26.387
SHME	SH	44.15	Brown	2158	150.25	4.43	17.843
BB2A1	BB2	44.49	Aposymbiotic	4954	203.15	4.09	19.613
BB2A3	BB2	44.49	Aposymbiotic	2424	127.57	3.72	16.511
BB2A4	BB2	44.49	Aposymbiotic	2475	102.5	3.83	14.972
BB2A5	BB2	44.49	Aposymbiotic	14440	103.48	3.15	13.641
BB2A6	BB2	44.49	Aposymbiotic	6489	70.55	2.87	11.091
BB2B1	BB2	44.49	Brown	10142	278.8	4.91	27.880
BB2B13	BB2	44.49	Brown	6277	192.1	4.37	20.587
BB2B15	BB2	44.49	Brown	5452	150.67	4.37	20.336
BB2B3	BB2	44.49	Brown	3131	145.75	4.13	19.887
BB2B7	BB2	44.49	Brown	2825	85	3.34	11.587
BB2G10	BB2	44.49	Green	5375	303.29	4.53	25.509
BB2G11	BB2	44.49	Green	43860	441.44	4.52	27.492
BB2G12	BB2	44.49	Green	46730	232.03	3.15	18.252
BB2G13	BB2	44.49	Green	9532	278.09	4.53	23.790
BB2G15	BB2	44.49	Green	13165	306.08	3.94	23.798

Appendix table 1: Sampling and alpha diversity data (Continued)

BBHA	BB	44.49	Brown	5579	122.11	3.3	14.842
BBHC	BB	44.49	Brown	17394	200.14	3.95	19.511
BBHD	BB	44.49	Brown	29404	530.74	5.23	35.711
BBHE	BB	44.49	Brown	3043	165.55	4.6	18.343
BBLA	BB	44.49	Brown	7678	185.12	4.02	20.598
BBLB	BB	44.49	Brown	62087	319.37	3.55	21.480
BBLC	BB	44.49	Brown	46859	384	4.7	29.261
BBLD	BB	44.49	Brown	42275	425.17	4.2	27.605
BBLE	BB	44.49	Brown	10821	138.33	4.21	16.122
BBMA	BB	44.49	Brown	8922	144.95	4.02	18.844
BBMB	BB	44.49	Brown	24352	209.21	4.12	22.615
BBMC	BB	44.49	Brown	40123	482.32	4.75	32.309
BBMD	BB	44.49	Brown	23393	292.25	4.26	26.708
BBME	BB	44.49	Brown	35717	323.47	4.43	27.188
CMHA	CM	45.29	Brown	3618	71	3.36	10.746
CMHB	CM	45.29	Brown	13413	234.75	4.48	24.990
CMHC	CM	45.29	Brown	29003	248.12	4.38	24.670
CMHD	CM	45.29	Brown	3940	96	3.19	14.797
CMHE	CM	45.29	Brown	15299	256.96	4.54	24.276
CMLA	CM	45.29	Brown	5725	90.43	3.47	12.898

Appendix table 1: Sampling and alpha diversity data (Continued)

CMLB	CM	45.29	Brown	43445	421.8	3.96	27.596
CMLC	CM	45.29	Brown	9530	211.17	4.33	25.284
CMLD	CM	45.29	Brown	3067	132.55	4.06	16.701
CMLE	CM	45.29	Brown	30167	250	4.02	22.608
CMMA	CM	45.29	Brown	8560	100	3.93	14.867
CMMB	CM	45.29	Brown	13288	221	4.21	22.331
CMMC	CM	45.29	Brown	28736	278.93	4.2	25.123
CMMD	CM	45.29	Brown	20104	264.66	3.96	24.649
CMME	CM	45.29	Brown	17335	345.47	4.97	30.243
HSHA	HS	45.93	Brown	5165	102.88	4.15	15.020
HSHB	HS	45.93	Brown	4283	206.04	4.6	22.225
HSHC	HS	45.93	Brown	3663	146.5	4.2	19.473
HSHD	HS	45.93	Brown	5536	199.18	4.38	23.339
HSHE	HS	45.93	Brown	25793	308.21	3.18	24.056
HSLA	HS	45.93	Brown	25472	516.69	4.83	34.485
HSLB	HS	45.93	Brown	6767	128.11	4.44	17.046
HSLC	HS	45.93	Brown	67185	385.73	4.16	24.974
HSLD	HS	45.93	Brown	5464	142.2	3.56	14.635
HSLE	HS	45.93	Brown	6293	266.5	4.87	27.226
HSMA	HS	45.93	Brown	8626	196.9	4.36	20.802

