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OYSTER-ASSOCIATED MICROBIAL COMMUNITY DYNAMICS

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BY

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ABSTRACT

Oysters are keystone species in the coastal environment, providing ecological, economic, and cultural services. A significant ecosystem service is their ability to improve water quality by filtration and denitrification. These ecological functions are made possible by oyster-associated microbes, but may also be threatened by microbial pathogens. This positions the oyster as a model system for the study of marine host-associated microbial diversity and function. However, studies of microbial diversity and function in shellfish are lacking, particularly in response to environmental perturbations. As coastal ecosystems change due to anthropogenic impacts and climate variability, it is important to understand how the oyster microbiome is affected and how this may impact the host. The aim of this dissertation is to determine the role of oyster associated microbiomes in response to selected environmental changes (*i.e.* probiotics, eutrophication, and nutrient enrichment).

Chapter 1 is a literature review describing the crosstalk between microbial community structure and function in marine host-associated microbiomes, and the importance of oyster microbiomes. The ecological need for both compositional and functional microbiome data is emphasized.

In Chapter 2, a survey of wild adult oyster gut microbiomes was performed to determine the effects of estuarine acidification and other environmental conditions. Oysters were collected at 5 sites along the north to south trophic gradient in Narragansett Bay, Rhode Island and the bacterial composition and function of their gut samples were analyzed using 16S rRNA amplicon sequencing and metatranscriptomics. Despite high variability in the bacterial community in oyster

samples within each site, we found that gut bacterial communities were selected from the seawater microbiomes and varied throughout the Bay. In addition, the transcriptionally most active taxa (as detected through metatranscriptome analysis) were not the most abundant (as detected by 16S rRNA amplicon sequencing), suggesting plasticity in function as a result of redundancy. These active bacteria showed significantly increased expression of genes in stress response and phosphorus metabolism pathways at the northern, most nutrient-rich and anoxic sites, as compared to the other sites. At the southern sites, characterized by higher dissolved oxygen and lower nutrient levels, the oyster microbiomes showed a significant upregulation of genes involved in nitrogen metabolism. These shifts in microbial community composition and function inform how estuarine conditions may affect host-associated microbiomes. This research also evaluated the potential relationship between the health status of each oyster, evaluated using histology and pathogen-specific qPCR, and oyster gut microbial community composition and function (Appendix A).

The influence of nutrient enrichment on farmed adult oyster microbiomes was investigated in Chapter 3. A field study was performed at two contrasting sites in Point Judith Pond, Rhode Island, where oysters were out-planted for 3 months. Half of the oysters at each site were treated with fertilizer pellets, while the other half were maintained at ambient site conditions. Gut, inner shell, and outer shell biofilm samples were collected and analyzed using both 16S rRNA amplicon and metatranscriptomic sequencing. We detected significant differences in microbial diversity between sample types, site, and treatment (nutrient enrichment). Nutrient enrichment caused significant differential expression of nitrogen metabolism genes, but this response varied

according to oyster sample type and field site. Overall nitrogen fixation and ammonia assimilation were upregulated in gut tissues, while denitrification, nitrogen fixation, and ammonia assimilation were downregulated in the outer shell samples. These results inform how oyster microbiomes perform coupled nitrification-denitrification, and how this might change with increased nutrients. In addition, Appendix B highlights the significant changes between microbial functions performed in each sample type.

In Chapter 4, we characterized bacterial community dynamics in an eastern oyster hatchery during the first 12 days after spawning and how it was affected by treatment with probiotic bacterium *Bacillus pumilus* RI06-95. Larvae, rearing water, and tank biofilm samples were collected from 3 separate probiotic trials and analyzed using 16S rRNA amplicon sequencing to determine the presence and relative abundances of bacteria. The bacterial community structures diverged by trial, sampling timepoint, and sample type, but there was no bulk effect of the probiotic. Instead, the probiotic acted by targeting selected taxa, amplifying *Oceanospirillales* in the rearing water and larvae, decreasing the relative abundance of *Vibrionales*, and increasing *Vibrionales* diversity. These targeted changes likely lead to a net decrease in potentially pathogenic species.

This dissertation emphasizes the significance of oyster-associated microbiomes and their importance to aquaculture disease prevention, wild fishery sustainability, and coastal restoration efficacy. As urbanization, coastal acidification, and disease outbreaks increase, it is important to understand these oyster-associated microbial community dynamics and how they might vary with environmental change.

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PREFACE

This dissertation was written in accordance with the manuscript format guidelines established by the Graduate School of the University of Rhode Island. The dissertation includes the following four manuscripts and a summary chapter:

1. “Microbial-Host Interactions in Coastal Ecosystems” prepared for submission to *Applied and Environmental Microbiology*.

2. “Functional Plasticity in Oyster Gut Microbiomes Along an Estuarine Gradient in Narragansett Bay, Rhode Island” prepared for submission to *The ISME Journal*.

3. “Nutrient Enrichment Affects Mechanisms of Nitrogen Cycling in Oyster-Associated Microbiomes” prepared for submission to *FEMS Microbiology Ecology*.

4. “Bacterial Community Dynamics in an Oyster Hatchery in Response to Probiotic Treatment” published in *Frontiers in Microbiology* in May 2019.

5. In the final summary chapter, the contributions made in this dissertation are explored within the context of each other and current literature.

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By

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Prepared for submission to *Applied and Environmental Microbiology*

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Abstract

Understanding the interplay between microbial community structures and the physiological/ecological functions they encode, is central to the understanding of how host-associated microbes affect host performance and ecosystem function, as well as how they may offset environmental perturbations. In this review, we discuss recent advances on the study of the interplay between community structure and function of host-associated microbiomes within the context of coastal ecosystems, by focusing on selected case studies that showcase the impact of environmental perturbation (*e.g.* seasonal cycles, eutrophication, chemical pollution). We also explore the use of microbe-microbe interactions (i.e. through the use of microbial supplements, such as probiotics) as a mechanism to manipulate microbial community structure and function, and how such changes may ultimately impact host resilience. These examples highlight how both the environment and composition of microbial community structure determine function, underlining the need for more structural and functional data from host-associated microbiome studies. This review ends with a discussion of the importance of host-associated microbiomes in oysters and an outline of this dissertation.

Importance of Host-Associated Microbiomes in Coastal Environments

There is increasing awareness that marine organisms interact with their associated microbiomes, and that these interactions influence many aspects of host and microbial fitness (Bahrndorff et al., 2016; McFall-Ngai et al., 2013). Several reviews have highlighted the importance and ubiquity of host-microbiome interactions across all trophic levels and domains of life, and elucidated their contributions to host fitness, performance, survival, etc. (Parfrey et al., 2018b; Thompson et al., 2017). It is estimated that humans host trillions of microbes in our gut and skin tissues alone and 37% of our ~23,000 genes have bacterial or archaeal homologs (derived from descent or horizontal gene transfer) (Domazet-Lošo and Tautz, 2008; Sender et al., 2016). In marine organisms, a review by O'Brien et al. (2019) highlights the abundance of and variation in microbiome complexity from a single symbiont (bobtail squid) to 3000 associated microbial taxa (sponges).

Host-associated microbiomes are made up of both permanent (symbionts) and transient members that may have a mutualistic, parasitic, or commensal relationship with the host, as determined by their impacts (beneficial, negative, or neutral) on host and microbial fitness (Hammer et al., 2019). Marine host-associated microbiomes perform many beneficial functions to their hosts, including nutrient sharing and cycling (Fiore et al., 2010; Kneip et al., 2007; Yellowlees et al., 2008; Zhang et al., 2015), protection against disease (Egan and Gardiner, 2016; Janssens et al., 2018; Longford et al., 2019; Vonaesch et al., 2018), acclimation to the environment (Carrier and Reitzel, 2018), and host access to their gene pool (Degnan, 2014). On the other

hand, marine microbes are also agents of disease, stress, and decay, especially to susceptible hosts (Groner et al., 2016; Lafferty et al., 2015). Microbial communities are abundant in the ocean and responsible for primary production, consequential nutrient cycling, and degradation of organic matter (Kirchman, 2008; Sogin et al., 2006). Both transient and resident host-associated microbiomes also contribute to ocean ecosystem function, since marine hosts have many key attributes that position them as hotspots of microbial activity in the ocean (Apprill, 2017). These attributes include unique substrates for growth and metabolite sharing, since the microbiome is in close proximity to the host (Beinart, 2019; Nyholm and Graf, 2012).

Coastal environments and their ecosystems are rapidly changing as urbanization, nutrient enrichment, toxin contamination, and other pollutants increase (Baumann et al., 2014; Wallace et al., 2014). These fluctuations have led to changes in benthic functioning (Hale et al., 2016; Linares et al., 2015), microbial diversity and activity (Paerl et al., 2002), algal toxicity (Hattenrath-Lehmann et al., 2015), and declined fisheries production (Haigh et al., 2015), which have widespread implications for coastal ecosystems. Since these changes are likely to increase, it is particularly important to understand the positive and negative interactions between marine hosts and their microbiomes (Melzner et al., 2013; Rheuban et al., 2018). In this review, we discuss how we might evaluate the impacts of environmental perturbation on host-associated microbial community structure and function, with a focus on the effects of nutrient enrichment and the addition of antibiotics or probiotics.

Studying the Impact of Environmental Perturbations on Host-Microbial Interactions: The Interplay between Microbial Community Structure and Function

In this review, we will focus on two axes of variation in host-associated microbiomes – structure and function – to characterize how environmental perturbations may affect microbe-host interactions in coastal ecosystems (Figure 1). Until now, due to the relatively high costs of sequencing and the lack of high-throughput functional assays that can be linked to specific members of the microbial community, few studies of host-associated microbiomes have attempted to dissect the functional responses of their resident microbial communities. A review of how microbiomes influence the ecosystems that surround them, proposed that microbial function is likely linked to a few select, "predictive" taxa that determine community aggregated traits (Hall et al., 2018). This would be particularly appropriate for disease phenotypes, where presence of a pathogen may predict the function and structure of an unbalanced, lower diversity microbiome (Lloyd and Pespeni, 2018; Longford et al., 2019; Vonaesch et al., 2018).

However, environmental microbiomes are notorious for breaking the rules (Hall et al., 2018) and there likely will be exceptions. For example, Graham et al. (2016) performed an extensive survey of the impact of including soil and sediment microbial community structures in global carbon and nitrogen cycling process predictions. They found that environmental conditions were the strongest predictor of metabolic rates, and only 29% of their 82 global datasets were improved with inclusion of microbial community composition data (16S rRNA amplicons). In this scenario, the microbial

function was much more influential than community structure (*i.e.* the same microbes may have been performing different functions or many microbes were performing the same function).

Perhaps the most well-studied marine host-microbe interaction is that of the squid *Euprymna scolopes* and its primary bacterial symbiont *Vibrio fischeri* (Nyholm and McFall-Ngai, 2004). This bacterial symbiont enables the light organ of the squid. A recent study of this system used metagenomics and metatranscriptomics to show that each light organ has a distinct microbial function due to colonization by different environmental microbes (Belcaid et al., 2019). In soils, a study by Rath et al. (2019) used salinity as the "filtering variable" to show how microbial respiration and growth is determined by the environment, and then predict how microbial community structure may change as a result. They determined that microbial respiration and growth depend on salinity and these changes are linked to specific taxa. Using this variable-specific approach in host-associated microbiomes may explain how environmentally-driven function depends on structure, and vice versa.

A system that is resilient to environmental change typically has high microbial diversity, functional redundancy, and high growth rates, which combine to promote functional plasticity (Nemergut et al., 2013; Shade et al., 2012). The key unknown in understanding how perturbations affect host-associated microbiomes is the identification of the factors that drive responses to a particular perturbation in a particular system. This may be driven by changes in microbial function because the same microbes are now performing new functions and/or by changes in community structure that allow for different physiological functions to be expressed (Louca et al.,

2018; Orland et al., 2019). This interplay between microbial community structure and ecological function is significant to the understanding of host-associated microbiomes. If we know these dynamics, then we can use the structure to predict the function of diverse microbial communities and how they may affect their hosts.

The composition and diversity of microbiomes is characterized using a variety of parameters. Microbial diversity is a function of the richness (*how many*) and evenness (*relative abundances*) of taxa in a microbial community. Host-associated microbial community structure is determined by the composition and diversity of microbial community members in the environment in which the host resides, as well as through selection exerted by the host itself (Webster et al., 2010). The host's life history, diet, feeding mechanisms, and development contribute to the selection of a host-specific microbiome (McFall-Ngai et al., 2013; Parfrey et al., 2018a). Currently, the most common method to determine microbial community structures is through the sequencing of a marker gene, particularly with the 16S rRNA subunit for bacteria (Zoetendal et al., 1998), the 18S rRNA subunit for eukaryotes (Moon-Van Der Staay et al., 2001), and internal transcribed spacer (ITS) region for fungi (Schoch et al., 2012). Increased affordability of high-throughput sequencing technologies have led to a deluge of new microbial community structure studies that characterize which microbes are present and their relative abundances (Pollock et al., 2018). However, marker gene (DNA) approaches cannot provide evidence of changes in microbial function.

Host-associated microbial functions are thus determined by the prevailing conditions within a given host compartment and by the type of microbes that occupy

these different niches within the host. The genomes of the microbial population (in other words, what genes are present in the microbiome) determine the potential for physiological and ecological function. Gene expression and protein and metabolite measurements can be used to characterize the microbial acclimation response to environmental change, and how their outcomes determine host phenotypes like disease state, percent survival, growth rates etc. (Dantas et al., 2013; Louca et al., 2018). Gene expression can be determined using a variety of methods, including qPCR, RNA microarrays, and metatranscriptomics. When a study is targeting a limited number of genes to explain a process, then qPCR and RNA microarrays are used. However, these gene expression-based methods are limited to known functional genes and databases.

Metatranscriptomic, metaproteomics, and metabolomic methods are used to characterize all coding mRNA, proteins or metabolites in a sample to determine total microbial activity (Gifford et al., 2014). Unfortunately, a major barrier to determining the function of microbial communities using metatranscriptomics, metaproteomics, or metabolomics is the lack of annotated protein databases. As of April 2019, experimentally confirmed protein functions made up less than 1% of the UniProt database (Bateman et al., 2017). The other 99% of the protein functions are either not characterized or, at best, their function is predicted for orthologs. Annotation of *Mycoplasma mycoides*, the bacterium with the smallest known genome, predicted only 324 protein from a total of 473 genes (68%), highlighting the need for improved protein annotation techniques (Antczak et al., 2019).

Based on the premise that an environmental perturbation affects the structure and function of a host-associated microbiome, then the structure and/or function will

change accordingly, leading to acclimation that seeks to offset the effects of perturbation (Louca et al., 2018; Robinson et al., 2010). The relative importance of the structure or the function depends on the perturbation, the environment, and the host. This model will be explained using 3 examples: nutrient enrichment and the addition of probiotics or antibiotics (Figure 2).

Impacts of Environmental Perturbation on Host-Microbial Interactions in Marine Species: Eutrophication

Marine organisms in coastal and estuarine environments are subjected to high levels of macronutrients (nitrogen, phosphorus) due to organic pollution and runoff (Nixon, 1995; Wallace et al., 2014). This nutrient enrichment phenomenon is becoming more prevalent due to increased urbanization and has a significant impact on trophic structures, particularly at the base of the food web (Meyer-Reil and Köster, 2000). When nutrient enrichment occurs, the microbial function changes to compensate for the influx of nutrients (Bricker et al., 2008; Fowler et al., 2013). This leads to changes in the microbial composition since the microbes are interacting and competing for nutrients. An extreme result of coastal eutrophication is an algal bloom: when excess nutrients stimulate algal growth, leading to reduced oxygen and therefore changes in overall ecosystem function and microbial community composition (Hudnell, 2008; Landsberg, 2002; Paerl et al., 2003). Studies of the microbial community dynamics during an algal bloom show that the competition for nutrients and anoxia lead to a different microbial composition in seawater after the bloom (Hattenrath-Lehmann and Gobler, 2017; Shin et al., 2018; Zheng et al., 2018).

Unfortunately, studies on the effects of nutrient enrichment on marine host-associated microbiomes are limited and there is no general consensus of its effects. Nutrient enrichment promoted the symbiosis between marine hydroid *Myrionema amboinese* and its dinoflagellate symbionts as a result of increased food supply to the host (Fitt and Cook, 2001). In the reef-building coral *Porites asteroides*, nutrient enrichment caused changes in symbiont function due to changes in prey abundance (Welsh et al., 2016). Microbial diversity of the staghorn coral decreased with nutrient enrichment and microbial communities were dominated by a *Rickettsia*-like organism (Shaver et al., 2017). A study of the effects of eutrophication on kelp microbiomes due to coastal urbanization uncovered differences in microbial community structure, but did not determine changes in microbial function (Marzinelli et al., 2018). Future studies should characterize how both the microbial community composition and function are affected by nutrient enrichment to fully understand the impact of increased eutrophication.

Impacts of Environmental Perturbation on Host-Microbial Interactions in Marine Species: Antibiotic Treatment

Antibiotics are substances used to kill bacteria or inhibit bacterial growth (Fajardo and Martínez, 2008); they have been used extensively for human therapy, agricultural, and farming applications, leading to high levels of antibiotic pollution in the environment (Knapp et al., 2008). As the use of antibiotics increases and their efficacy decreases, antimicrobial resistance is gaining awareness as a global health crisis (Ventola, 2015). As antimicrobial resistance grows, it is important to study their

effects on host-associated microbiomes, particularly in coastal environments (Stepanauskas et al., 2006). When antibiotics are added to a system, the microbial composition changes as targeted susceptible microbes are eradicated, leading to microbial community structure changes (Eckert et al., 2019; Nogales et al., 2011). This ultimately alters the function of the community after antibiotic treatment, since there will be a new microbial community structure and different microbes present after colonization of open niches by existing or new species.

The overall effect of antibiotics on host microbial community structures and corresponding effects on the host physiology varies considerably between studies. Treatment with prophylactic antibiotics caused increased mortality after bacterial challenge in black molly fish, but no detectable effect on the microbial diversity (Schmidt et al., 2017). Antibiotic treatment with *Streptomycin* in zebrafish larvae or oxytetracycline in juvenile Atlantic salmon caused significant decreases in alpha-diversity and increased larval mortality (Navarrete et al., 2008; Pindling et al., 2018). In adult Atlantic salmon intestinal samples treated with florfenicol and oxolinic acid, changes in beta-diversity and key taxa were observed (Gupta et al., 2019). Measurements of succession in seaweed microbiomes after antibiotic disturbance showed that functions and interactions within a microbiome were restored, leading to host protection (Longford et al., 2019). Despite the variability between studies, these observations in marine hosts confirm that antibiotics lead to microbial community composition (and likely function) changes that have impacts on the hosts. A better understanding of these compositional and functional changes in the microbial

community of the host would aid in managing the potential negative impacts of antibiotics on hosts.

Exploiting Microbe-Microbe Interactions to Increase Host Resilience: Probiotics

Probiotics have been used to improve host health and protection against disease in human, aquaculture, and agricultural settings (Chauhan and Singh, 2018; Pandey et al., 2015; Pérez-Sánchez et al., 2018). Probiotics are beneficial microorganisms that protect the host by pathogen interference, immunomodulation, or improvement of barrier function in the resident host microbiota (Sánchez et al., 2017). In humans, the benefits of probiotics have been widely studied, and shown to increase hormone regulation, alter cell proliferation, promote vitamin absorption, and promote immune cells (Reid et al., 2019). These impacts are modulated by the host microbiome: probiotics alter the microbial community composition to promote host health (Sánchez et al., 2017).

In aquatic systems (marine and freshwater), probiotics have been shown to exert their effects through a variety of mechanisms of action, including competition for nutrients, improvement of water quality, pathogen inhibition, secretion of antimicrobials, and immunomodulation (Kesarcodi-Watson et al., 2012; Prado et al., 2010). Many *in vitro* studies suggest that probiotics inhibit or decrease pathogens in the system, leading to a reduced chance of disease outbreak (Sohn et al., 2016). Little is known, however, about how probiotics affect the function of the microbial communities in the culture systems in which they are used.

The impact of probiotics on marine microbiomes has been studied in a variety of marine hosts to determine shifts in microbial community structure, often with emphasis on specific taxa. A study of the impact of *Phaeobacter inhibens* treatment on the microalga *Emiliana huxleyi* and the European flat oyster *Ostrea edulis* found significant changes in the relative abundance of *Vibrios* and *Pseudoalteromonadales*, with the most substantial impact observed in the oyster samples (Dittmann et al., 2019). Probiotic treatment of juvenile Kumamoto oysters with *Streptomyces* N7 and NL8 resulted in increased species diversity and changes in *Bacteriovorax* and *Vibrio* taxa (García Bernal et al., 2017). A study of the effect of probiotic *Bacillus pumilus* RI06-95 on larval eastern oysters established that probiotics did not affect overall diversity, but affected key members of the microbiome to increase larval protection (Stevick et al., 2019, Chapter 2 of this dissertation). In shrimp, treatment with a probiotic mixture caused significant shifts in beta-diversity and the detection of 12 new species in one study (Vargas-Albores et al., 2017), but this finding could not be confirmed in a parallel study (Huerta-Rábago et al., 2019). A multi-species probiotic applied in tilapia aquaculture caused decreased microbial diversity in the digestive gland (Merrifield and Carnevali, 2014). Altogether, these studies indicate that probiotic effects on microbial community structure are highly dependent on the host species, the probiotic, and the 16S rRNA amplicon analysis methods used. Notwithstanding detection of changes in microbial community structure, little is known about the effects of probiotics on microbial function, and how those changes in microbial community structure and function may protect and improve the health of the host. Future studies should address these questions, as well as the potential impact of

variability observed in marine host microbiomes on probiotic activity, using high-resolution functional surveys.

Conclusions and Perspectives: Factors to be Considered in Future Studies of Host-Microbial Associations

Previous studies of host responses to environmental perturbations show high variability and differential responses that may be explained by aspects of the system (i.e. host variability, tissue type, ambient environmental conditions) other than microbial community structure and function. The role of host genotype, genetic potential, and the resulting phenotype and physiology is likely an important determinant of interactions within a microbiome. Many studies have alluded to the relationship between host genetics and associated microbiomes (Dishaw et al., 2014; Morrissey et al., 2019), but these interactions have not been characterized in marine systems. However, it is known that host genetics and lifestyle play a role in vertical microbiome transmission between generations for some species, especially in marine broadcast spawning organisms, such as corals (Gundel et al., 2011; Sharp et al., 2007). Additionally, the host is constantly placing pressure on its microbial community through immunomodulation and metabolism (Muñoz et al., 2019; Nyholm and Graf, 2012; Pindling et al., 2018). This may lead to variation in microbiomes between hosts in a community, and differences in host success.

In addition to host genetics, tissue function and physiology may also play a key role in determining the structure and function of its associated microbiome. Each compartment within an organism provides a distinct microbial niche that promotes a

certain microbiome, depending on its anatomy, physiology, and isolation. These are sub-environments that are significantly different than the host's ambient environment. For example, different tissues give off different metabolites, maintain different acidity and pH, and have variable oxygen content (Belcaid et al., 2019; Lynch et al., 2019).

In order to understand the importance and role of host-associated microbiomes, it is essential to determine both the structure *and* function of the microorganisms. Increased functional studies using a combination of approaches able to measure microbial community function (*e.g.* qPCR, metatranscriptomics, metaproteomics, metabolomics) and relate it to changes in host function (*e.g.* growth, survival, health status, host transcriptomics) in the context of controlled environmental perturbations are necessary to supplement microbial surveys and provide context for changes in community structures and their potential impacts on their hosts.

Oysters as a Model System in the Study of the Impact of Environmental Perturbation on Host-Microbial Associations

Oysters are keystone species in marine ecosystems, providing economic growth in terms of seafood harvest and ecological services (Barbier et al., 2011; Grabowski et al., 2012; Wijsman et al., 2018). A 2019 survey by the Food and Agriculture Organization of the United Nations (FAO) valued global wild oyster fishery exports at $148 million USD and aquaculture at $6.8 trillion USD (FAO, 2019). This value has increased exponentially in the past decade due to continually increasing seafood demands (FAO, 2018). In Rhode Island, USA, total aquaculture products were valued at $6.09 million USD in 2018, and oysters were the largest aquaculture export, with a

total sale of 8,515,950 specimens (Beutel, 2018). Besides the commercial value of the shellfish, the Rhode Island oyster shellfishery comprises an industry that adds historical and cultural value (Schumann, 2015).

Wild and restored oyster reefs provide a multitude of ecosystem services including water filtration, erosion control, and habitat provision (Grabowski et al., 2012; Kellogg et al., 2014; La Peyre et al., 2014; Rodriguez et al., 2014). A one-acre oyster reef can clean the equivalent of up to 36 Olympic swimming pools per day, clearing out excess nutrients, chlorophyll-*a*, toxic compounds, metals, and particles in the coastal environment (Coen et al., 2007). Oysters are also hosts to nitrogen metabolizing bacteria, which remove environmental nitrogen in both wild and restored populations (Humphries et al., 2016). Oyster reefs can provide a physical barrier to stabilize shorelines against storm surge or erosion (Meyer et al., 1997). Finally, oyster reefs provide habitat for fish, other benthic invertebrates, and epibenthic fauna. The value of these ecosystem services has been estimated to range from $5,500 to $99,000 per hectare of oyster reef per year (Grabowski et al., 2012).

Oysters have long been used as a model system for studying host-microbial interactions (King et al., 2019; Le Roux et al., 2016; Pierce and Ward, 2018; Robledo et al., 2018). Oysters are commonly found in coastal waters around the globe and, due to their commercial value, their genetics have been studied extensively (Gómez-Chiarri et al., 2015; Guo et al., 2008). As filter feeders that ingest large volumes of water containing phytoplankton and microbes from the water, oysters are especially susceptible to pathogens and thus to changes in their overlying bacterial communities (Burge et al., 2016). This includes protozoan, bacterial, and viral diseases that all have

significant economic and ecological impacts (Burge et al., 2016; Lafferty et al., 2015). Many studies have investigated the specific mechanisms of infection (especially *Vibrio* spp, *Perkinsus* spp., and *Alliroseovarius crassostreae*) in oysters using targeted functional assays (Zannella et al., 2017). However, current knowledge of oyster-associated microbiomes is largely limited to purely descriptive studies during ambient conditions in adult oysters. This knowledge is summarized below.

Microbial Community Structure in Oysters

There has been a surge in oyster microbiome projects over the past decade, likely due to increased accessibility and affordability of high-throughput sequencing technologies (Pollock et al., 2018). Many recent studies have used 16S rRNA amplicon sequencing to characterize the presence and relative abundances of bacteria in oyster tissue(s) in a variety of conditions. Oyster microbiomes differ by location, season, and overall environmental conditions (Khan et al., 2018; King et al., 2012; Lokmer et al., 2016b; Pierce et al., 2016; Wendling et al., 2014). Oyster microbial communities also vary with tissue type and developmental stage (Dubé et al., 2019; Green and Barnes, 2010; Hernández-Zárate and Olmos-Soto, 2006; Lokmer et al., 2016b; Trabal Fernández et al., 2014).

Despite overall variability in community structure, there are abundant bacterial taxa shared across all oyster microbiome studies, particularly in the phyla *Proteobacteria* (mainly *Vibrionales*, *Oceanospirillales*), *Bacteroidetes*, and *Tenericutes* (Pierce and Ward, 2018). Not surprisingly, these are bacteria that are commonly found in the marine environment (Fuhrman et al., 2006; Logares et al.,

2014). An abundance of *Cyanobacteria* is often observed in oyster stomach and gut samples from shallow waters, likely due to a combination of 16S amplicon region bias, transient algal food consumed by the oyster, and environmental conditions (Trabal Fernández et al., 2014). The oyster microbiome structure is, however, distinct from microbial communities in the seawater and sediment, implying oyster hosts select for bacteria that have the ability to survive within their tissues (Lokmer and Wegner, 2015; Pujalte et al., 1999; Stevick et al., 2019; Thomas et al., 2014; Vezzulli et al., 2018); as determined by host-microbe interactions described in sections above. Moreover, oyster tissue samples collected from the same site have diverse microbiomes, despite their identical environments. Factors influencing variability in oyster microbiomes within a site include differences in an individual oyster's filter feeding behavior and subsequent ingestion or rejection of food as pseudofeces (host selection) (Kramer et al., 2016; Ward and Shumway, 2004); the presence of tissue microenvironments within oysters (King et al., 2012; Trabal Fernández et al., 2014); and host genetics and physiological status (Lokmer et al., 2016a; Wegner et al., 2013).

The crosstalk between the host, its environment, and its microbiota is largely unexplored in oysters. For example, in an aquaculture setting, four microbiome studies of *Crassostrea gigas* larvae found that, even though the microbial community in the rearing water changes throughout the year, there is little effect of UV treatment, algae, and other rearing conditions (Asmani et al., 2016; Laroche et al., 2018; Powell et al., 2013; Trabal Fernández et al., 2014). A study that tested the effect of algal feed on larval Pacific oyster fecal microbiomes found that they change as a function of algal feed and its associated microbes (Simons et al., 2018). A recent study compared the

microbiomes of the extrapallial fluid from *Crassostrea gigas* and *Ostrea lurida* and found no effect of chalky deposits (Banker and Vermeij, 2018). There is a need for more information on the biotic and abiotic drivers influencing microbial community structure in oysters, and their relative contribution to determining that structure (*e.g*. host health, nutrient load, probiotics). Moreover, studies of direct environmental manipulation to determine oyster microbiome responses to nutrients, oxygen levels, disease, and other factors are lacking.

Another relatively unexplored area of oyster-associated microbiome research is the impact of parasitic infection and disease (King et al., 2019). Many studies have focused on *Vibrio* diversity, since it is a taxon of commonly studied bivalve pathogens (Le Roux et al., 2016; Lemire et al., 2015; Lokmer and Wegner, 2015; Preheim et al., 2011; Wendling et al., 2014). However, few studies have considered microbial community-wide responses to disease in oysters. A study of Sydney rock oysters infected with the protozoan parasite *Marteilia sydneyi* discovered disease-induced shifts in diversity and changes in abundant Operational Taxonomic Units (OTUs) relative to non-infected specimens (Green and Barnes, 2010). Changes in the *Vibrio* taxa and relative abundances were observed in oysters diseased with "summer mortality" or OsHV-1 (King et al., 2018; Pathirana et al., 2019). Given that these infections and diseases are environmentally-driven, it is difficult to determine if the change in oyster microbial community structure is due to the environment or the disease (King et al., 2019). Controlled experiments are necessary to tease apart the relationship between the oyster microbial community structure and disease, independent of environmental conditions. For example, it would be advantageous to

study the effects of diseases like Dermo (caused by the protozoan parasite *Perkinsus marinus*) or Juvenile Oyster Disease (caused by the bacterial pathogen *Alliiroseovarius crassostreae)* on eastern oyster associated microbial composition and function through challenges experiments performed in an environmentally controlled system.

Microbial Function in Oysters

The function of targeted oyster-associated microbes has been broadly studied, particularly in the context of economically and ecologically relevant issues such as infectious disease and ecosystem function (*e.g.* nitrogen metabolism). Microbial function in oysters is an outcome of the environment, host biology (*e.g.* genetics, physiology, immunity), and overall microbial diversity (Bachère et al., 2015; Bruto et al., 2017; Le Roux et al., 2016; Lozupone, 2018). For example, the pathogenicity of *Vibrio* spp. in oysters changes depending on the resident population of *Vibrios* in oysters and the oysters' ability to fight off the infection (Bruto et al., 2017; Lemire et al., 2015). Studies of nitrogen gas production in live oysters versus naked shells found differences in denitrification and nitrification rates, suggesting that microbial nitrogen metabolism differs between the gut microbiota and outer shell biofilms (Caffrey et al., 2016; Ray et al., 2019).

There have been, however, few studies of the oyster-associated overall microbiome function using high-throughput sequencing technologies. Recent studies have used qPCR and functional inference from 16S rRNA sequencing to estimate the genetic potential of the oyster gut, shell, and/or sediment bacteria (Arfken et al., 2017;

Dubé et al., 2019; Murphy et al., 2019). These studies found that the functional diversity of the oyster microbiome is tissue-specific and the predicted function changes between bivalves as well as field locales. However, these methods are based on orthologous functional genes among phylogenetically related species and ignore genome-level variation within a species or genus. The majority of functional traits are not monophyletic, and there can be high levels of variation in protein-coding genes within a species (Aguilar et al., 2004; Martiny et al., 2013, 2015). To our knowledge, no studies have so far determined potential functional roles of oyster microbiomes using shotgun metagenomics, RNA-based microarrays, or shotgun metatranscriptome methods.

Goals of this Dissertation

Despite an abundance of historic and emerging literature on the oyster microbiome, there are many unanswered questions. These questions touch on many aspects of the microbiome, particularly spatial and temporal dynamics, evolutionary and phylogenetic traits, and environmental impacts. For example, how does the oyster microbiome structure and function change within an oyster, within a field site, between sites in an estuary, between estuaries, or on a global scale? What effect do host, population, species, or genus genetics have on the selection of an oyster's microbiome? Integrating descriptive and functional microbiome analyses will help in addressing these questions, and improve the understanding of marine microbial-host interactions.

The overall goal of this dissertation is to characterize microbiomes of farmed and wild specimens of the eastern oyster (*Crassostrea virginica*) in Narragansett Bay, Rhode Island and determine how they change with environmental perturbation (i.e. eutrophication and probiotics). Changes in microbial community structure and function will be measured to assess how these perturbations impact the microbial community function (Figure 2). This analysis will allow for the prediction of changes in ecosystem function and host health, characterization of microbiomes in climate change scenarios, and a greater understanding of host-associated microbial ecology in marine systems.

Chapters 2 and 3 address environmental impacts on the microbiome of adult eastern oysters through evaluation of: a) the influence of the estuarine acidification gradient in Narragansett Bay, Rhode Island on the composition of gut microbiomes in oysters and oyster health, and b) the effect of nutrient enrichment (eutrophication) on oyster-associated microbial communities within the context of nitrogen cycling. A combination of metatranscriptomics, 16S rRNA amplicon sequencing, histology, and qPCR methods were used to address these goals. In Chapter 4, we explored the mechanisms of action of probiotic *Bacillus pumilus* RI06-95 to protect larval eastern oysters through associated microbial activity in the hatchery setting. An extensive 16S rRNA amplicon study was performed using control and treated larvae, tank biofilm, and rearing water samples collected from at least 2 timepoints during 3 separate hatchery probiotic trials.

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Figures

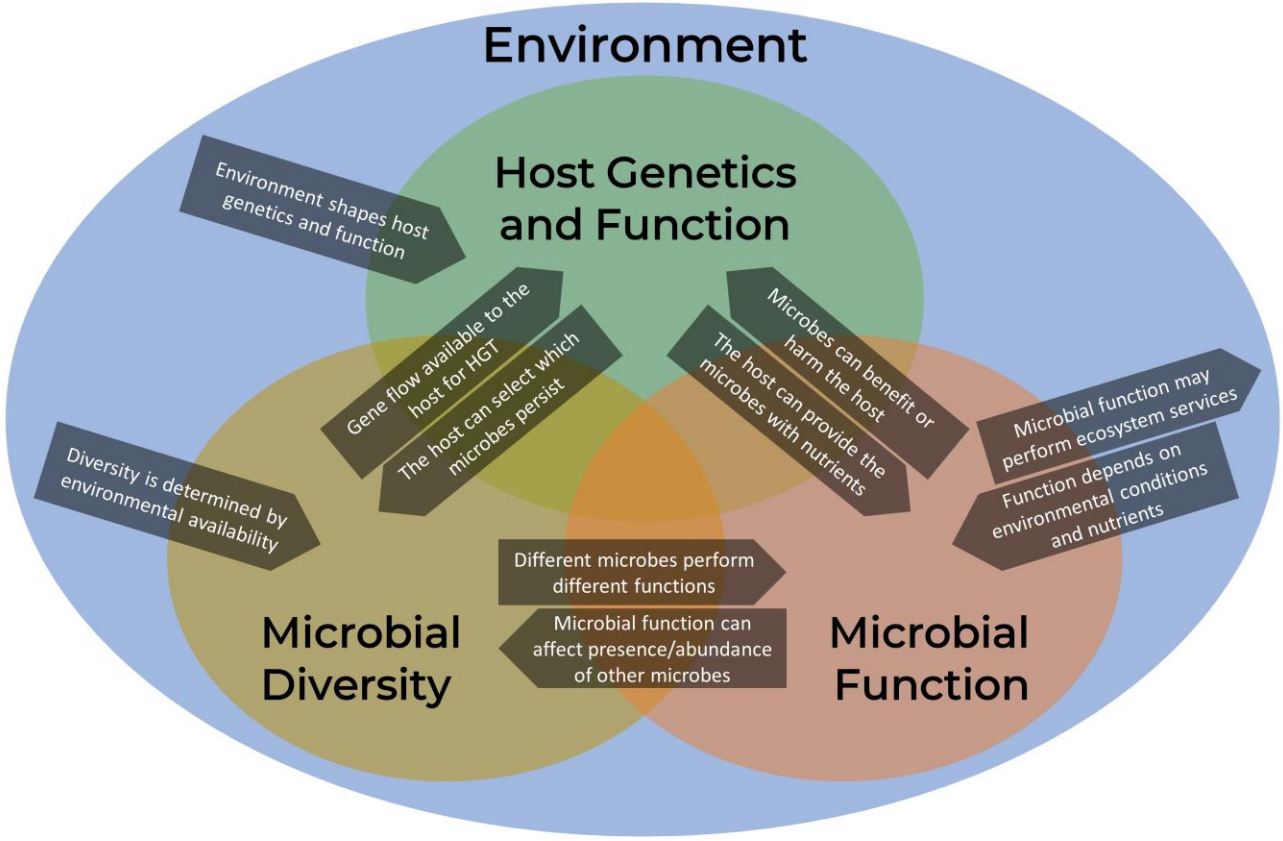

Figure I-1. Examples of interactions between the environment, host, and microbial diversity or function.

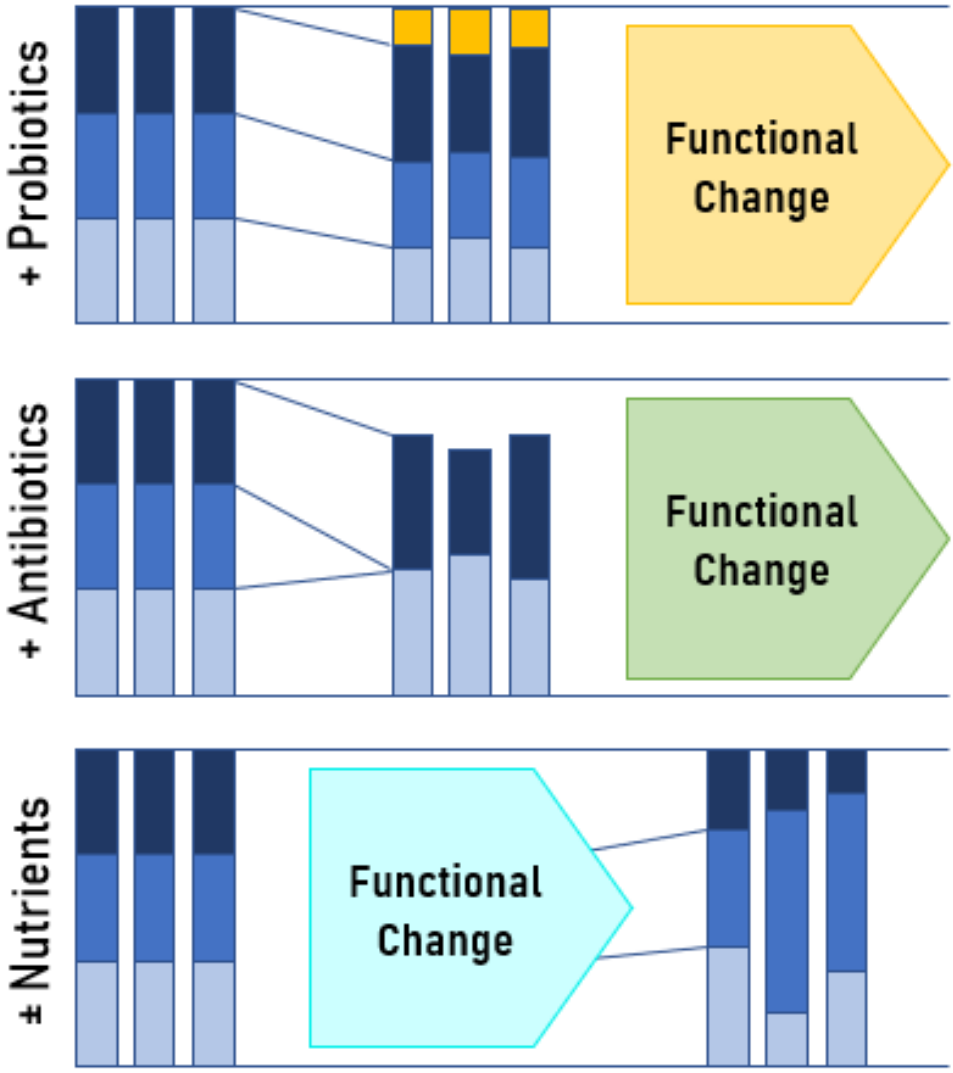

Figure I-2. Working model of how perturbations affect microbial community composition and function.
The hypothesized effects of antibiotics, probiotics, and nutrients are shown. A functional change is denoted with an arrow, and a change in structure is denoted by changes in the percent abundance bar plots.

CHAPTER II: Functional Plasticity in Oyster Gut Microbiomes Along an Estuarine Gradient in Narragansett Bay, Rhode Island

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Abstract

Oysters in coastal and estuarine environments are subject to fluctuating environmental conditions, including nutrient loading, runoff, pollution, and anoxia, that may impact their health and the ecosystem services they provide. We propose that these variations in environmental conditions cause changes in their associated microbial community structure and function. Adult wild oyster gut and seawater samples were collected at 5 sites along an estuarine nutrient gradient in Narragansett Bay, Rhode Island, USA in August 2017. Samples were analyzed by 16S rRNA sequencing of the V6 region to characterize bacterial community structures and metatranscriptomes were sequenced to determine oyster gut microbial function. A North to South estuarine gradient was observed, with increasing salinity, pH, and dissolved oxygen, and decreasing nitrate, nitrite and phosphate concentrations. There were significant differences in bacterial composition between the oyster gut and water samples (ASV level, Bray-Curtis k=2), suggesting niche selection of certain taxa by the oyster host. The community structure of the most transcriptionally active bacterial taxa was similar at each site, but expression of genes involved in nutrient utilization varied throughout the Bay, based on nutrient availability, pH, and dissolved oxygen level at each site. At the northern sites, characterized by higher nutrients and anoxia, the oyster gut microbial community showed significant upregulation of genes associated with stress response and phosphorus metabolism. This response was opposite to the southern sites, where the oyster gut microbiomes showed upregulation in genes associated with nitrogen metabolism and downregulation of stress response genes. The most transcriptionally active bacteria in oyster gut samples were not the

most abundant, suggesting plasticity due to functional redundancy. Microbial gene expression varied according to the eutrophication gradient, providing insight into how environmental conditions shape host-associated microbial functions.