Appendix table 1: Sampling and alpha diversity data (Continued)

HSMB	HS	45.93	Brown	21541	326.23	4.39	26.667
HSMC	HS	45.93	Brown	40751	238.34	3.73	20.052
HSMD	HS	45.93	Brown	20282	344.89	4.79	29.406
HSME	HS	45.93	Brown	18793	364.3	4.21	24.885
TWHA	TW	47.52	Brown	5989	160.18	4.36	19.536
TWLA	TW	47.52	Brown	3327	110.11	3.69	15.658
TWLD	TW	47.52	Brown	10937	212.97	3.98	22.415
TWMA	TW	47.52	Brown	6252	172.26	3.74	19.592
TWMB	TW	47.52	Brown	7248	300.89	4.87	27.797
TWMC	TW	47.52	Brown	3934	114.88	4.27	15.066
TWMD	TW	47.52	Brown	6389	299.04	4.8	26.783
PAHB	PA	48.14	Brown	9006	277.14	4.4	25.565
PAHC	PA	48.14	Brown	14213	322.6	4.89	30.713
PAHD	PA	48.14	Brown	7938	183.21	4.47	21.466
PAHE	PA	48.14	Brown	11573	266.02	4.74	26.181
PALA	PA	48.14	Brown	5704	179.14	3.78	19.532
PALB	PA	48.14	Brown	11010	235.12	4.36	25.570
PALC	PA	48.14	Brown	17555	406.13	4.89	33.115
PALD	PA	48.14	Brown	4234	215.5	4.61	24.611
PALE	PA	48.14	Brown	13711	268.06	4.46	26.233

Appendix table 1: Sampling and alpha diversity data (Continued)

PAMA	PA	48.14	Brown	6263	203.87	4.68	22.529
PAMB	PA	48.14	Brown	6295	198.68	4.35	21.130
PAMC	PA	48.14	Brown	11970	238.43	4.54	24.525
PAMD	PA	48.14	Brown	6288	74	3.34	11.348
PAME	PA	48.14	Brown	5400	56	3.52	9.3066