Introduction

Coastal ecosystems, and estuaries in particular, serve as habitat for highly diverse communities that contribute up to 77% of worldwide ecosystem services (Costanza et al., 1997; Martínez et al., 2007). Humans directly rely on these environments for ecosystem functions and biodiversity but also for human activities like tourism and fisheries (Bulleri and Chapman, 2010; Firth et al., 2016; Liquete et al., 2013). Environmental conditions in estuarine ecosystems fluctuate rapidly due to changes in nutrient loading, river runoff, and other physical, chemical, and biological factors (Nixon, 1995; Sunda and Cai, 2012; Waldbusser and Salisbury, 2014). For example, pH values in coastal waters can vary by as much as one pH unit over daily and seasonal cycles, reflecting changes in biological inputs, microbial activity, ambient dissolved oxygen, and pCO_2 (Alexandre et al., 2012; Baumann et al., 2014). These frequent changes in estuarine water chemistry (known as estuarine acidification) are also affected by human activity and coastal geomorphology, and these influences will likely increase over the coming decades (Wallace et al., 2014). In particular, increased coastal urbanization has disrupted natural shorelines and concentrated sewage effluent and nutrient inputs near estuaries (Duarte et al., 2008; McKinney, 2006). This increased pollution may lead to surges in algal blooms and invasive species, and decreased ecosystem and human health (Duh et al., 2008; Firth et al., 2016).

Estuaries such as Narragansett Bay, Rhode Island, USA, provide a natural gradient to study the impacts of eutrophication and coastal acidification. The head of the Bay, located in a highly urbanized area, is highly eutrophic while trophic levels at the mouth are more similar to those found over the continental shelf (Calabretta and

Oviatt, 2008; Oczkowski et al., 2008). Previous studies have shown eutrophication in Narragansett Bay affects many physical and biological systems, including nitrification rates (Berounsky and Nixon, 2006), primary productivity (Oviatt, 2008), animal physiology (e.g. Widdows et al., 1981), and benthic biodiversity (Hale et al., 2016; Pelletier et al., 2017). Over the last 20 years, Narragansett Bay has undergone dramatic changes as a result of targeted efforts in reducing nutrient impacts and improving overall water quality. This has led to changes in the ecosystem functioning and position Narragansett Bay as an model estuary for the study of eutrophication (Oviatt et al., 2017).

Marine microbial communities play a central role in ecosystem function by forming a base for primary production and nutrient cycling. Microbial communities in coastal seawater and sediment exhibit plastic responses to environmental changes or gradients (Highton et al., 2016; Meyer and Riebesell, 2015; Nogales et al., 2011; Paerl et al., 2002). This may lead to changes in primary productivity, and therefore coastal ecosystem functioning (Paerl et al., 2003). Studies of bacterial community structures and nitrogen cycling in several coastal lagoons found that physical gradients and nutrients affect sediment microbial interactions and function (Highton et al., 2016; Kieft et al., 2018). In marine sediments, studies have detected no significant difference in microbial community structure when exposed to high nutrients, but reported dramatic changes in ecological function (Bowen et al., 2011; Bulseco et al., 2019; Chen et al., 2019).

Host-associated microbiomes are gaining importance as major contributors to ecosystem services and host functioning (Beinart, 2019; Fiore et al., 2010; Harris,

1993; Nyholm and Graf, 2012). However, little is known about how fluctuating environmental conditions affect the composition and function of microbes associated with filter feeders like oysters, or how changes in their host-associated microbiomes change host physiology or ecosystem function (Apprill, 2020; Moulton et al., 2016; Pfister et al., 2014). Various studies have found that environmental conditions affect microbial community structures in marine hosts, including corals (Shaver et al., 2017), sponges (Cleary et al., 2019), eelgrass (Lin et al., 1996), seagrass (Crump et al., 2018), oysters (Pierce et al., 2016) and mussels (Li et al., 2019). Varying pollution levels change the microbial community structures and susceptibility to chemicals in Manila clams (Milan et al., 2018). A study of the effects of urban pollution on kelp showed changes in microbial community structures (Marzinelli et al., 2018). Studies that examine host-associated microbial functional responses to environmental change are very limited and focus on model organisms (i.e. corals or zebrafish) in lab-based studies (Rocca et al., 2018).

In Narragansett Bay, as in other coastal estuaries, oysters are an integral part of the local history, culture, and fishing industries. In addition to making up a large percentage of annual seafood consumption, oysters, as keystone species, provide many ecosystem functions, including clearing of overlying waters, coastal erosion prevention, and nutrient cycling (Grabowski et al., 2012). Oyster-associated microbiomes are responsible for many of the ecosystem services provided by oysters. As oysters filter out bacteria, plankton, algae, and other microorganisms from the water, they retain and provide a habitat for specific bacteria that perform denitrification and assimilate excess phosphorus (Caffrey et al., 2016; Kellogg et al.,

2014). Microbes may also aid in maintaining oyster health and homeostasis by controlling infection, performing nutrient removal, or providing metabolites (Lokmer and Wegner, 2015; Trabal Fernández et al., 2014; Wegner et al., 2013). However, oysters are also susceptible to accumulating bacterial and protozoan pathogens that impact host and human health (King et al., 2019a; Lafferty et al., 2015; Romalde and Barja, 2010).

Previous studies of microbial ecology in oysters have been limited to surveys of microbial community structures in different compartments of the oyster. The makeup of the microbiome in adult oysters, as determined by 16S rRNA amplicon sequencing or other genetic markers, varies with location, season, tissue type, disease status, and environmental conditions (King et al., 2019a; Pierce and Ward, 2018). Some studies have attempted to infer oyster-associated microbial function from 16S rRNA amplicon sequencing (Arfken et al., 2017; Murphy et al., 2019). However, the effects of eutrophication on the response of the oyster microbiome using RNA-based methods have not been reported so far. *In situ* studies of combined environmental effects on host-associated microbiomes are needed to accurately predict ecosystem functions and how they might change in the future.

In this study, we evaluated the oyster gut microbiome structure and functional response using 16S rRNA amplicon sequencing and metatranscriptomics at 5 sites along the trophic gradient in Narragansett Bay. This survey provides a snapshot of the oyster microbiomes in a relatively small geographic area in a temperate coastal estuary affected by eutrophication, and how these host-associated microbiomes are affected by their local environment. We hypothesized that the environmental gradient in

Narragansett Bay will cause differential responses in oyster gut microbial community structure, stress response, and nutrient cycling. The results of this study inform how environmental stressors may affect ecosystem services provided by oysters and understanding of host-associated microbial function.

Methods

Sample Collection

Five sites were selected along the Western coastline of Narragansett Bay: 1.PVD (Providence River: Bold Point Park), 2.GB (Greenwich Bay: Goddard Memorial State Park), 3.BIS (Bissel Cove: Rome Point), 4.NAR (Narrow River), and 5.NIN (Ninigret Pond) (Figure 1). These sites are representative of a diversity of environmental conditions (i.e. nutrients, dissolved oxygen, pH, salinity) within a coastal estuary and varying levels of anthropogenic inputs (Table 1). Wild oysters were collected from the northern 4 sites, and farmed oysters were collected from 5.NIN (no wild oysters were found). Environmental data for temperature, pH, DO, salinity, and chlorophyll-*a* were collected using a YSI 6 Series Multiparameter Water Quality Sonde (Model 6920VS) probe at all sites every 30 seconds for 15 minutes during the morning and afternoon hours on one day of the week of sampling.

Sample collections were completed from August 17-25, 2017 and consisted of oyster and water samples at each of the 5 sites with scientific collector's permit #212 granted by the RI Department of Environmental Management. A total of 150 oysters were collected from 5 sites (30 per site) and processed on the day of collection. The oysters were weighed and measured, and samples of gut tissues (around 300 mg) were

preserved in RNAlater for RNA/DNA extractions. All preserved tissue samples were stored at -80 °C until nucleic acid extractions. Up to 1 L of seawater from each site was filtered onto a 0.22 μm Sterivex filter, filled with RNAlater, and then stored at -80 °C until DNA extraction. An additional sample of seawater (30 mL) was filtered through a 0.22 μm syringe-top PES filter and frozen at -80 °C for nutrient analyses. Nutrient concentrations (nitrite, nitrate, ammonium, and phosphate) in three replicate samples of seawater per site collected at the time of oyster collection were analyzed using a Lachat QuickChem QC8500 automated ion analyzer operated by the University of Rhode Island Marine Sciences Research Facility.

Gut DNA and RNA Extraction

Total nucleic acids (TNA) were extracted from 150-200 mg of gut tissue (n=10 oysters per site; 50 total) using the Qiagen Allprep PowerViral DNA/RNA extraction kit with modifications as follows. The gut tissue sample was added directly to a 0.1 mm glass bead tube (Qiagen), along with 600 μL of Solution PV1 and 6 μL of sterile β-mercaptoethanol to minimize RNA degradation. The samples were subjected to bead beating for 5 minutes, followed by proteinase K digestion at 55 °C for 1 hour in a shaker at 300 rpm. The supernatant was transferred to a new microcentrifuge tube and the protocol continued according to the manufacturer's recommendations. Following TNA extraction, the concentration was quantified using a Nanodrop 2000 instrument (ThermoFisher), and 5 μL was allocated for RNA and 30 μL was allocated for DNA.

RNA purification from the 5 μL TNA aliquot was performed using the DNase Max I kit according to the manufacturer's protocol in a 50 μL reaction volume. DNA purification of the 30 μL TNA aliquot was performed using an adapted version of the

DNeasy PowerLyzer PowerSoil Kit. In brief, the TNA aliquot was transferred to a new 2 mL microcentrifuge tube and 1200 μL of Solution C4 was added, then vortexed to mix. Next, 4 μL of RNase A solution was added to the sample and incubated for 2 minutes at room temperature. The treated DNA was then purified using the spin column and an ethanol wash with Solution C5. The final DNA sample was eluted in 50 μL of Solution C6. Following extraction, DNA and RNA concentrations were quantified with both a Nanodrop 2000 instrument (ThermoFisher) and Qubit Fluorometer High-Sensitivity reagents (Invitrogen).

Seawater DNA extraction

Total DNA from water samples was extracted from the Sterivex filters using the Qiagen Allprep PowerViral DNA/RNA and DNeasy PowerLyzer PowerSoil kits with modifications as follows. The RNAlater was flushed out of the filters using a sterile syringe, then the filters were rinsed with 2 mL of 1X sterile nuclease-free Phosphate Buffer Saline (PBS, pH 7.4, Invitrogen). Solution PV1 (1800 μL) and 18 μL of sterile β-mercaptoethanol were added directly to the filter cartridge and incubated at 37 °C for 30 minutes. Next, 20 μL of proteinase K was added to the filter and digested at 55°C for 1 hour. The supernatant was flushed from the filter, transferred to 3 new microcentrifuge tubes and the protocol continued according to the manufacturer's recommendations. DNA was purified from the entire TNA product using the methods described above. Following extraction, DNA concentrations were quantified with both a Nanodrop 2000 instrument (ThermoFisher) and Qubit Fluorometer High-Sensitivity reagents (Invitrogen).

Nucleic Acid Amplification and Sequencing

In order to obtain a comprehensive representation of the gut microbial community and their activities, 2 types of sequencing were performed: 16S rRNA amplicon of the V6 region (DNA, a measure of overall composition) and whole shotgun metatranscriptomes (RNA, a snapshot of functional activity at the time of collection) (Graham et al., 2016). 16S rRNA gene amplicons were prepared using 967F/1064R primers to amplify the V6 region in the 50 gut DNA samples (10 per site) and the 5 water samples, along with a mock community and blank control. A two-step PCR reaction using 300ng of gut DNA or 10ng of water DNA was performed in triplicate following protocols from the Keck Sequencing Center at the Marine Biological Laboratory (https://vamps.mbl.edu/resources/primers.php). The PCR products were analyzed with 75bp paired-end sequencing to obtain overlapping reads on an Illumina MiSeq at the Genomics and Sequencing Center at the University of Rhode Island.

The metatranscriptomic libraries were prepared from 2μg of gut RNA (n=5 per site), fragmented at 500nt using Covaris ultrasonification, and treated with the Illumina Ribo-Zero Gold rRNA Removal Epidemiology Kit prior to library prep to remove both host and bacterial rRNA. Illumina TruSeq PCR-free library kits were used to prepare the libraries, and then verified using both a KAPA library quantification kit and an Agilent Bioanalyzer. The resulting metatranscriptomic libraries were sequenced on an Illumina NovaSeq S4 to obtain 2x150bp paired-end reads at the Yale Center for Genome Analysis.

Processing and Analysis of Sequencing Data

16S rRNA amplicon sequences were demultiplexed and quality filtered using DADA2 (v1.6.0) implemented in R (v3.4.1) in QIIME2 (v2018.4.0) with parameters --p-trunc-len-r 65 \ --p-trunc-len-f 76 \ --p-trim-left-r 19 \ --p-trim-left-f 19 to determine analysis sequence variants (ASVs) (Callahan et al., 2016; Caporaso et al., 2010). All ASVs were analyzed with the QIIME2 pipeline (v2018.4.0) and classified directly using the SILVA database (99% similarity, release #132) (Bokulich et al., 2018; Bolyen et al., 2019). Taxonomy data was normalized by percentage to the total ASVs in each sample and then exported as a matrix for analysis in R (v3.4.1). All descriptive and statistical analyses were performed in the R statistical computing environment with the *vegan* v2.5.5 and *phyloseq* v1.28.0 packages (Dixon, 2003; McMurdie and Holmes, 2013). Additional visualizations were computed using the *ComplexHeatmap* v3.9 and *UpSetR* v1.4.0 packages (Conway et al., 2017; Gu et al., 2016)

Raw reads from the microbial community metatranscriptomes were first quality controlled with Trimmomatic software v0.36 (Bolger et al., 2014). Metatranscriptomic analysis was performed using scripts from the SAMSA2 pipeline (Westreich et al., 2018), with the following modifications. The quality-controlled paired-end reads were combined using PEAR v0.9.10 and then rogue rRNA reads were removed from the merged reads using SortMeRNA v2.1 (Kopylova et al., 2012; Zhang et al., 2014). These cleaned, merged reads were classified to the Species level using Kraken2 v2.0.7 and relative percent abundances per Phylum and Order were calculated using Bracken v2.2 (Lu et al., 2017; Wood and Salzberg, 2014). Functional annotation of the data was performed against SEED Subsystem databases using DIAMOND v0.9.23

(Buchfink et al., 2014). Finally, custom scripts using DESeq2 v1.14.1 were used to calculate differential expression between sites or groups (Love et al., 2014). The resulting changes in expression were exported to R for analysis and visualization using *ggplot2* v3.2.1 and *cowplot* v1.0.0 (R Development Core Team, 2011; Wickham, 2009; Wilke, 2019).

Statistical Analysis

All statistical analyses of environmental and sequencing data were performed in R (v3.4.1 R Development Core Team, 2011) as follows. The environmental principal component analysis (PCA) was calculated using the prcomp(scale=TRUE) command implemented in base *stats* v3.6.1, and then plotted using autoplot() enabled by *ggfortify* v0.4.7 (Tang et al., 2016). Significant differences in environmental parameters between sites were determined using all raw data subset by site and parameter. The data per site was compared using the compare_means() command from the *ggpubr* v0.2.2 package (Kassambara, 2019). The method for each comparison was defined as "anova" for initial testing, then "t.test" for pairwise comparisons. Adjusted *p*-values were calculated by adding "p.adjust.method = BH" to the command, to determine using the Benjamini-Hochberg method (Love et al., 2014). Statistical summary tables are included as Supplementary Tables S2-S7.

Results

Sampled Sites showed Variability in Environmental Conditions

A summary of all measurements collected and correlations with latitude is presented in Table 1. A North-South estuarine gradient was detected, especially in nutrient concentrations. Salinity, pH, and DO increased down the Bay from Providence (1.PVD; North) to Ninigret Pond (5.NIN; South), as coastal eutrophication and the influence of river inputs decreased (Table 1, Spearman's Correlation Coefficients, SCC =-0.8, -0.8, -0.9). Nitrite, nitrate, and phosphate concentrations decreased along the North-South gradient (Table 1, SCC=0.7, 0.6, 0.9), with 1. PVD showing significantly higher concentrations of nitrite, nitrate, and phosphate than all other sites (t-test, p <0.01). A PCA analysis showed that environmental factors, each averaged per site, explained 80% of the variation between sites (Figure 2). Each site was characterized by a subset of environmental factors over the sampling period (Figure 2). The 1. PVD site was characterized by the highest nutrient levels (nitrite, nitrate, phosphate, p <0.01, compared to all other sites), 2. GB by the highest chlorophyll-a, 3. BIS by the highest ammonium concentrations (t-test, p<0.001), 4.NAR site by a higher temperature (NS) and significantly lower salinity than all other sites (t-test, p<0.045), and 5.NIN by significantly higher pH than all other sites (t-test, p=0.023). The average mass, length, and width of oysters at each site decreased down the Bay, with the exception of oysters from 3.BIS, which were significantly heavier and larger than oysters collected at other sites (Table 1, SCC=0.7,0.7,0.8; t-test, p<0.001).

Differences in Microbial Community Structures were Observed between Sites and Sample Types

A total of 2,217,804 quality-controlled, bacterial 16S rRNA sequences were analyzed from 50 gut samples and 10 water samples from 5 sites (Table S1). Sequence variant analysis and taxonomic classification resulted in the detection of 304 bacterial Orders across 45 Phyla across all samples. The most dominant phyla in the oyster gut samples, averaged for all oysters at all sites, were *Cyanobacteria* (38±18%) *Proteobacteria* (21±13%), *Tenericutes* (6±12%) and *Actinobacteria* (3±2%). The most dominant phyla in the water column, averaged from all sites, were *Proteobacteria* (62±10%), *Cyanobacteria* (15±12%), *Bacteroidetes* (15±7%), and *Actinobacteria* (3±2%). Differences in bacterial community structures were observed between the oyster gut and water samples, in addition to between sites for both sample types (gut and water) (Figures 3 and S1).

Effect of sample type on microbial community structures

The structure of the gut microbiome was distinct from the water microbial community, regardless of the sampling site (Figure S1B, adonis2 p=0.001). Of the 304 Orders detected in the 16S amplicon data, the water and gut samples had 135 (44.4%) Orders in common, while 8 (2%) were exclusively found in the water and 161 (52.9%) were found only in the oyster gut, suggesting selection by the host (Figure 3A). *Flavobacteriales*, *Rhodobacterales*, *Rhodospirillales*, and *Oceanospirillales* were proportionally more abundant in all of the water samples than the gut samples (Figure 3B, t-test, p<0.001). Conversely, *Corynebacteriales*, *Vibrionales*, *Desulfobacterales*, and *Mycoplasmatales* were relatively more abundant in the gut samples (Figure 3B, t-

test, $p<0.05$). Significantly more unknown bacterial Orders were detected in the oyster gut samples, compared to the water (Figure 3B, t-test, $p<0.001$).

Effect of site on microbial community structures

The oyster gut bacterial communities from each site were significantly different at the Analysis Sequence Variant (ASV) level (Figures S1B, adonis2 $p=0.001$), with samples from 1.PVD, 2.GB and 3.BIS showing significantly lower alpha-diversity (Figure S1A, Simpson's Index; $p<0.01$) than samples at other sites. This appears to be driven by specific microbial signatures found at each site. For example, *Corynebacteriales* and *Synechoccales* were significantly more abundant in the water and gut samples from 4.NAR and 5.NIN than at other sites, and *Verrumicrobia* were significantly more abundant at 4.NAR than in others sites (Figure 3, t-test, $p<0.05$). Oyster gut samples at 2.GB showed higher percentages of chloroplast-associated 16S rRNA amplicons (50±27%), which is consistent with high chlorophyll-a levels measured at this site (Figure 2). Oyster gut samples from all sites share 105 Orders (34.5% of 304 total), while 9-31 (3-10%) Orders were distinct to gut samples at certain sites (Figure S2). For example, 2.GB has the fewest number of distinct orders (9), which belong to diverse phyla, including *Bacteroidetes*, *Latescibacteria*, and *Proteobacteria*.

Comparison of Transcriptionally Active Microbial Community Structures

A total of 409 million metatranscriptomic 150 bp-long, quality-controlled paired-end reads were obtained from 25 gut samples (n=5 per site; Table S1). Direct taxonomic annotation of these merged paired-end reads classified 32±15% of the

reads, which is comparable with other studies (Antczak et al., 2019; Crump et al., 2018; Kieft et al., 2018). This level of annotation is most probably due to incomplete taxonomic coverage in reference databases. Marker gene classification was not possible due to the rRNA depletion performed during library prep and subsequent biased removal of common taxonomy markers (Petrova et al., 2017). Of the taxonomically annotated reads, 68 bacterial Orders across 29 Phyla were detected, of which 36 (53%) were also detected in the gut 16S amplicon data. The most active annotated phyla in the gut samples (all oysters) were *Firmicutes* (35±1%) and *Proteobacteria* (27±1%) (Figure 3B). The most active taxa (*Bacillales*, *Pseudomonadales*, and *Rhizobiales*; as detected in the metatranscriptomes) were not the most abundant taxa (as detected by 16S rRNA amplicon analysis) (Figure 3B). There were 260 (out of 296, 87%) Orders detected in the oyster gut 16S amplicons that were not detected in the metranscriptomes (Figure 3A). While microbiome structures of oyster gut samples (as detected by 16S rRNA amplicon analysis) showed differences by site (Figure S1B), microbiome structures of the active taxa (as determined by taxonomic annotation of metatranscriptomic reads) in the gut samples were not different between sites (Figure S3).

Transcriptional Responses in the Oyster Gut Microbial Community Reflect the Estuarine Gradient in Narragansett Bay

Although no significant differences were detected between sites on the taxonomy of the transcriptionally active microbial taxa in the gut tissue (Figure S3), their transcriptional responses varied based on the environmental conditions at each

site (Figure 4). In order to increase statistical power in the identification of pathways to be targeted for further analysis, the microbial transcriptional response at the more eutrophic northern sites (1-3) was compared to that of the southern sites (4-5; considered as the "control group"). This resulted in eleven SEED Level-1 pathways that showed significantly differential gene expression between northern and southern sites, including a significant upregulation of stress responses and general metabolic activities (carbohydrates, respiration, amino acids, fatty acids, lipids etc.) in northern sites, as well as a downregulation of photosynthesis, metabolic transport, and motility and chemotaxis (Benjamini-Hochberg adjusted $p<0.05$; Figure 4A).

Stress Response

Based on the focus of this research on the effects of anthropogenic factors (*e.g.* eutrophication, urbanization) on oyster microbial community and function, a more in-depth analysis of differences in the expression of genes involved in stress responses and nutrient cycling was performed (SEED level 2 annotation). Differential expression of genes in stress response and nutrient pathways at each of the sites was compared to the mean level of expression at all sites (Figures 4B and 5). A significant upregulation in the expression of microbial genes involved in dealing with osmotic stress was detected in samples from 2.GB (as compared to the mean of all sites), as well as a significant downregulation in genes involved in periplasmic stress ($p<0.05$). Conversely, a significant upregulation in genes involved in periplasmic stress (*e.g. rseA, degS, deQ*) and downregulation in genes involved in osmotic stress (*e.g.* genes coding for betaine aldehyde dehydrogenase and choline dehydrogenase) and oxidative stress (*e.g.* genes coding for NAD G3P dehydrogenase) was detected in oyster gut

samples from 5.NIN (p<0.05, Figure 4B, S4, and S5). Expression of microbial genes involved in other acute stress responses, including acid stress, cold shock, and heat shock, was not significantly different between sites.

Nitrogen Metabolism

Nutrient cycling is central to ecosystem services provided by oysters. Nitrogen and phosphorus are especially important, since they are the major components of eutrophication and often limiting factors to primary production (Howarth, 1988; Wallace et al., 2014). Overall, no significant changes in expression of genes involved in nitrogen metabolism (SEED level 2 annotation) was observed in the gut oyster microbiome from the different sites (Figure 5A top), despite the significant differences in levels of nitrate, nitrite, and ammonium levels detected between sites (Table 1). High levels of variability in the expression of genes involved in the different pathways involved in nitrogen metabolism were observed between oysters within sites. However, significant differences between sites were observed in the patterns of expression of genes from specific pathways involved in nitrogen metabolism (Figure 5A bottom), reflecting differences in the responses of oyster gut microbes to the environmental conditions at each site. At the northernmost site (1.PVD), there was a significant downregulation of denitrification genes (*e.g. nosF* and cytochrome c-dependent nitric oxide reductase (*cNor*)) compared to the mean of all sites, while at the southernmost site (5.NIN), a significant upregulation of genes involved in ammonia pathways (*e.g.* genes coding for NR(I), *GlnE,* and nitrate reductase) and a downregulation of nitrilase genes was observed (p<0.05, Figure S6).

Phosphorus Metabolism

Expression of genes in the oyster gut microbiome involved in phosphorus metabolism decreased down the Bay, with microbial communities in the guts of oysters from the most southern (5. NIN) and northern (1. PVD) sites respectively showing significantly lower and higher levels of expression of genes involved in phosphorus metabolism than the mean of the sites ($p<0.01$*;* Figure 5B top). An upregulation of genes involved in the phosphate pathway (*e.g.* alkaline phosphatase) was observed in the gut microbiome of oysters from the northernmost site (1.PVD), as well as an upregulation of genes in the phosphonate pathway in oysters from 2.GB (*e.g.* phosphonoacetaldehyde hydrolase) (Figure S7). These two sites also showed the highest concentrations of phosphate in water (Table 1). Conversely, there was a significant downregulation of phosphate and phosphonate pathways at the southernmost site compared to the mean of all sites (5.NIN, $p<0.01$, Figure S7).

Discussion

A better understanding of the effect of environmental conditions on both the structure and function of oyster-associated microbes is important for the management of oyster populations and optimization of the ecosystem services they provide. In this study, we have characterized the composition and function of oyster-associated microbiomes at sites within a temperate, urbanized estuary. We found that oyster gut microbiomes during the summer were diverse in composition and differed between sites. Differences between the structure of microbiomes between water and oyster gut were consistent with selection and amplification of taxa from the water environment

by the oyster hosts. Although no significant differences in oyster gut community structure of the most active taxa was observed between sites throughout Narragansett Bay, significant differences in gene expression of several gene pathways (stress response, nutrient utilization) was observed between sites, reflecting the environment at each of the sites. In particular, the gut microbial community of oysters collected at the northern sites, which were characterized by high levels of nutrients and anoxia, showed upregulation of genes associated with stress response and phosphorus metabolism, as compared to southern sites. Microbes in the gut of southern oysters showed a relative upregulation of genes associated with nitrogen metabolism. These responses varied according to the eutrophication gradient, indicating that the responses of oyster gut-associated microbiomes reflect the local environment, despite the fact that they are located within the host (*i.e.* the oxygen and nutrient status of the water is pervasive in the oyster gut). This study also confirms the power of a metatranscriptomic analysis to provide insights into how estuarine acidification may shape host-associated microbial functions (Figure 6).

Surprisingly, overall expression of genes involved in nitrogen metabolism in oyster-associated microbiomes was significantly higher at sites with the lowest levels of nutrients (NO_2^-, NO_3^-, NH_4^+) in the water at the southern range of the estuarine gradient than at the more eutrophic northern sites. These results are consistent with previous findings showing that oxygen conditions control nitrogen and phosphorus cycling in the sediments by limiting nutrient availability (Testa et al., 2013), with high oxygen concentrations promoting nitrogen removal (Alzate Marin et al., 2016). This interaction between oxygen concentration (or redox state) and nitrogen metabolism

has been well-documented in marine sediments: higher DO and low NO_3^- concentrations will stimulate denitrification, while the opposite occurs with high NO_3^- concentrations (Rysgaard et al., 1994; Smith and Tiedje, 1979). Therefore, our findings indicate that the environment in the oyster gut reflects the overall environmental conditions at the site, consistent with expectations from sediment and water column observations. In the more oxygen-rich waters of the southern sites, oyster gut-associated microbes would use nitrogen as an electron donor for dissimilatory nitrate reduction to ammonium (DNRA) and ammonia-related pathways (Enrich-Prast et al., 2016), while in the more eutrophic, anoxic, and acidic waters of the Providence River where there is no available ammonia, oyster-associated microbiomes would instead upregulate pathways using the phosphate available from the sediment (Gomez et al., 1999; Lam and Kuypers, 2010). Alternatively, oyster-associated gut metatranscriptomes in these northern sites may have been enriched in genomes adapted to prefer phosphate over nitrogen.

Microbiomes in the gut of oysters collected at each of the sites also reflected potential stressors at each of the sites. For example, the increase in microbial oxidative stress observed at 1.PVD and 2.GB has been widely observed in microbial communities in response to anoxia, pollution, and toxins (Alves de Almeida et al., 2007; Lesser, 2006). The upregulation of periplasmic stress response (due to stressors within the inner bacterial membrane) observed in samples collected at site 5.NIN is likely coupled with increased nitrogen metabolism and transport (Raivio and Silhavy, 2002; Reyes et al., 2017; Spiro, 2012). In general, as eutrophic conditions worsen, bacteria will expend more energy on stress response and metabolic activities, a trend

that has also been shown in marine sediment microbiomes (Meyer-Reil and Köster, 2000; Zhang et al., 2015). Other stressors, including pathogens, toxins, or chemical pollutants may have contributed to the differential expression in stress response pathway between sites and require further study.

These results suggest that oysters select and amplify certain bacterial species, showing selection and niche colonization, as shown in other host species (Parfrey et al., 2019). The fact that bacterial composition in gut samples does not completely reflect the existing community in the water samples may indicate that oysters amplify rare members in the water community and/or retain bacteria previously acquired through time horizontally from the water or vertically from parents. Consistent with the hypothesis of amplification, certain bacterial taxa that are known intracellular anaerobes were relatively more abundant in gut than water samples. These include members of the *Mycoplasmatales*, *Actinobacteria*, *Mollicutes*, *Clostridiales*, and *Desulfobacterales*. *Mycoplasmatales* have been identified as common invertebrate symbionts and are avid biofilm-formers, allowing them to survive and replicate in the host (Fraune and Zimmer, 2008; McAuliffe et al., 2006). *Proteobacteria* formed the most abundant and active phylum in the overall community as determined by the 16S rRNA and metatranscriptomic analyses, consistent with published literature in oysters (King et al., 2019a; Pierce et al., 2016; Stevick et al., 2019). High variability among oysters within sites in the relative abundances of certain taxa (i.e. *Mycoplasmatales* or *Caulobacterales*) suggest that not just host selection by filter feeding plays a role in shaping community structure, but that factors like oyster health and/or host genetics may play a role (King et al., 2019b; Wegner et al., 2013). Further examination of

within-site variability and their relationship with other host parameters (*e.g.* health and physiological status, genetics) may reveal how certain taxa are promoted in each oyster. Studies have shown decreased microbial diversity in health-compromised hosts, which may limit their ability to respond to environmental change (King et al., 2019a; Kinross et al., 2011). The interplay between the environment at each site, oyster-associated microbiomes, and host health will be the focus of further study. (See Appendix A for data on the health status of oysters collected for this study, as determined by histology and qPCR. Sample sizes were not enough in this study to establish a relationship between bacterial community composition and function and oyster health status.)

Comparisons between the 16S rRNA amplicon data with metatranscriptomic analysis of the oyster-associated microbial community may also provide some initial insights into identification of which of the microbes show a symbiotic relationship with the oyster host, versus those that are transient food in the gut (*i.e.* accumulate in the oyster gut through association with food selectively ingested by oysters through filter feeding) (Newell and Jordan, 1983; Pierce and Ward, 2018). In particular, of the selected taxa shown to be relatively more abundant in the gut samples as compared to the water, a subset was detected to be transcriptionally active (suggesting that are not being immediately digested as food), particularly *Bacillales,* and *Vibrionales*. These taxa are commonly found in oysters, and known for their biofilm-forming abilities (King et al., 2019a; Pierce and Ward, 2018; Rampadarath et al., 2017; Riiser et al., 2018). Conversely, despite the high relative abundance of *Synechococcales* and other *Cyanobacteria* detected in oyster gut samples in the southern sites through 16S rRNA

amplicon sequencing, the gut metatranscriptomes do not show a relative enrichment in levels of expression of genes involved in photosynthesis. Increased abundances of *Cyanobacteria* were also observed in the water samples at the southern sites, confirming that the relative abundance of these taxa in gut samples was a reflection of recent feeding activity. Further experiments in feeding and non-feeding conditions should be done to confirm the transient or resident nature of these taxa.

Conclusion

In summary, the estuarine gradient affected oyster-gut associated microbial communities by causing changes in community composition, microbial stress responses, and microbial metabolic responses. Estuarine acidification and other stressors increased microbial stress response pathways and changed expression of microbial genes involved in nutrient utilization. Changes in the function of the oyster gut microbiome mainly reflected local environmental conditions, within the context of a diverse microbial community structure. Our results suggest that the microbial community in the oyster host functions similarly to microbes in water and sediment (although we did not measure if perhaps the host amplifies these functions, compared to the water or sediments). Additional research is needed to probe how microbial functions respond to specific environmental stressors, particularly within coastal marine species. Combined, these results have implications for environmentally-driven changes in oyster microbial acclimation and potential ecosystem services. The environmental conditions and presence of a functionally diverse community, along

with site- and host- driven microbial community structures, determine the function and contributions of the oyster microbiome.

Oyster gut microbial communities in these studies showed high levels of structural and functional diversity. Microbial functional plasticity coincides with functional redundancy in the microbiome: many different taxa encoding the same diverse functions or many taxa each encoding a distinct function (Louca et al., 2018). Functional plasticity as a result of functional redundancy has also been observed in microbial communities in humans (Gomez et al., 2019), soils (Espenberg et al., 2018; Glassman et al., 2018), marine sediments (Bulseco-McKim et al., 2017), and other host-associated microbiomes (Apprill, 2017; Rivest et al., 2018). Based on our results, we expect that oysters transplanted to other locations would show similar function as resident oysters, due to a diverse microbiome and functional redundancy (Antczak et al., 2019; Graham et al., 2016). This functional plasticity allows for microbial acclimation to changing estuarine conditions, perhaps also benefiting the host (Apprill, 2020; Carrier and Reitzel, 2018).

This study also has implications for quantification of ecosystem services provided by oyster restoration and aquaculture. In Narragansett Bay, oyster fisheries were a dominant industry in the late 1880s, but a combination of pollution, overfishing, and dredging lead to the collapse of oyster populations in the 1940s (Schumann, 2015). In recent years, numerous efforts have been made to renew oyster reefs and restore their ecosystem services in Narragansett Bay. A common goal of oyster restoration projects is improvement of water quality by stimulation of environmental denitrification (Grabowski et al., 2012; Kellogg et al., 2014). Our

findings support that removal of bioavailable nitrogen by denitrification, an important ecosystem service provided by oysters, declines in low oxygen, nutrient rich environments (Howarth et al., 2011; Lam and Kuypers, 2010; Zehr et al., 2006). Enhanced denitrification would occur at high dissolved oxygen and nutrient rich environments, such as the conditions observed at 4.NAR during the summer. This implies that if the environmental microbial community does not have the genes necessary for the nitrogen pathway and/or the environmental conditions do not favor the process, then the addition of oysters to the site will not promote the ecosystem service. The prevailing environmental conditions and function of the resident environmental microbial community should be considered when selecting sites for oyster farming and restoration. In this study, 4.NAR and 5.NIN would provide the greatest return on investment for a restoration project, if only the benefits of denitrification are considered.

The results of study address knowledge gaps in oyster biology and ecology that may be explained by the effect of environmental factors on microbial communities associated with the host. As estuarine acidification increases, it is important to determine how microbial communities in oysters will change with environmental parameters, and determine relationships between microbial community structure and environmental conditions. The results presented here form a baseline for future studies that explore how human-driven estuarine acidification changes overall oyster health and its impacts on oyster farming.

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Figures and Tables

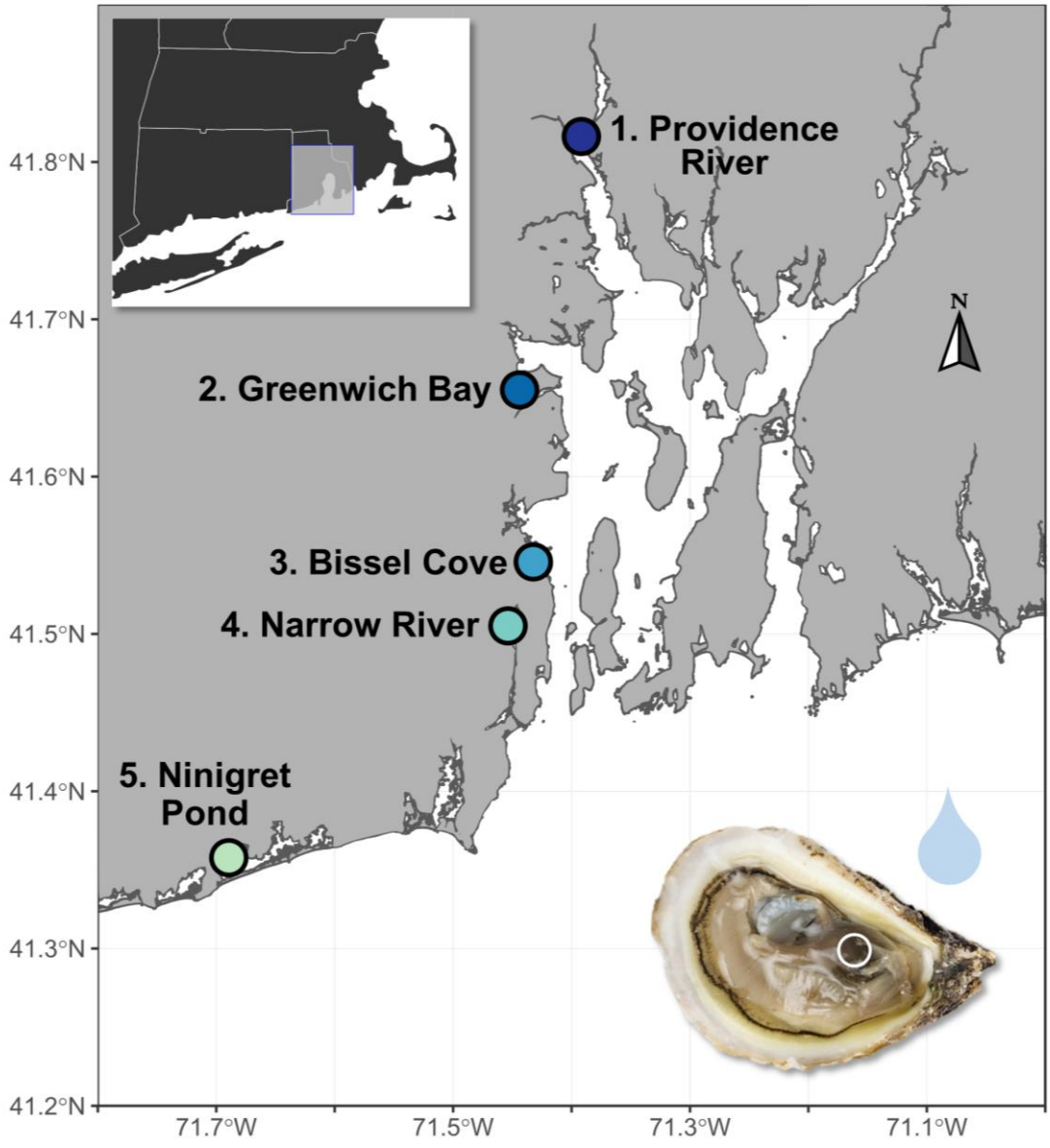


Figure II-1. Map of study area with 5 sampling locations.
A schematic of the samples collected from each site is show in the bottom right.