Appendix table 2: Core orders of the microbiome of A. elegantissima

Order	Prevalence			Abundance quantiles				
	50%	75%	95%	0.000	0.250	0.500	0.750	1.000
o__Thiotrichales	X	X	X	0.000	0.016	0.027	0.045	0.143
o__Alteromonadales	X	X	X	0.000	0.026	0.043	0.082	0.296
o__Verrucomicrobiales	X	X	X	0.000	0.010	0.017	0.032	0.095
o__[Saprospirales]	X	X	X	0.000	0.014	0.025	0.039	0.129
o__Flavobacteriales	X	X	X	0.001	0.096	0.141	0.210	0.386
o__Acidimicrobiales	X	X	X	0.000	0.010	0.017	0.024	0.097
o__Actinomycetales	X	X	X	0.000	0.002	0.008	0.018	0.157
o__Rhizobiales	X	X	X	0.007	0.028	0.044	0.068	0.232
o__Rhodobacterales	X	X	X	0.002	0.048	0.086	0.121	0.405
o__Xanthomonadales	X	X	X	0.000	0.001	0.002	0.004	0.053
o__Burkholderiales	X	X	X	0.000	0.009	0.020	0.045	0.285
o__Rhodocyclales	X	X	X	0.000	0.001	0.003	0.009	0.161
o__Sphingomonadales	X	X	X	0.002	0.010	0.017	0.029	0.148
o__Pseudomonadales	X	X	X	0.000	0.003	0.008	0.018	0.302
o__Chromatiales	X	X		0.000	0.005	0.014	0.027	0.212
o__Enterobacteriales	X	X		0.000	0.000	0.001	0.003	0.043
o__Legionellales	X	X		0.000	0.001	0.004	0.017	0.280
o__[Marinicellales]	X	X		0.000	0.000	0.002	0.006	0.022
o__Myxococcales	X	X		0.000	0.003	0.007	0.012	0.070
o__Chroococcales	X	X		0.000	0.000	0.003	0.015	0.254
o__Pirellulales	X	X		0.000	0.002	0.005	0.010	0.036
o__Desulfuromonadales	X	X		0.000	0.001	0.003	0.010	0.075
o__Spirochaetales	X	X		0.000	0.000	0.002	0.008	0.059
o__Lactobacillales	X	X		0.000	0.001	0.003	0.009	0.133
o__Clostridiales	X	X		0.000	0.001	0.005	0.013	0.069
o__Kiloniellales	X	X		0.000	0.001	0.003	0.008	0.056
o__Sva0725	X	X		0.000	0.000	0.002	0.005	0.038
o__Rhodospirillales	X	X		0.000	0.002	0.010	0.034	0.117
o__Desulfobacterales	X	X		0.000	0.004	0.014	0.029	0.166
o__Bacillales	X	X		0.000	0.002	0.005	0.013	0.097
o__Bacteroidales	X	X		0.000	0.002	0.005	0.013	0.328
o__Campylobacterales	X	X		0.000	0.001	0.004	0.014	0.288
o__Thiohalorhabdales	X	X		0.000	0.003	0.011	0.018	0.193

Appendix table 2: Core orders of the microbiome of A. elegantissima (continued)

o__HTCC2188	X			0.000	0.000	0.001	0.003	0.033
o__Vibrionales	X			0.000	0.005	0.015	0.042	0.250
o__Pasteurellales	X			0.000	0.000	0.000	0.002	0.051
o__Oceanospirillales	X			0.000	0.003	0.008	0.019	0.215
o__Fusobacteriales	X			0.000	0.000	0.001	0.004	0.109
o__Chlamydiales	X			0.000	0.000	0.000	0.001	0.040
o__Bdellovibrionales	X			0.000	0.000	0.002	0.004	0.012
o__Cenarchaeales	X			0.000	0.000	0.001	0.002	0.097
o__Mycoplasmatales	X			0.000	0.000	0.001	0.014	0.208
o__Z20	X			0.000	0.000	0.000	0.002	0.020
o__Oscillatoriales	X			0.000	0.000	0.000	0.002	0.392
o__Pseudanabaenales	X			0.000	0.000	0.001	0.010	0.188
o__Deinococcales	X			0.000	0.000	0.000	0.001	0.061
o__Lentisphaerales	X			0.000	0.000	0.000	0.002	0.016
o__Rickettsiales	X			0.000	0.000	0.001	0.002	0.046
o__Phycisphaerales	X			0.000	0.000	0.000	0.002	0.007
o__Planctomycetales	X			0.000	0.000	0.001	0.003	0.066
o__CL500-15	X			0.000	0.000	0.000	0.001	0.005
o__agg27	X			0.000	0.000	0.000	0.002	0.008
o__Ignavibacteriales	X			0.000	0.000	0.000	0.001	0.010
o__[Rhodothermales]	X			0.000	0.000	0.000	0.002	0.014
o__Cytophagales	X			0.000	0.002	0.005	0.009	0.033
o__Sphingobacteriales	X			0.000	0.000	0.001	0.002	0.011
o__Nitrospirales	X			0.000	0.000	0.001	0.007	0.076
o__DRC31	X			0.000	0.000	0.000	0.001	0.027
o__FS117-23B-02	X			0.000	0.000	0.001	0.007	0.078
o__Kordiimonadales	X			0.000	0.000	0.000	0.003	0.074
o__BD7-3	X			0.000	0.000	0.003	0.006	0.014
o__NB1-j	X			0.000	0.000	0.001	0.004	0.024
o__GMD14H09	X			0.000	0.000	0.000	0.001	0.010
o__Methylophilales	X			0.000	0.000	0.000	0.003	0.012
o__Nitrosomonadales	X			0.000	0.000	0.001	0.006	0.031