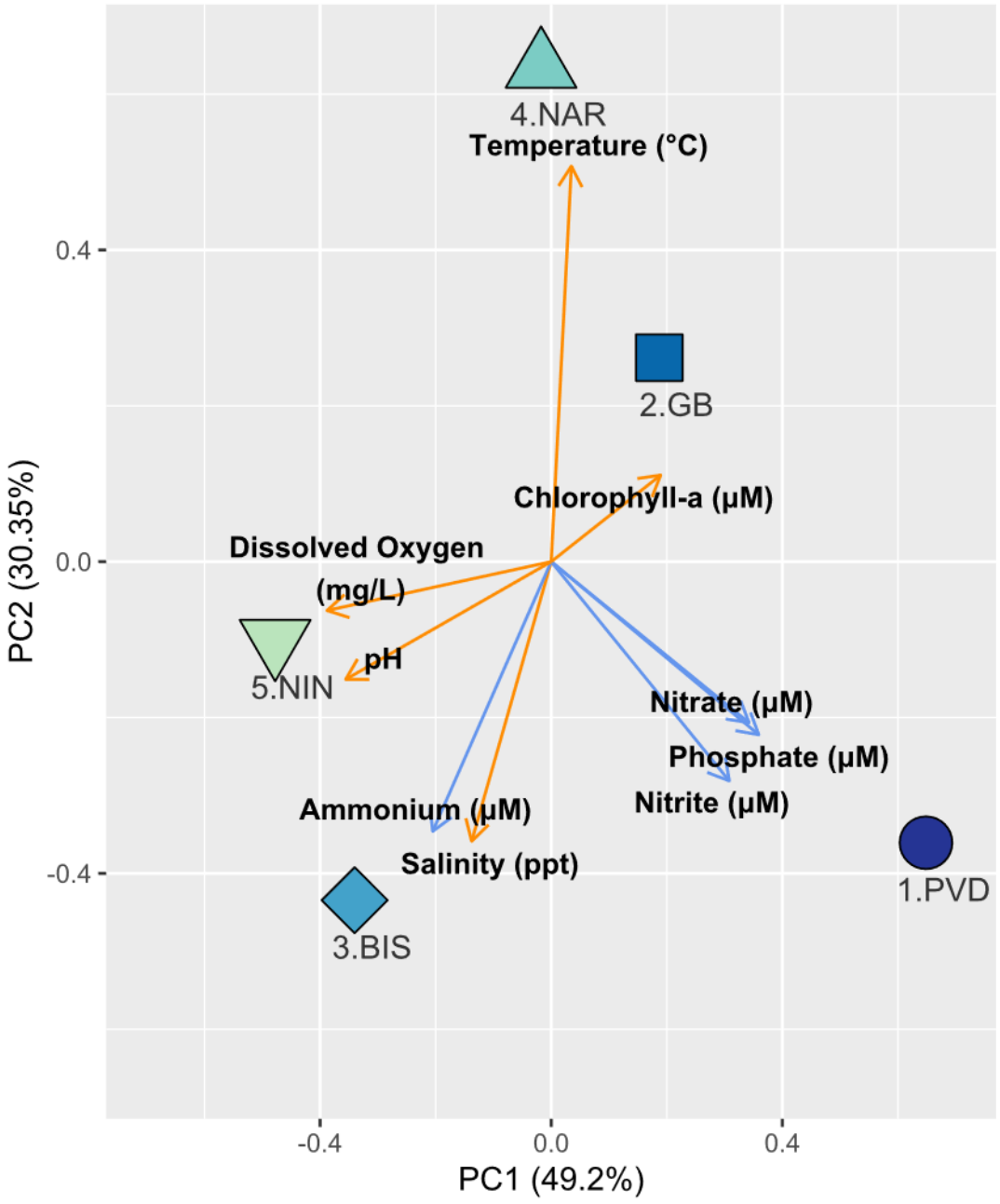


Figure II-2. PCA of environmental factors, averaged per site.
Each site is represented by a colored symbol and each environmental factor is represented with an arrow. Orange arrows indicate average environmental values measured *in situ* during the sampling week (n=2); light blue arrows are nutrient concentrations measured from water samples (n=3).

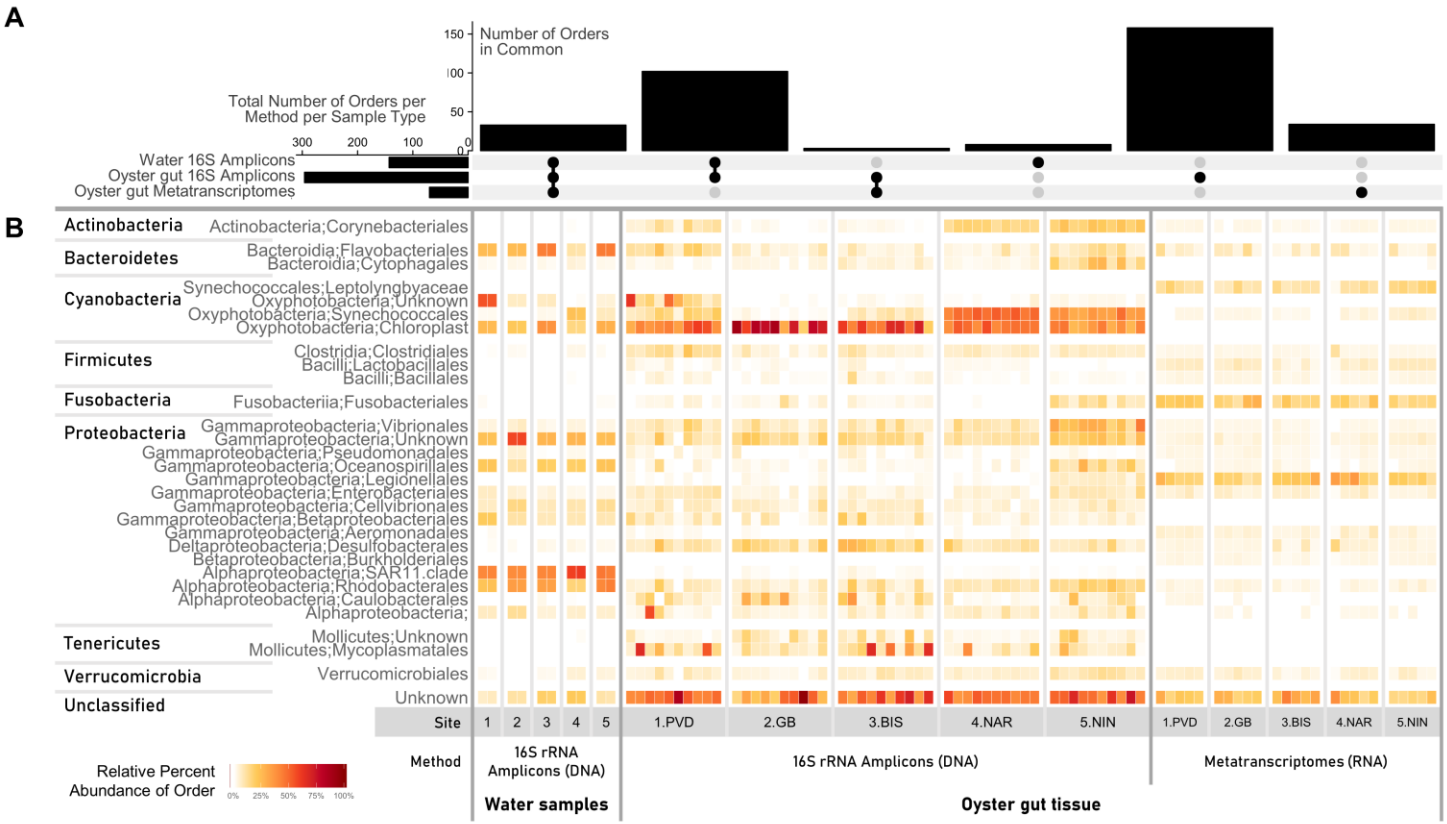


Figure II-3. Effect of site and sample type on present and active bacterial community structures.
(A) Number of bacterial Orders shared between the water 16S rRNA amplicons, oyster gut 16S rRNA amplicons, and oyster gut metatranscriptomes (vertical bars). The total number of Orders found in each group is shown in the horizonal bar graph on the left. (B) Relative percent abundances of top 30 bacterial Orders associated with seawater samples (n=2) or oyster gut tissue (n=10 or 5), per site. The most abundant bacteria (16S rRNA amplicons, middle, n=10) and the most transcriptionally active bacteria (metatranscriptomes, right, n=5) in the oyster gut are shown.

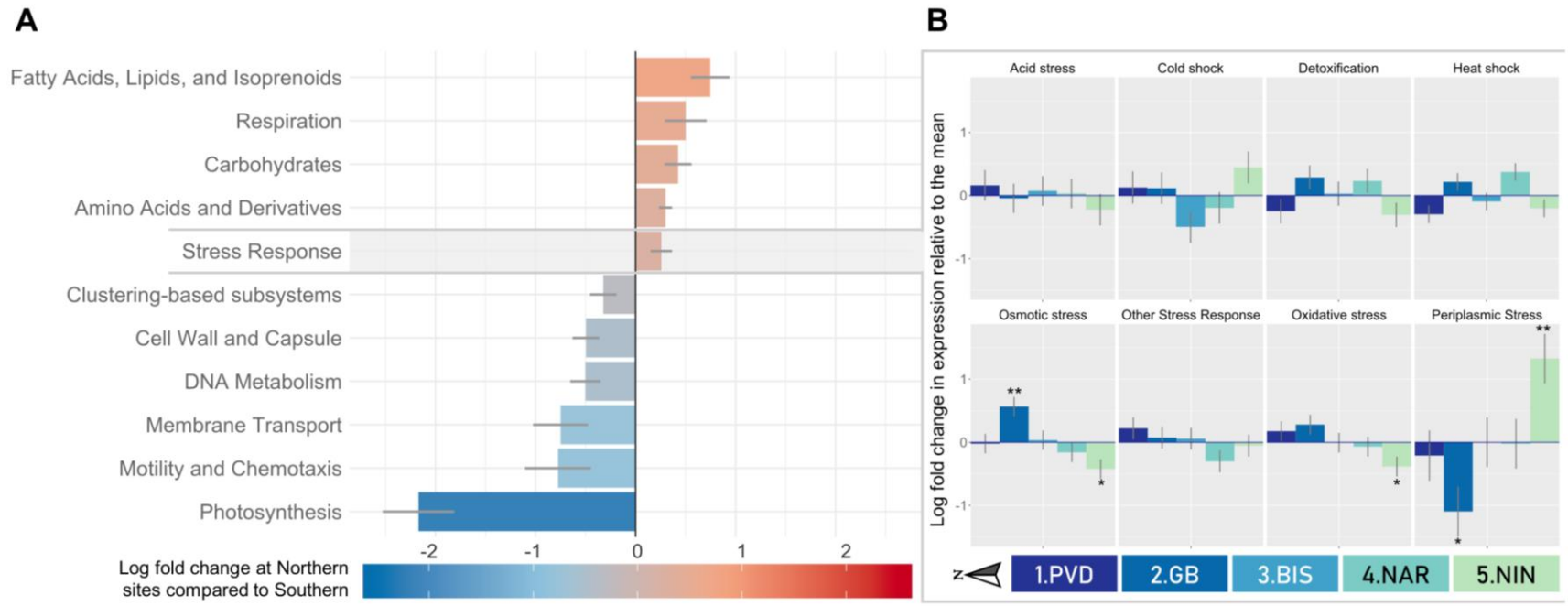


Figure II-4. Differential expression of significant and stress response pathways.
(A) Differential expression of all significant (padj<0.05) Level 1 pathways at the Northern sites (1-3, n=15), compared to Southern sites (4-5, n=10). A red bar (fold change>0) indicates upregulation in the North and a blue bar (fold change<0) indicates downregulation in the North. (B) Differential expression of Level 2 Stress Response pathways at each site, relative to the mean of all other sites (n=5, Significance: *padj<0.05, **padj<0.01).

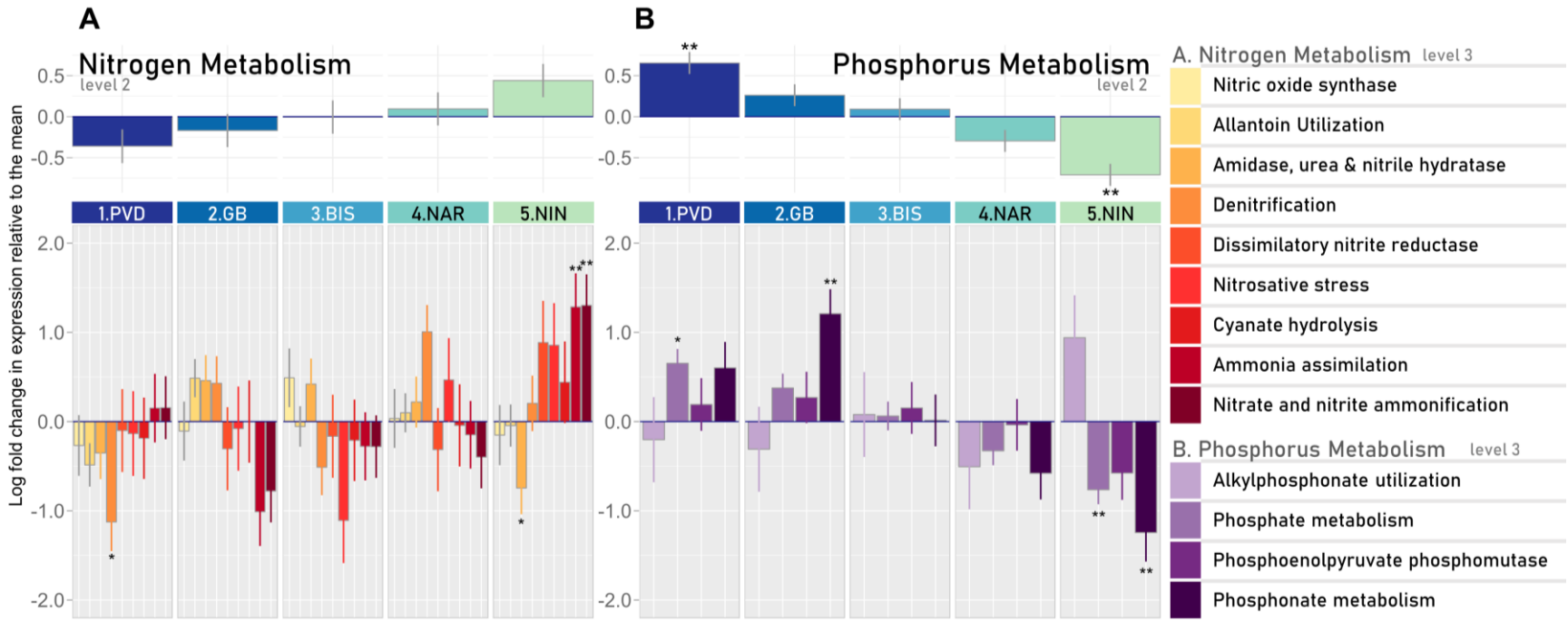


Figure II-5. Nitrogen and phosphorus metabolism at each site.
(n=5, Significance: *padj<0.05, **padj<0.01). (A) Differential expression of Nitrogen pathways at all sites, relative to the mean of the others. (top) Total differential expression of overall nitrogen metabolism, indicated with the blue-green colors. (bottom) Relative log fold change in nitrogen metabolism pathways, indicated with yellow-red colors. (B) Differential expression of Phosphorus pathways at all sites, relative to the mean. (top) Total differential expression of overall phosphorus metabolism, indicated with the blue-green colors. (bottom) Relative log fold change in phosphorus metabolism pathways, indicated with purple colors.

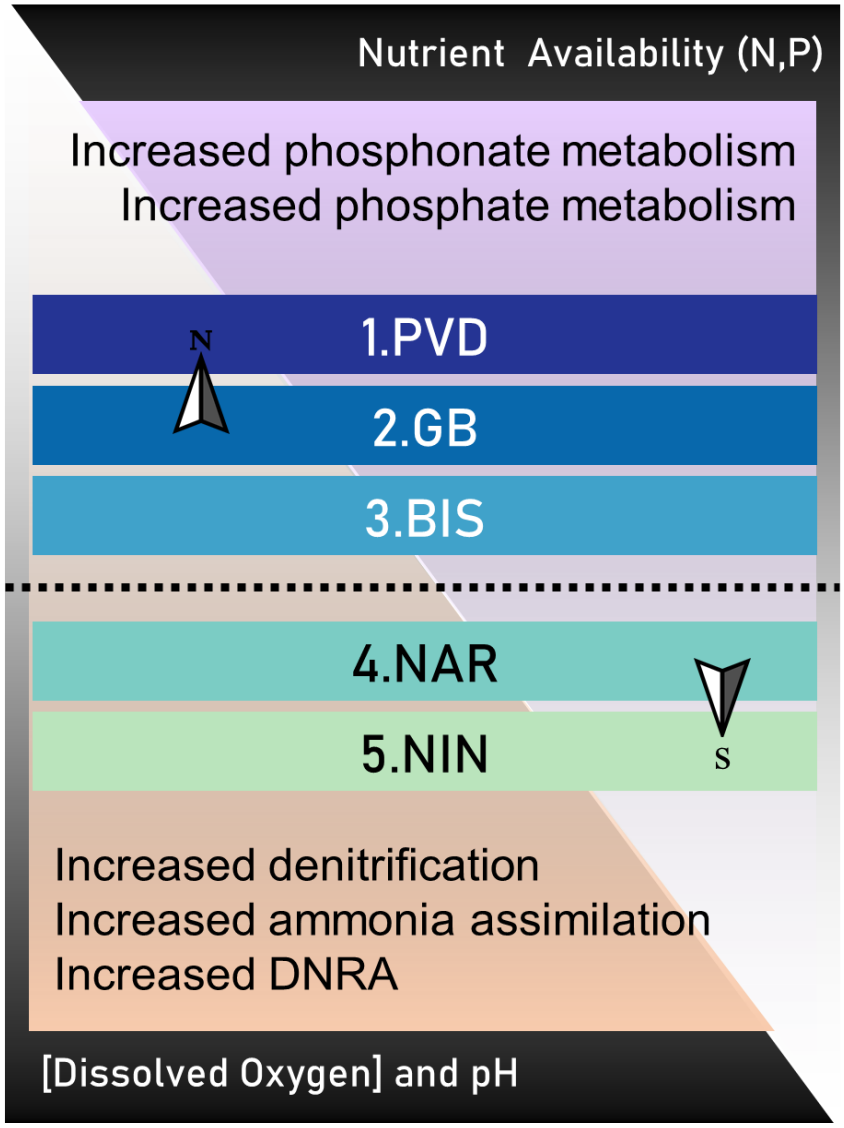


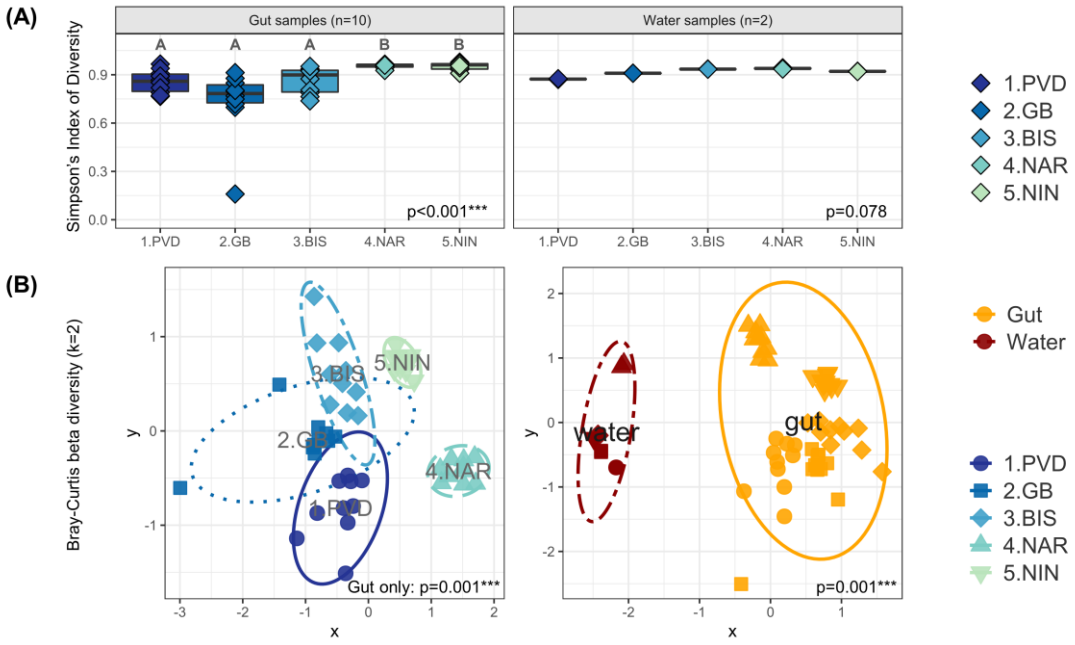
Figure II-6. Summary of changes in nutrient cycling according to relevant environmental factors.

Table II-1. Summary of all measurements collected per site.
Environmental values are daily averages ± standard deviation measured at each site during week of collection. Nutrient values are averages of three-point samples collected from each site at time of oyster collection. Spearman's correlation coefficient (-1 to 1) was calculated for the association between each parameter and Latitude. The most significant SCC values (|≥0.8|) are shaded green. A value closer to 1 indicates that the parameter decreases from North-South (1.PVD to 5.NIN) and a value closer to -1 indicates that the parameter increases from North-South. A correlation coefficient of 0 means there is no linear association and that the value does not consistently change along the estuarine gradient. Significantly different measurements to all other sites as determined by a pairwise Student's T-test or Wilcox rank-sum test are indicated in bold (Tables S2-S4).

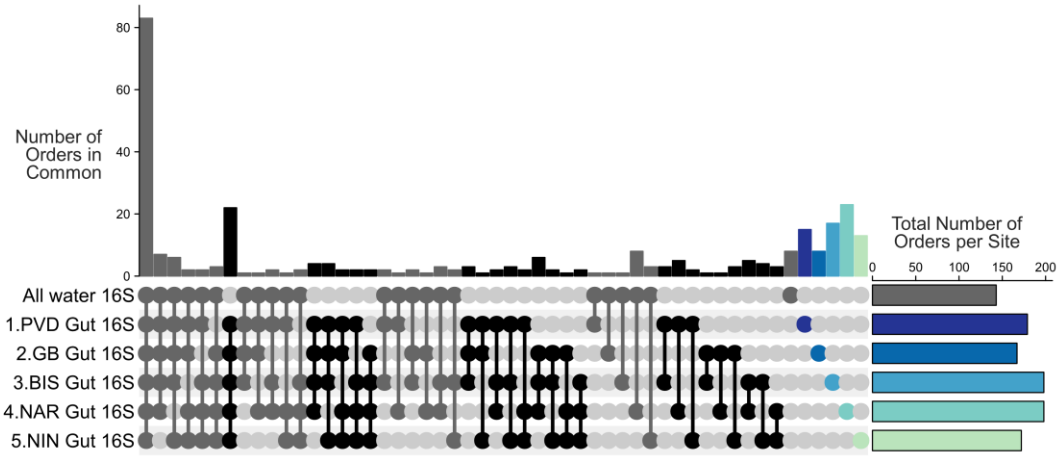
	1.PVD	**2.GB**	**3.BIS**	**4.NAR**	**5.NIN**	**SCC**
Location (GPS coordinates)	41.816, -71.391	41.654, -71.445	41.545, -71.431	41.505, -71.453	41.358, -71.689	1
Environmental						
Temperature (°C)	23.0±0.8	24.3±1.3	22.7±1.5	25.4±0.3	23.3±1.6	-0.3
Salinity (psu)	24.8±2.1	28.5±0.2	30.5±0.1	**18.0±0.4**	28.9±0.9	-0.2*
pH	7.4±0.0	7.4±0.2	7.9±0.0	7.6±0.2	**8.2±0.0**	-0.9
Chlorophyll- *a* (µg/L)	8.1±4.0	18.8±7.5	4.9±2.8	4.6±1.3	3.8±0.4	0.9
Dissolved Oxygen (mg/L)	4.9±1.5	5.7±3.1	8.2±1.0	7.0±1.9	9.5±3.5	-0.9
Nutrients						
Ammonium (µM)	7.6±0.1	5.6±0.9	**45.8±0.8**	1.6±1.1	13.9±0.1	-0.1
Nitrite (µM)	**0.7±0.0**	0.0±0.0	0.1±0.2	0.0±0.0	0.0±0.0	0.7
Nitrate (µM)	**9.7±0.1**	1.9±0.2	2.1±0.3	2.3±0.1	0.9±0.1	0.6
Phosphate (µM)	**3.7±0.1**	1.6±0.1	0.7±0.1	0.1±0.0	0.2±0.0	0.9
Oyster Characteristics (n=30)						
Average mass (g)	124.6±35.9	93.8±32.7	**165.8±83.0**	52.9±16.5	44.2±8.4	0.7
Average length (mm)	98±21	84±11	**104±21**	76±9	71±7	0.7
Average width (mm)	68.2±8.6	63.0±6.4	**67.2±9.5**	47.8±7.5	51.7±6.7	0.8

*Spearman's correlation coefficient for Salinity without 4.NAR is -0.8.

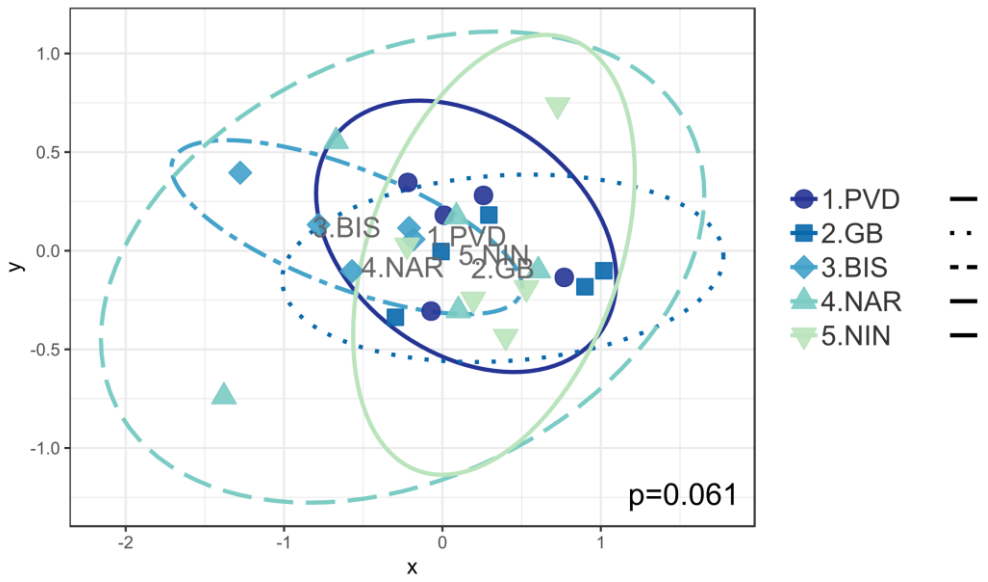
Supplementary Material



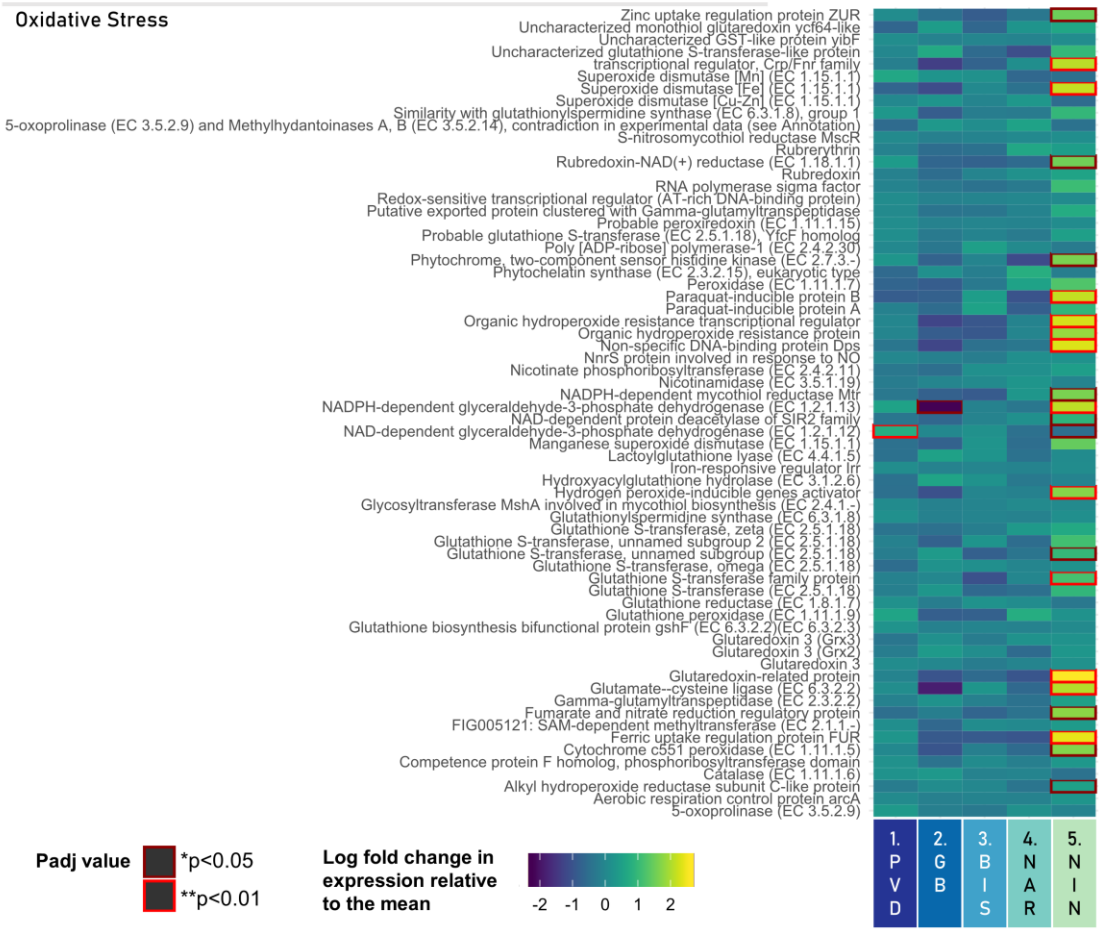
Supplementary Figure 1. (A) Simpson's Index of Diversity calculated using ASV-level 16S rRNA amplicons for gut samples (left, n=10) and water samples (right, n=2). Global p-values were calculated using the Kruskal-Wallis rank-sum test, and pairwise p-values were calculated with the Wilcox rank-sum test (*p<0.05). (B) NMDS plot visualization of Bray-Curtis beta-diversity (k = 2) at the ASV level for gut samples by Site (left) and all samples by Type (right). The ellipse lines show the 95% confidence interval (standard deviation). p-values indicate significance of grouping with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test.



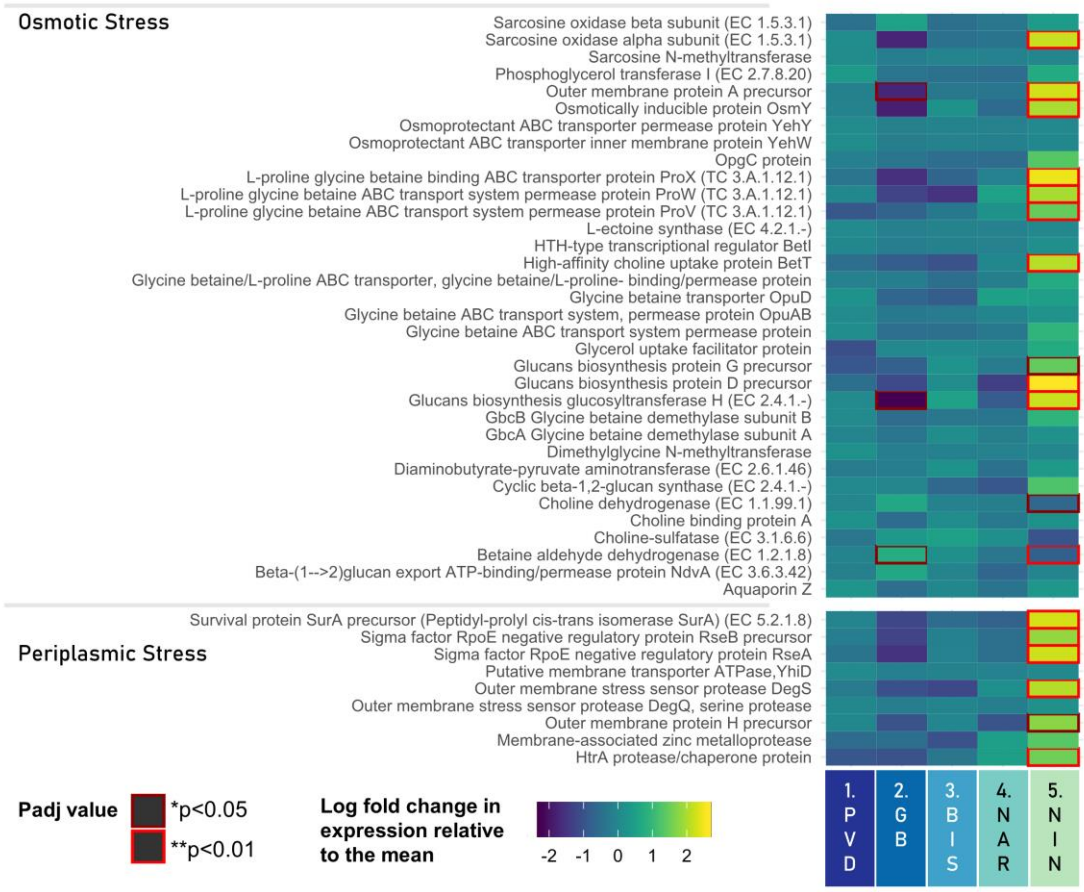
Supplementary Figure 2. Number of bacterial Orders shared between the oyster gut and seawater 16S rRNA amplicons at each site (vertical bars). The total number of Orders found in each group is shown in the horizontal bar graph on the right. Intersections in gray denote comparisons that include the water samples.



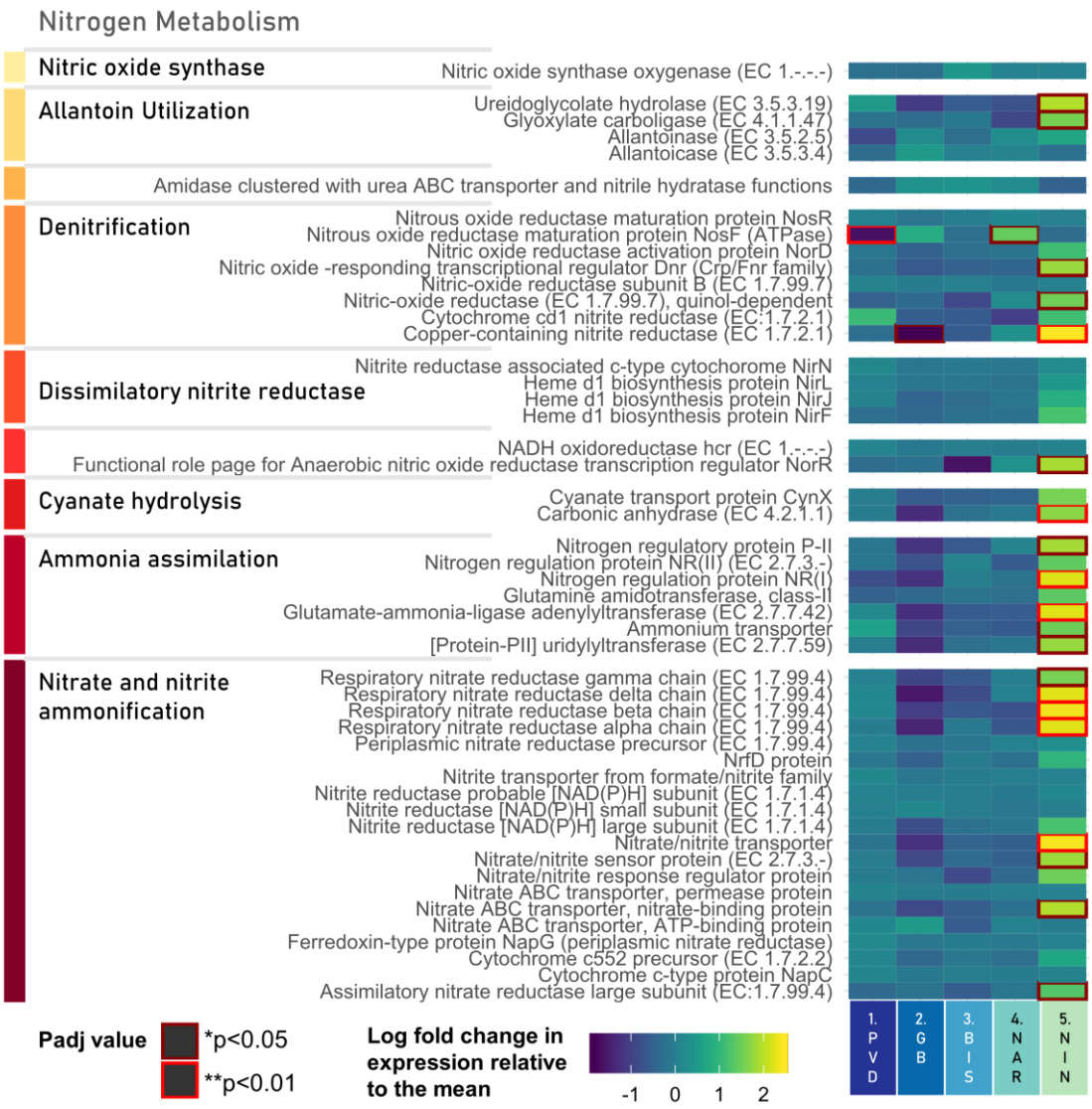
Supplementary Figure 3. NMDS plot visualization of Bray-Curtis beta-diversity (k = 2) at the Species level for gut metatranscriptomic samples by Site. The ellipse lines show the 95% confidence interval (standard deviation). p-values indicate significance of grouping with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test.



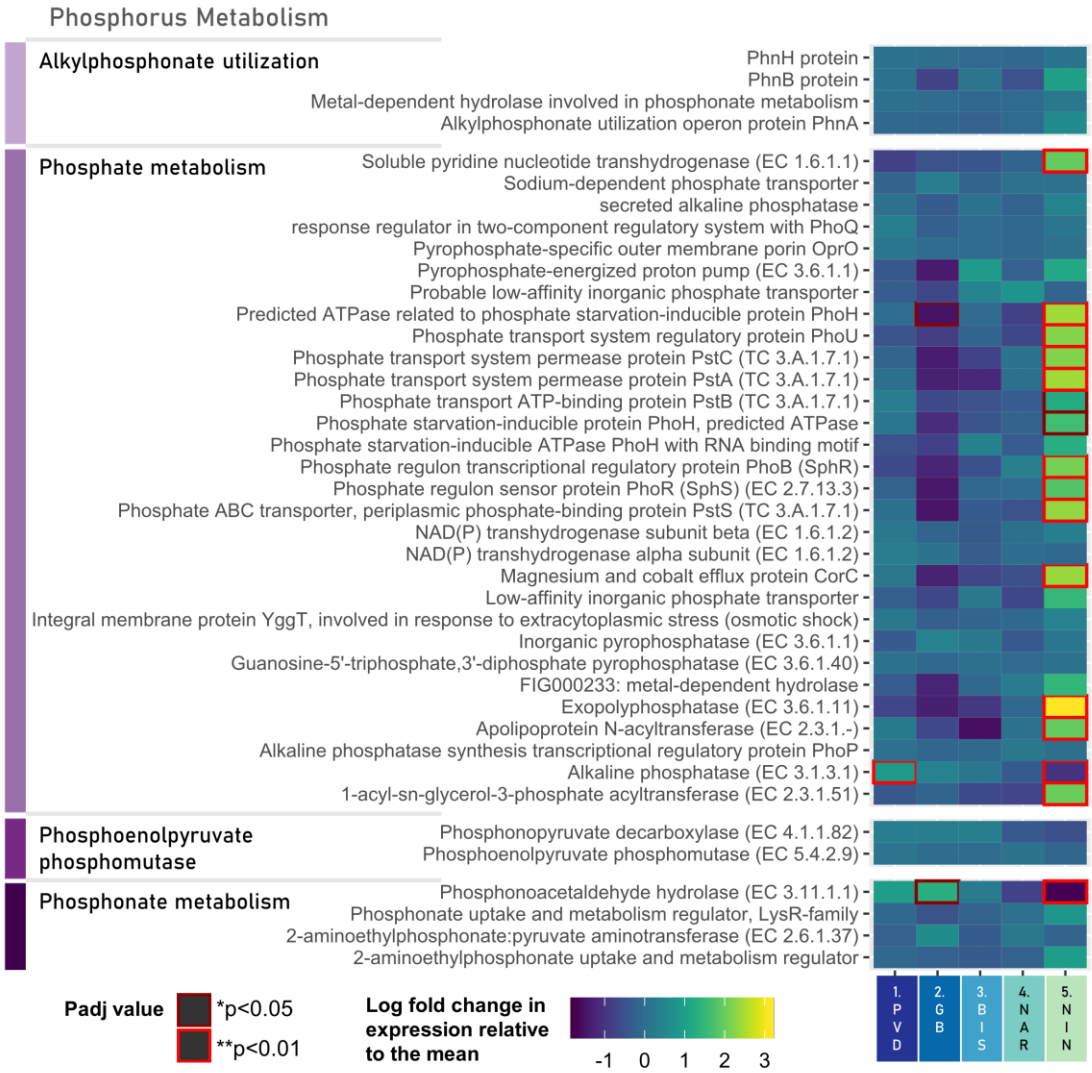
Supplementary Figure 4. Differential expression (log fold change) of Level 4 gene annotation of Oxidative stress response groups at each site, relative to the mean of the others. All significantly regulated genes are outlined in red.



Supplementary Figure 5. Differential expression (log fold change) of Level 4 gene annotation of Osmotic and Periplasmic stress response groups at each site, relative to the mean of the others. All significantly regulated genes are outlined in red.



Supplementary Figure 6. Differential expression (log fold change) of level 4 gene annotation of nitrogen metabolism pathways at each site, relative to the mean of the others. All significantly regulated genes are outlined in red.



Supplementary Figure 7. Differential expression (log fold change) of level 4 gene annotation of phosphorus metabolism pathways at each site, relative to the mean of the others. All significantly regulated genes are outlined in red.

Supplementary Table 1. Sequencing summary statistics, including the number of reads that passed quality control (QC) in each 16S rRNA amplicon and metatranscriptomic sample. No metatranscriptomes were sequenced for water samples.