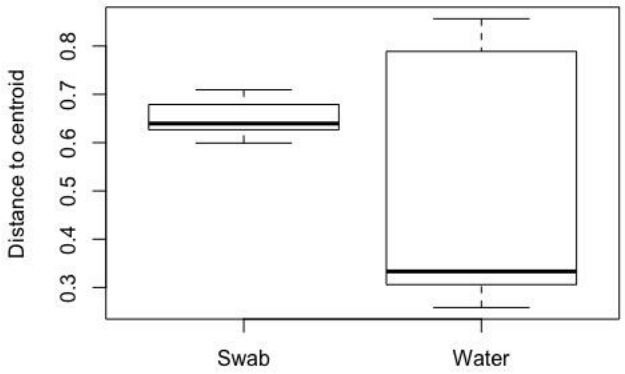
Appendix table 3: Taxa indicators of symbiotic state

Phyla	Indicator of	Phi	FDR q-value
SR1	Green	1.000	0.014
Bacteroidetes	Green	0.806	0.014
Proteobacteria	Green	0.814	0.014
Verrucomicrobia	Green	0.799	0.014
Fusobacteria	Not green	0.728	0.058
Spirochaetes	Symbiotic	0.756	0.058
Firmicutes	Not brown	0.619	0.105
Planctomycetes	Green	0.609	0.105
Classes			
Unassigned SR1	Green	1.000	0.020
Spirochaetes	Not green	0.866	0.020
Flavobacteriia	Green	0.843	0.020
Alphaproteobacteria	Green	0.833	0.020
Verrucomicrobiae	Green	0.799	0.024
Gammaproteobacteria	Green	0.770	0.024
Dehalococcoidetes	Not green	0.866	0.029
Deltaproteobacteria	Green	0.720	0.044
[Saprospirae]	Green	0.692	0.051
Epsilonproteobacteria	Symbiotic	0.756	0.063
Fusobacteriia	Green	0.711	0.063
Bacteroidia	Symbiotic	0.690	0.067
Bacilli	Not brown	0.673	0.067
Acidimicrobiia	Green	0.620	0.112
Planctomycetia	Green	0.580	0.145
Orders			
Alteromonadales	Green	0.842	0.030
Spirochaetales	Not green	0.866	0.030
Flavobacteriales	Green	0.834	0.030
Rhodobacterales	Green	0.843	0.030
Rhizobiales	Green	0.824	0.030
Verrucomicrobiales	Green	0.799	0.042
FS117-23B-02	Not green	0.866	0.050
Thiohalorhabdales	Green	0.795	0.073

Appendix table 3: Taxa indicators of symbiotic state

Kiloniellales	Symbiotic	0.742	0.078
[Saprospirales]	Green	0.692	0.082
Thiotrichales	Green	0.714	0.091
Fusobacteriales	Brown	0.756	0.101
Legionellales	Symbiotic	0.756	0.108
Bacteroidales	Symbiotic	0.690	0.123
[Marinicellales]	Green	0.657	0.139
Methylophilales	Green	0.620	0.181
Acidimicrobiales	Green	0.644	0.181
HTCC2188	Green	0.622	0.183
Chromatiales	Green	0.589	0.183
Lactobacillales	Green	0.707	0.183
Sphingomonadales	Not brown	0.645	0.183
Desulfovibrionales	Green	0.707	0.183
GMD14H09	Green	0.605	0.184

Appendix B: Supplementary figure for Chapter 3



Appendix figure 2: distances to the centroid of biofilm (Swab) and water dissimilarity matrices.