Sample Name	*Sample Type*	*Number of QC'd 16S Amplicon Reads*	*Number of QC'd Metatranscriptomic Reads*
1.PVD.1	gut	44017	
1.PVD.2	gut	23147	
1.PVD.3	gut	12526	15,492,554
1.PVD.4	gut	48164	21,147,198
1.PVD.5	gut	12269	12,693,972
1.PVD.6	gut	18405	19,559,038
1.PVD.7	gut	15334	
1.PVD.8	gut	39536	17,838,913
1.PVD.9	gut	8302	
1.PVD.10	gut	44423	
2.GB.1	gut	35784	20,572,807
2.GB.2	gut	41874	20,247,959
2.GB.3	gut	57064	
2.GB.4	gut	66328	
2.GB.5	gut	14269	15,391,203
2.GB.6	gut	30679	7,342,452
2.GB.7	gut	39547	21,364,544
2.GB.8	gut	42135	
2.GB.9	gut	36380	
2.GB.10	gut	11366	
3.BIS.1	gut	27638	
3.BIS.2	gut	31156	
3.BIS.3	gut	24509	17,534,930
3.BIS.4	gut	34760	16,169,419
3.BIS.5	gut	34530	
3.BIS.6	gut	58360	
3.BIS.7	gut	28715	20,216,183
3.BIS.8	gut	48028	
3.BIS.9	gut	9891	18,440,372
3.BIS.10	gut	30931	18,960,268
4.NAR.1	gut	41807	10,663,545
4.NAR.2	gut	35900	14,799,611
4.NAR.3	gut	50945	
4.NAR.4	gut	65248	

4.NAR.5	gut	44783	
4.NAR.6	gut	28186	14,708,519
4.NAR.7	gut	32686	13,545,684
4.NAR.8	gut	54944	
4.NAR.9	gut	22411	13,950,925
4.NAR.10	gut	57266	
5.NIN.1	gut	52428	
5.NIN.2	gut	24707	15,180,735
5.NIN.3	gut	78086	
5.NIN.4	gut	37135	15,447,506
5.NIN.5	gut	57846	
5.NIN.6	gut	42666	
5.NIN.7	gut	26419	17,064,359
5.NIN.8	gut	4467	
5.NIN.9	gut	30093	13,277,188
5.NIN.10	gut	50503	17,421,647
1.PVD.W1	water	26702	
1.PVD.W2	water	35604	
2.GB.W1	water	33545	
2.GB.W2	water	26888	
3.BIS.W1	water	48289	
3.BIS.W2	water	31249	
4.NAR.W1	water	65630	
4.NAR.W2	water	33277	
5.NIN.W1	water	57394	
5.NIN.W2	water	50633	

Supplementary Table 2. Results of One-Way ANOVA for environmental conditions measured by the YSI Sonde probe per site. Pairwise p-values were calculated with a Student's T-test, adjusted p-value with Benjamini-Hochberg method.

Environmental Condition		***All Sites***	***1.PVD: 2.GB***	***1.PVD: 3.BIS***	***1.PVD: 4.NAR***	***1.PVD: 5.NIN***	***2.GB: 3.BIS***	***2.GB: 4.NAR***	***2.GB: 5.NIN***	***3.BIS: 4.NAR***	***3.BIS: 5.NIN***	***4.NAR: 5.NIN***
Temperature (°C)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	0.27	0.366	0.827	0.104	0.851	0.37	0.429	0.555	0.225	0.741	0.301
	P adj	0.27	0.71	0.85	0.71	0.85	0.71	0.71	0.79	0.71	0.85	0.71
Salinity (psu)	DF	**4**	1	1	1	1	1	1	1	1	1	1
	P value	**3.97E-04**	0.244	0.162	0.127	0.186	**0.0181**	**0.00258**	0.638	**0.00657**	0.233	**0.0177**
	P adj	**4.00E-04**	0.27	0.27	0.25	0.27	**0.045**	**0.026**	0.64	**0.033**	0.27	**0.045**
		*******					*****	******		******		*****
pH	DF	**4**	1	1	1	1	1	1	1	1	1	1
	P value	**0.0108**	0.994	**0.00488**	0.486	**0.00227**	0.21	0.552	0.129	0.295	0.0177	0.154
	P adj	**0.011**	0.99	**0.024**	0.61	**0.023**	0.35	0.61	0.31	0.42	0.059	0.31
		*****		******		******						
Chlorophyll- a (µg/L)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	0.0583	0.254	0.453	0.42	0.363	0.2	0.218	0.214	0.923	0.673	0.523
	P adj	0.058	0.64	0.65	0.65	0.65	0.64	0.64	0.64	0.92	0.75	0.65
Dissolved Oxygen (mg/L)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	0.4	0.793	0.133	0.363	0.282	0.436	0.671	0.362	0.522	0.695	0.482
	P adj	0.4	0.79	0.75	0.75	0.75	0.75	0.77	0.75	0.75	0.77	0.75

Supplementary Table 3. Results of One-Way ANOVA for nutrient concentrations measured from seawater samples with the Lachat nutrient analyzer. Pairwise p-values were calculated with a Student's T-test, adjusted p-value with Benjamini-Hochberg method. Note that the Nitrite concentrations measured at 2.GB, 4.NAR, and 5.NIN were 0±0.

Measurement		*All Sites*	*1.PVD: 2.GB*	*1.PVD: 3.BIS*	*1.PVD: 4.NAR*	*1.PVD: 5.NIN*	*2.GB: 3.BIS*	*2.GB: 4.NAR*	*2.GB: 5.NIN*	*3.BIS: 4.NAR*	*3.BIS: 5.NIN*	*4.NAR: 5.NIN*
Ammonium (µM)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	< 2E-16	0.023	2.8E-10	0.011	1.2E-07	3.6E-10	0.0078	0.00031	6.8E-06	1.1E-09	0.0026
	P adj	< 2E-16	0.023	1.8E-09	0.012	2.9E-07	1.8E-09	0.0097	0.00052	1.4E-05	3.8E-09	0.0038
		****	*	****	*	****	****	**	***	****	****	**
Nitrite (µM)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	1.0E-04	1.4E-04	2.6E-03	1.4E-04	1.4E-04	0.36			0.36	0.36	
	P adj	1.0E-04	3.2E-04	4.5E-03	3.2E-04	3.2E-04	0.36			0.36	0.36	
		***	***	**	***	***						
Nitrate (µM)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	< 2E-16	6.3E-08	4.5E-10	4.3E-06	1.8E-05	0.20	0.039	0.0032	0.34	0.00020	3.5E-05
	P adj	< 2E-16	3.1E-07	4.5E-09	1.4E-05	4.6E-05	0.22	0.049	0.0045	0.34	0.00033	7.0E-05
		****	****	****	****	****		*	**		***	****
Phosphate (µM)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	< 2E-16	1.5E-06	1.3E-05	9.2E-05	2.5E-05	1.1E-04	8.1E-05	3.8E-05	4.5E-07	5.5E-07	0.079
	P adj	< 2E-16	5.1E-06	3.3E-05	1.1E-04	4.9E-05	1.2E-04	1.1E-04	6.3E-05	2.7E-06	2.7E-06	0.079
		****	****	****	****	****	***	****	****	****	****	

Supplementary Table 4. Results of One-Way ANOVA for oyster measurements. Pairwise p-values were calculated with a Student's T-test, adjusted p-value with Benjamini-Hochberg method.

Measurement		*All Sites*	*1.PVD: 2.GB*	*1.PVD: 3.BIS*	*1.PVD: 4.NAR*	*1.PVD: 5.NIN*	*2.GB: 3.BIS*	*2.GB: 4.NAR*	*2.GB: 5.NIN*	*3.BIS: 4.NAR*	*3.BIS: 5.NIN*	*4.NAR: 5.NIN*
Mass (g)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	< 2E-16	0.0010	0.017	1.9E-12	2.3E-13	8.1E-05	2.5E-07	2.9E-09	3.1E-08	7.3E-09	0.013
	P adj	< 2E-16	0.0012	0.017	9.4E-12	2.3E-12	1.2E-04	4.2E-07	9.6E-09	6.1E-08	1.8E-08	0.015
		****	***	*	****	****	****	****	****	****	****	*
Length (mm)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	< 2E-16	0.0016	0.30	4.3E-06	1.1E-07	3.4E-05	0.0033	2.9E-06	7.5E-08	2.6E-09	0.032
	P adj	< 2E-16	0.0023	0.30	8.6E-06	3.8E-07	5.6E-05	0.0041	7.2E-06	3.7E-07	2.6E-08	0.036
		****	**		****	****	****	**	****	****	****	*
With (mm)	DF	4	1	1	1	1	1	1	1	1	1	1
	P value	< 2E-16	0.010	0.66	6.8E-14	2.7E-11	0.053	1.2E-11	9.6E-09	4.9E-12	1.7E-09	0.036
	P adj	< 2E-16	0.015	0.66	6.8E-13	6.8E-11	0.058	3.9E-11	1.6E-08	2.4E-11	3.4E-09	0.045
		****	*		****	****		****	****	****	****	*

Supplementary Table 5. Kruskal-Wallis Rank Sum Test for the Simpson's Index of Diversity values calculated using ASV-level 16S rRNA amplicons by Sample Type and Site. Pairwise p-values were calculated with the Wilcox rank-sum test.

All Sites – *Gut*				
	DF	**Chi-Squared**	**P value**	
All Sites	4	29.528	6.11E-06	***
1.PVD: 2.GB	1		0.079	
1.PVD: 3.BIS	1		0.912	
1.PVD: 4.NAR	1		0.0042	**
1.PVD: 5.NIN	1		0.0018	**
2.GB: 3.BIS	1		0.062	
2.GB: 4.NAR	1		0.00011	**
2.GB: 5.NIN	1		0.00022	***
3.BIS: 4.NAR	1		0.00069	***
3.BIS: 5.NIN	1		0.00648	**
4.NAR: 5.NIN	1		0.76	
All Sites – *Water*				
	DF	**Chi-Squared**	**P value**	
All Sites	4	8.4	0.0780	

Supplementary Table 6. Welch Two Sample T-Test for select Orders detected in 16S rRNA amplicons by Sample Type (gut vs. seawater), adjusted p-value with Benjamini-Hochberg method.

Gut vs. Water Samples			
Taxa	**P value**	**P adj**	
Actinobacteria;Actinobacteria;Corynebacteriales	8.25E-08	5.80E-07	****
Bacteroidetes;Bacteroidia;Cytophagales	8.31E-03	1.20E-02	**
Bacteroidetes;Bacteroidia;Flavobacteriales	3.92E-03	6.10E-03	**
Cyanobacteria;Oxyphotobacteria;Chloroplast	1.68E-08	1.60E-07	****
Cyanobacteria;Oxyphotobacteria;Synechococcales	4.50E-05	1.40E-04	****
Cyanobacteria;Oxyphotobacteria	2.87E-01	3.10E-01	
Firmicutes;Bacilli;Bacillales	2.95E-02	3.60E-02	*
Firmicutes;Bacilli;Lactobacillales	4.52E-04	9.70E-04	***
Firmicutes;Clostridia;Clostridiales	1.04E-06	4.90E-06	****
Fusobacteria;Fusobacteriia;Fusobacteriales	6.42E-04	1.30E-03	***
Proteobacteria;Alphaproteobacteria;Caulobacterales	2.82E-04	6.90E-04	***
Proteobacteria;Alphaproteobacteria;Rhodobacterales	8.91E-04	1.70E-03	***
Proteobacteria;Alphaproteobacteria;SAR11.clade	1.16E-05	4.60E-05	****
Proteobacteria;Alphaproteobacteria	5.38E-01	5.60E-01	
Proteobacteria;Deltaproteobacteria;Desulfobacterales	6.35E-09	8.90E-08	****
Proteobacteria;Gammaproteobacteria;Aeromonadales	9.03E-02	1.00E-01	
Proteobacteria;Gammaproteobacteria;Betaproteobacteriales	4.53E-02	5.30E-02	*
Proteobacteria;Gammaproteobacteria;Cellvibrionales	5.56E-03	8.20E-03	**
Proteobacteria;Gammaproteobacteria;Enterobacteriales	2.72E-02	3.50E-02	*
Proteobacteria;Gammaproteobacteria;Legionellales	1.34E-03	0.0024	**
Proteobacteria;Gammaproteobacteria;Oceanospirillales	1.91E-05	0.000067	****
Proteobacteria;Gammaproteobacteria;Pseudomonadales	8.30E-01	0.83	
Proteobacteria;Gammaproteobacteria;Vibrionales	1.85E-04	0.00052	***
Proteobacteria;Gammaproteobacteria	2.45E-02	0.033	*
Tenericutes;Mollicutes;Mycoplasmatales	1.92E-03	0.0032	**
Tenericutes;Mollicutes	2.98E-04	6.90E-04	***
Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales	1.00E-06	4.9E-06	****
Unknown	5.01E-13	1.4E-11	****

Supplementary Table 7. Welch Two Sample T-Test for select Orders detected in 16S rRNA amplicons from gut samples by Sampling Site, adjusted p-value with Benjamini-Hochberg method.

Taxa		1.PVD: 2.GB	1.PVD: 3.BIS	1.PVD: 4.NAR	1.PVD: 5.NIN	2.GB: 3.BIS	2.GB: 4.NAR	2.GB: 5.NIN	3.BIS: 4.NAR	3.BIS: 5.NIN	4.NAR: 5.NIN
Actinobacteria;	p	0.0083	0.0092	0.0077	0.00093	0.32	5.3E-05	8.9E-05	5.7E-05	9.3E-05	0.062
Actinobacteria;	p.adj	0.052	0.056	0.051	0.015	0.51	0.003	0.003	0.003	0.003	0.19
Corynebacteriales					*		**	**	**	**	
Bacteroidetes;	p	0.046	0.014	0.078	0.0065	0.057	0.99	0.0073	0.060	0.010	0.0073
Bacteroidia;	p.adj	0.15	0.071	0.21	0.049	0.18	1	0.051	0.18	0.059	0.051
Cytophagales					*						
Bacteroidetes;	p	0.15	0.75	0.0040	0.52	0.37	0.19	0.23	0.18	0.81	0.10
Bacteroidia;	p.adj	0.33	0.86	0.04	0.68	0.54	0.38	0.4	0.37	0.89	0.25
Flavobacteriales				*							
Cyanobacteria;	p	0.017	0.017	0.016	0.017	0.72	0.37	0.98	0.20	0.67	0.089
Oxyphotobacteria	p.adj	0.077	0.077	0.077	0.077	0.84	0.55	0.99	0.38	0.78	0.23
Cyanobacteria;	p	0.017	0.078	0.79	0.17	0.14	0.021	0.0060	0.11	0.0084	0.082
Oxyphotobacteria;	p.adj	0.077	0.21	0.88	0.35	0.3	0.089	0.047	0.25	0.052	0.22
Chloroplast								*			
Cyanobacteria;	p	0.002	0.022	3.6E-06	0.00024	0.0038	2.0E-06	6.3E-05	2.5E-06	9.6E-05	0.0030
Oxyphotobacteria;	p.adj	0.027	0.094	0.00034	0.0055	0.04	0.00034	0.003	0.00034	0.003	0.033
Synechococcales		*		***	**	*	***	**	***	*	*
Firmicutes;	p	0.10	0.40	0.037	0.045	0.23	0.26	0.39	0.19	0.20	0.54
Bacilli;	p.adj	0.25	0.56	0.13	0.15	0.4	0.43	0.56	0.38	0.38	0.7
Bacillales											
Firmicutes;	p	0.12	0.64	0.21	0.051	0.27	0.24	0.10	0.47	0.12	0.00089
Bacilli;	p.adj	0.27	0.76	0.39	0.16	0.45	0.41	0.25	0.63	0.27	0.015
Lactobacillales											*

Taxa		1.PVD: 2.GB	1.PVD: 3.BIS	1.PVD: 4.NAR	1.PVD: 5.NIN	2.GB: 3.BIS	2.GB: 4.NAR	2.GB: 5.NIN	3.BIS: 4.NAR	3.BIS: 5.NIN	4.NAR: 5.NIN
Firmicutes;	p	0.014	0.031	0.034	0.032	0.33	0.085	0.12	0.78	0.88	0.85
Clostridia;	p.adj	0.071	0.12	0.13	0.12	0.51	0.22	0.27	0.88	0.92	0.92
Clostridiales											
Fusobacteria;	p	0.33	0.79	0.022	0.0044	0.29	0.14	0.016	0.033	0.0041	0.0027
Fusobacteriia;	p.adj	0.51	0.88	0.093	0.041	0.47	0.31	0.077	0.13	0.04	0.033
Fusobacteriales					*					*	*
Proteobacteria;	p	0.34	0.32	0.36	0.39	0.51	0.66	0.41	0.12	0.18	0.54
Alphaproteobacteria	p.adj	0.51	0.51	0.53	0.56	0.67	0.77	0.57	0.27	0.36	0.7
Proteobacteria;	p	0.13	0.24	0.059	0.78	0.76	0.044	0.18	0.085	0.32	0.11
Alphaproteobacteria;	p.adj	0.3	0.42	0.18	0.88	0.87	0.15	0.37	0.22	0.51	0.26
Caulobacterales							*				
Proteobacteria;	p	0.60	0.34	0.61	0.067	0.75	0.42	0.20	0.20	0.29	0.044
Alphaproteobacteria;	p.adj	0.73	0.51	0.73	0.2	0.86	0.59	0.38	0.38	0.46	0.15
Rhodobacterales											*
Proteobacteria;	p	0.95	0.99	0.50	0.87	0.93	0.47	0.82	0.50	0.88	0.58
Alphaproteobacteria;	p.adj	0.96	0.99	0.66	0.92	0.95	0.63	0.9	0.67	0.92	0.71
SAR11.clade											
Proteobacteria;	p	0.21	0.046	0.88	0.46	0.25	0.22	0.038	0.048	0.014	0.29
Deltaproteobacteria;	p.adj	0.39	0.15	0.92	0.62	0.42	0.4	0.14	0.16	0.071	0.46
Desulfobacterales			*						*	*	
Proteobacteria;	p	0.14	0.35	0.74	0.0048	0.20	0.16	0.39	0.56	0.063	0.018
Gammaproteobacteria	p.adj	0.3	0.52	0.85	0.042	0.38	0.34	0.56	0.7	0.19	0.079
					**						*
Proteobacteria;	p	0.025	0.0096	0.29	0.0069	0.43	0.28	0.24	0.13	0.34	0.094
Gammaproteobacteria	p.adj	0.1	0.057	0.46	0.051	0.6	0.46	0.41	0.29	0.51	0.24
Aeromonadales		*	**		**						

Taxa		1.PVD: 2.GB	1.PVD: 3.BIS	1.PVD: 4.NAR	1.PVD: 5.NIN	2.GB: 3.BIS	2.GB: 4.NAR	2.GB: 5.NIN	3.BIS: 4.NAR	3.BIS: 5.NIN	4.NAR: 5.NIN
Proteobacteria; Gammaproteobacteria Betaproteobacteriales	p	0.097	0.88	0.032	0.040	0.15	0.21	0.32	0.055	0.068	0.66
	p.adj	0.25	0.92	0.12	0.14	0.32	0.39	0.51	0.18	0.2	0.78
				*	*						
Proteobacteria; Gammaproteobacteria Cellvibrionales	p	0.21	0.87	0.25	0.35	0.22	0.082	0.58	0.18	0.39	0.10
	p.adj	0.38	0.92	0.42	0.52	0.4	0.22	0.71	0.36	0.56	0.25
Proteobacteria; Gammaproteobacteria Enterobacteriales	p	0.0003	0.00014	0.00013	0.80	0.61	0.55	0.012	0.95	0.0081	0.0076
	p.adj	0.0068	0.0036	0.0036	0.89	0.74	0.7	0.066	0.95	0.052	0.051
		**	**	**							
Proteobacteria; Gammaproteobacteria Legionellales	p	0.62	0.33	0.24	0.0019	0.24	0.20	0.0021	0.85	0.0016	0.0016
	p.adj	0.74	0.51	0.42	0.027	0.42	0.38	0.027	0.92	0.024	0.024
					*			*		*	*
Proteobacteria; Gammaproteobacteria Oceanospirillales	p	0.20	0.61	0.57	0.042	0.39	0.41	0.0029	0.96	0.013	0.011
	p.adj	0.38	0.73	0.71	0.15	0.56	0.58	0.033	0.97	0.068	0.062
								*			
Proteobacteria; Gammaproteobacteria Pseudomonadales	p	0.90	0.48	0.11	0.072	0.64	0.26	0.21	0.076	0.058	0.55
	p.adj	0.93	0.64	0.25	0.21	0.76	0.43	0.39	0.21	0.18	0.7
Proteobacteria; Gammaproteobacteria Vibrionales	p	0.51	0.82	0.95	0.0060	0.65	0.45	0.0046	0.84	0.0054	0.005
	p.adj	0.67	0.9	0.97	0.047	0.77	0.62	0.042	0.91	0.046	0.047
					*			*		*	*
Tenericutes; Mollicutes	p	0.031	0.094	0.45	0.16	0.86	0.021	0.90	0.074	0.80	0.13
	p.adj	0.12	0.24	0.62	0.34	0.92	0.089	0.93	0.21	0.88	0.29
Tenericutes; Mollicutes; Mycoplasmatales	p	0.14	0.60	0.23	0.16	0.06	0.37	0.55	0.10	0.072	0.56
	p.adj	0.3	0.73	0.41	0.34	0.19	0.54	0.7	0.25	0.21	0.7

Taxa		1.PVD: 2.GB	1.PVD: 3.BIS	1.PVD: 4.NAR	1.PVD: 5.NIN	2.GB: 3.BIS	2.GB: 4.NAR	2.GB: 5.NIN	3.BIS: 4.NAR	3.BIS: 5.NIN	4.NAR: 5.NIN
Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales	p	0.081	0.54	0.79	0.013	0.010	0.00070	0.0004	0.25	0.033	0.0040
	p.adj	0.22	0.7	0.88	0.07	0.059	0.013	0.0081	0.42	0.13	0.04
							*	**			*
Unknown	p	0.63	0.92	0.56	0.79	0.56	0.90	0.47	0.44	0.86	0.33
	p.adj	0.75	0.94	0.7	0.88	0.7	0.93	0.63	0.6	0.92	0.51

CHAPTER III: Nutrient Enrichment Affects Mechanisms of Nitrogen Cycling in Oyster-Associated Microbiomes

By

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Abstract

As keystone species in the coastal environment, oysters play a crucial role in nitrogen cycling, improving water quality for other marine organisms and humans. It is understood that host-associated microbes are responsible for the oyster's ability to promote nitrogen cycling within the environment. However, the composition of oyster-associated microbiomes and their physiological mechanisms driving nitrogen cycling and gas production are unknown. Adult oysters (30 oysters per container, 6 containers) were deployed for three months in the summer of 2017 at two sites in Point Judith Pond, Rhode Island, with different levels of ambient nitrogen loading. Within each site, three of the six containers were spiked with fertilizer to simulate nutrient runoff. Tissues (gut, inner and outer shell biofilms) from a subsample of oysters (n = 36, 3 per container) were collected for analysis of microbial community composition and function. The microbial community structures were determined by DNA amplification and sequencing of the 16S rRNA V6 region, and metatranscriptomes were sequenced to determine the transcriptional response of the oysters to nutrient enrichment. Both sample type and environmental conditions had a significant effect on microbial community structure and function in oysters. Expression of nitrogen metabolism genes was significantly different in each sample type and site, with significant changes in response to nutrient enrichment. Changes in the microbial community composition of outer shell biofilms likely led to a significant effect of location and nutrient enrichment on the denitrification ability of oysters when exposed to a high nutrient load. This study informs nitrogen cycling abilities of

microbial communities in oysters, and how this function is affected by nutrient enrichment.

Introduction

Oysters are keystone species in the coastal environment, and provide a range of ecosystem services, including habitat provision for other marine species, protection against erosion, and improvement of water quality by filtration (Burge et al., 2016; Grabowski et al., 2012). Furthermore, oysters play a crucial role in nitrogen cycling and removal, due to their effective filter feeding mechanisms and concentration of nutrients (Coen et al., 2007; Kellogg et al., 2014; Stief, 2013). Organisms in coastal ecosystems are threatened by many factors, including increased nutrient loading, increased anthropogenic inputs (toxins or heavy metals), warming temperatures, and decreased dissolved oxygen (Haigh et al., 2015; Wallace et al., 2014). This has led to altered ecosystem interactions due to increased mortality, compromised growth, disease outbreaks, and many other consequences (Groner et al., 2016; Hendriks et al., 2010; Kroeker et al., 2012; Ross et al., 2011).

Nitrogen is a particularly important nutrient in coastal ecosystems since it controls biomass at the bottom of the food web and may limit primary production (Howarth, 1988). In the coastal environment, nitrogen is constantly transformed by microbes as a main electron donor for metabolism (Enrich-Prast et al., 2016; Kellogg et al., 2013). These processes can be grouped intro reducing and oxidizing reactions as nitrogen is converted from inorganic species to gas or ammonium (Albright et al., 2018). Denitrification is defined as the anaerobic microbial reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to gaseous nitrous oxide (N_2O), nitric oxide (NO), and finally dinitrogen gas (N_2), effectively removing the nitrogen from the system (Knowles, 1982). This

process is a key ecosystem service provided by oysters, valued at $1385–$6716 USD per hectare of oyster reef per year in 2011 dollars (Grabowski et al., 2012).

Oysters and other benthic organisms remove both particulate and dissolved nitrogen in the coastal environment by assimilation into their tissues or shells or biodeposit sediment burial, and recycle bioavailable nitrogen to N_2 gas by coupled nitrification-denitrification (Kellogg et al., 2014). It is understood that host-associated bacteria are responsible for this ability to cycle dissolved nitrogen, particularly through denitrification by gut microbes (Caffrey et al., 2016a; Humphries et al., 2016; Smyth et al., 2015). Oyster-associated microbes are also known to produce nitrous oxide (N_2O) as a byproduct of nitrification, incomplete denitrification, or during decomposition by outer shell biofilms (Heisterkamp et al., 2013; Ray et al., 2019a; Stief et al., 2009). It is estimated that bivalves can reduce suspended particle concentrations by 45%, and remove 1-25% of annual nitrogen loads (Carmichael et al., 2012). However, the relative contribution of oysters and other bivalves to nitrogen removal by benthic ecosystems is highly variable across different regions and dependent upon the method used (Kellogg et al., 2013).

The effect of biotic and abiotic factors on nitrogen gas production in the marine environment, particularly in sediments and the water column, has been the focus of extensive research. Sediment N_2 and N_2O gas production is not controlled by NO_3^- concentrations, but by organic matter mineralization, dissolved oxygen, and substrate availability (Enrich-Prast et al., 2016; Highton et al., 2016; Seitzinger, 1988). A survey of water column nitrification rates at various pH values in Narragansett Bay, RI found that nitrification rates were negatively correlated with pH (Fulweiler et al.,

2011). In oxygen minimum zones, overall nitrogen loss occurs by combined anammox and heterotrophic denitrification (Lam and Kuypers, 2010; Penn et al., 2019). In bivalves, denitrification rates are determined by biofilm thickness (Suarez et al., 2019), nutrient loading (Grabowski et al., 2012; Lunstrum et al., 2018), habitat density (Smyth et al., 2015), and many other factors (Jetten, 2008). A comparison of gas production from oysters with and without their outer shell biofilm found that the outer shell releases low levels of N_2O, NO_2^-, and NH_4^+, while the oyster gut produces N_2-N and N_2O (Ray et al., 2019b). N_2O and N_2 production are known to be positively correlated with shellfish biomass (Heisterkamp et al., 2010; Stief et al., 2009).

A study by Gárate et al. (2019) found that a combination of warming and added nitrogen led to increased N_2O production in eastern oysters, compared to ambient conditions. A follow-up field study was conducted in Point Judith Pond, Rhode Island, USA (a well-mixed estuary in southern Rhode Island where many eastern oyster leases are held) (Hamilton, 2018; Hamilton et al., *in prep*). Farmed oysters were out planted at contrasting ends of the estuarine gradient of the Pond and half were treated with increased nutrients for 3 months. The oysters were brought to the laboratory and incubated with 100µM $NH_4^+NO_3^-$ (enrichment above seawater) to determine the impact of preexisting field conditions on gas production rates (N_2 and N_2O) under high nutrient loading (*i.e.* measurement of denitrification potential under high nutrient loading). Overall denitrification rates were higher at the southern site, where there was lower DO, higher temperature and chlorophyll-*a*. At the southern location, N_2 measurements shifted from net denitrification to nitrogen fixation under enriched

conditions (Hamilton, 2018). These changes are likely due to differences in the oyster microbiomes composition and/or function.

Nutrient enrichment possibly affects microbial communities in coastal environments, which likely alters gas production rates (Stevick et al., *in prep* (Chapter 2); Bulseco-McKim et al., 2017; Bulseco et al., 2019; Murphy et al., 2019). A study of genes involved in nitrous oxide flux and microbial community structure in salt marsh sediments determined that changes in gas production are due to microbial community structure changes, rather than just changes in microbial gene expression (Angell et al., 2018). On the other hand, increased nutrient levels did not affect sediment microbial community composition, but affected abundance of nitrogen cycling genes in a eutrophic lagoon (Highton et al., 2016). Yet, the natural mechanisms and controls of nitrogen cycling and gas production by the oyster commensal bacteria are mostly unknown. The oyster outer shell, inner shells, and tissues such as the gut provide significantly different environmental niches facilitating different microbiome structures and likely different contributions to nitrogen metabolism (Arfken et al., 2017; Barillé et al., 2017; King et al., 2019). A recent study by Arfken et al. (2017) used functional inference from 16S rRNA sequencing to estimate the denitrification potential of the oyster gut, shell, and sediment bacteria in relation to gas production measurements, and found that bacteria containing the *nosZI* gene (nitrous oxide reductase) are important for facilitating denitrification and differ between sample types. There is, however, no comprehensive characterization of the effects of nutrient enrichment on oyster gut, inner, and outer shell microbial community composition and function using high-throughput methods. There is a need to accurately characterize the

bacteria and transcribed genes that are responsible for nitrogen cycling by oysters, which may change in response to increased nutrient loading.

In this study, we used samples collected from a previous field study (Hamilton, 2018) to explore changes in oyster-associated microbial community structure and function in response to nutrient enrichment, within the context of nitrogen cycling. We used 16S rRNA amplicon and metatranscriptomic sequencing to assess: (a) the effect of site, nutrient enrichment within site, and tissue type (shell biofilms and gut) on microbial composition and function in oysters; and (b) which gene pathways associated to nitrogen cycling processes respond to nutrient loading in oyster-associated microbiomes. The results of the study inform how oysters contribute to estuarine ecosystems and how this may change in response to increased eutrophication and nutrients.

Methods

Field Experiment and Sample Collection

Farmed eastern oysters, *Crassostrea virginica*, were obtained from a farm located in Point Judith Pond, RI and deployed at Billington Cove (Northern, 41°25'17"N; 71°30'09"W) and Bluff Hill Cove (Southern, 41°23'24"N; 71°30'36"W), Point Judith Pond, RI, for 3 months from June-August 2017, as described in Hamilton (2018). At both locations, there were 6 buckets containing 30 oysters each, 3 maintained ambient conditions and 3 at enriched nutrient conditions (Figure 1). Enriched treatments were performed with Milorganite slow release pellet fertilizer (Worm et al., 2000) to target 20 µM inorganic nitrogen (~2% phosphorus). General environmental measurements

(dissolved oxygen - DO, salinity, chlorophyll-*a*, temperature, and pH) were taken every 15 minutes at each site throughout the 3-month experiment. Seawater dissolved inorganic nitrogen (DIN: NO_2^-, NO_3^-, NH_4^+) measurements were collected at high and low tide in each bucket the week before the experiment ended (Figure 1; Hamilton 2018).

Following the field incubations (Aug 2017), three oysters were randomly selected from each bucket for microbial community analysis (36 total). Field oysters collected for microbial community analysis were processed as follows. After sterile DI water rinse of the outer shell of the oysters, a sterile cotton swab was used to sample the microbial biofilm. The oysters were then shucked, and another sterile swab was used to sample the microbial biofilm in the inner shell of the top valve of the oyster. Finally, sterile dissection scissors were used to cut a >30 mg section of gut tissue from a consistent location. All swab and tissue samples were stored in 1.5 mL RNAlater (Invitrogen) for later analysis.

The reminder of the oysters were used to determine the effect of site and nutrient enrichment on denitrification potential under high nutrient loading. Briefly, oysters from each of the field bucket forts were transferred to tanks containing 100µM $NH_4^+NO_3^-$, incubated at 18°C and 24°C, and gas rates were determined. A summary of relevant environmental data and gas measurements previously reported in Hamilton (2018) is reported in Figure 1.

Nucleic Acid Extractions

DNA and RNA were co-extracted from each sample (inner and outer shell biofilm, and gut samples) using the Qiagen Allprep PowerViral DNA/RNA extraction

kit with modifications as follows. The swab samples were removed from the RNAlater and each washed with 1 mL of sterile, filtered 1X nuclease-free Phosphate Buffer Saline (PBS, pH 7.4, Invitrogen). The remaining RNAlater was centrifuged to pellet the excess cells, then washed with 1X PBS. The cell pellet was suspended in 600 µL of Qiagen Solution PV1 and added directly to a 0.1mm glass bead tube, along with the washed swab and 6 µL of sterile β-mercaptoethanol. The gut samples were each weighed and 30 mg was added to a 0.1 mm glass bead tube, in addition to 600 µL of Solution PV1 and 6 µL of sterile β-mercaptoethanol. All samples were then vortexed horizontally for 5 minutes for mechanical disruption, then 10 µL of proteinase K (Qiagen) was added and the samples were incubated at 55°C for 1 hour in a shaker at 300 rpm for chemical lysis. Following lysis of the tissues and cells, the supernatant was transferred a new 2 mL microcentrifuge tube and the protocol continued based on the manufacturer's protocol. The total nucleic acids in the samples were quantified using a Nanodrop 2000 instrument (ThermoFisher) and divided into equal volumes for RNA and DNA purification.

RNA was purified from a 30 µL total nucleic acid aliquot using the DNase Max 1 kit in a 50 µL reaction volume following the manufacturer's protocol (Qiagen). DNA was purified using the final steps of the DNeasy PowerLyzer PowerSoil Kit (Qiagen). The 30 µL total nucleic acid aliquot was combined with 1200 µL of Solution C4, then vortexed to mix. Digestion of RNA was then performed by adding 4 µL of RNase A solution and incubating for 2 minutes at room temperature. The treated DNA was then washed and purified on the spin column, and eluted into a 50 µL volume. Purified RNA and DNA concentrations and quality were quantified with both a Qubit

Fluorometer High-Sensitivity reagents (Invitrogen) and Nanodrop 2000 instrument (ThermoFisher).

Nucleic Acid Amplification and Sequencing

Bacterial targeted 16S rRNA amplicons and metatranscriptomes were sequenced from the DNA and RNA samples, respectively. PCR reactions were performed in triplicate using 967F/1064F primers with partial Illumina tails (V6 region, custom IDT primers), Phusion High-Fidelity DNA Polymerase (ThermoScientific), nuclease-free water (FisherScientific), and template DNA according to protocols from the Keck Sequencing Center at the Marine Biological Laboratory (Huse et al., 2014). Due to high levels of oyster DNA in the gut samples, 300 ng of input DNA was used from gut samples and 5 ng was used per swab sample to obtain equivalent bacterial amplification, as determined by gel electrophoresis. A bacterial DNA mock community (10 ng) and blank control were also amplified and included in the analysis (Zymo Research). All PCR products (110 total: 36 per sample type and 2 controls) were analyzed with 75 bp paired-end sequencing to obtain overlapping reads on an Ilumina MiSeq at the University of Rhode Island Genomics and Sequencing Center.

Triplicate RNA samples (n=36) were pooled in equimolar concentrations per experimental bucket and sample type to avoid pseudo-replication and align with environmental measurements in each bucket. Pooled RNA samples were fragmented at 300 nt using Covaris ultrasonification and verified using an Agilent Bioanalyzer. 500 ng of each sample was treated with the Ribo-Zero Gold rRNA Removal Epidemiology Kit (Illumina) to remove eukaryotic and bacterial DNA, and then libraries were prepared using the Illumina TruSeq PCR-free kit. Libraries were

verified using both a KAPA library quantification kit and an Agilent Bioanalyzer, then normalized to 1 nM for sequencing. The 36 metatranscriptomic libraries were sequenced using a half lane of Illumina NovaSeq S4 chemistry to obtain 2x150 bp paired-end reads at the Yale Center for Genome Analysis.

16S Amplicon Sequence Processing and Statistical Analysis

The paired-end 16S rRNA amplicon sequences were demultiplexed, then quality filtered and merged with DADA2 (v1.6.0) executed in QIIME2 (v2018.4.0) to calculate analysis sequence variants (ASVs) (Bolyen et al., 2019; Callahan et al., 2016). The resulting ASVs were analyzed with default parameters in QIIME2 and directly classified using the SILVA database at 99% similarity (release #132) (Bokulich et al., 2018). The ASV and taxonomy data was exported as a matrix for further analysis in R (v3.6.1) and normalized by percentage per sample (R Development Core Team, 2011). Percentage data per ASV was averaged for replicate samples from the same sample type and bucket (n=3), and this averaged amplicon data was used for further statistical testing.

All statistical analyses of the 16S amplicon data were performed in the R statistical environment (v3.6.1) and visualized using *ggplot2* v3.2.1 and *cowplot* v1.0.0 (Wickham, 2009; Wilke, 2019). ASV data was organized and cleaned using the *dplyr* and *tidyr* v0.8.3 packages (Wickham et al., 2019; Wickham and Henry, 2019). Significant changes between taxa were calculated using the Kruskal-Wallis and Wilcox rank sum tests with the compare_means() command from the *ggpubr* v0.2.2 package (Kassambara, 2019). Non-Metric Dimensional Scaling (NMDS) plots were generated with ASV-level percent abundances using metaMDS(distance = "bray", k =

2) and significance was determined using adonis2() from the *vegan* v2.5.5 package (Dixon, 2003). A Principle Components Analysis (PCA) of the correlation between major phyla and environmental data was calculated using prcomp(scale.=TRUE) and plotted using autoplot() within the *ggfortify* v0.4.7 package (Tang et al., 2016). Distances between total community samples based on the Manhattan similarity metric were calculated using ASV-level percent abundances with dist(method="manhattan") and plotted using the *pheatmap* v1.0.12 package (Kolde, 2019).

Metatranscriptome Processing and Statistical Analysis

Quality filtering of the demultiplexed raw paired-end shotgun metatranscriptomic reads was performed with Trimmomatic software v0.36 and visualized with FastQC v0.11.5 (Andrews, 2010; Bolger et al., 2014). The quality-controlled PE reads were then mapped to the eastern oyster, *C. virginica*, genome (NCBI GCA 002022765.4-3.0) using HISAT2 v2.0.4 with option –un-conc to remove the host transcripts and save the non-aligned (non-oyster) reads (Pertea et al., 2016). These non-aligned reads were then analyzed for microbial taxonomy and function using scripts adapted from the SAMSA2 pipeline and summarized with MultiQC v1.7 (Ewels et al., 2016; Westreich et al., 2018). PEAR v0.9.10 software was used to merge the non-aligned PE reads for downstream analysis with parameter --min-overlap 1 (Zhang et al., 2014). Species-level taxonomy was assigned to the merged reads using Kraken2 v2.0.7 and summarized by percent abundance per taxonomic level (Phylum, Order) using Bracken v2.2 (Lu et al., 2017; Wood and Salzberg, 2014). Finally, DIAMOND v0.9.23 was used to annotate the reads according to RefSeq functional taxonomy and cluster genes into SEED pathway subsystems (Buchfink et al., 2014).

Taxonomic and functional annotation tables were exported for statistical analysis in the R statistical environment (v3.6.1), using *ggplot* v3.2.1 visualization and other packages listed above. A heatmap of the distance between sample taxonomy in the metatranscriptomes was calculated using species-level percent abundance with the command dist(method="manhattan") and plotted with *pheatmap*. Normalized functional abundance and differential expression were calculated with custom scripts using DeSeq2 v1.24.0, with the *apeglm* shrinkage method: lfcShrink(type="apeglm") (Love et al., 2014; Zhu et al., 2019). The rlog() transformation was used on all genes with counts >5 to observe overall trends in the metatranscriptomes. Sample distances based on normal-transformed gene counts (normTransform()) was calculated using dist(method="manhattan"). In order to observe bulk trends between sample types, the $\log_2$ fold change of genes and pathways expressed by each group (gut, outer shell, inner shell) was compared to the mean of the other 2 groups, regardless of site or treatment. The effect of nutrient enrichment was calculated by using each ambient sample group (site, type) as a control group for the corresponding enriched samples. Significance in differential expression was calculated in DeSeq2 with the Benjamini-Hochberg adjustment method.

Results

Sequencing and Annotation of 16S rRNA Amplicons and Metatranscriptomes

The sequencing of 108 16S rRNA V6 amplicon libraries resulted in 5.4 million paired-end (PE) reads in total. Quality filtering using DADA2 implemented in QIIME2 created 37,600 ± 9,000 merged and annotated amplicon reads per sample,

spread across 14,050 ASVs (Figure 2A). A total of 1.7 billion quality-controlled PE sequencing reads were obtained from 36 metatranscriptomic samples, averaging 47,500,000 (±22,000,000) PE reads per sample (Table S1). An average of 11±16% of the quality-controlled reads per sample mapped to the *C. virginica* genome and were removed prior to downstream microbial analyses. There were significantly more reads that mapped to the oyster genome in the gut and inner shell samples than the outer shell samples (Wilcox, $p<0.001$; Figure S1). Paired-end read merging resulted in 41,200,000 (±22,000,000) sequences per sample that were annotated using Kraken for taxonomy and DIAMOND/SEED for function. Microbial taxonomy was assigned to 35±7% of the metatranscriptomic reads, and function annotation was assigned to 5±2% of the reads (~2±1.6million reads per sample) (Table S1). This low annotation rate is comparable with other environmental metatranscriptomic studies (Broberg et al., 2018; Crump et al., 2018; Jiang et al., 2016) and is probably due to incomplete reference databases.

Microbial Community Structures Differ Between Oyster Tissues, Field Site, and Nutrient Enrichment

The 14,050 ASVs generated from the 16S rRNA amplicon sequences were classified into 260 Orders across 53 Phyla. The bacterial communities detected in the gut, inner shell, and outer shell were significantly different to each other (Bray-Curtis, k=2, adonis2 $p<0.001$; Figures 2B and S2A). This was due to distinct patterns in the bacterial community structures between the sample types. At the phylum level, gut samples contained significantly more *Tenericutes* (17±12%), *Verrucomicrobia* (4±2%), and Unknown taxa (23±13%) than the shell biofilms (*Tenericutes*: 0.1±0.2%;

Unknown: 11±6%) (Wilcox, $p<0.001$; Figure 2B). Orders that were significantly more abundant in the gut samples include *Mycoplasmatales* (12±11%), *Entomoplasmatales* (5±8%), *Verrucomicrobiales* (4±2%), and *Corynebacteriales* (2±2%) (Wilcox, $p<0.001$; Figure S3). The outer and inner shell samples contained significantly more *Proteobacteria* (62±9%; 56±11%) and the inner shell samples contained increased *Bacteroidetes* (11±9%) compared to the gut samples (Wilcox, $p<0.001$; Figure 2B).

The overall bacterial community structures (all sample types combined) were also significantly different by field site and enrichment treatment at the ASV level (Bray-Curtis, k=2, adonis2 $p<0.05$; interaction $p<0.001$; Figure S2B). Furthermore, the bacterial communities for each sample type (inner and outer shell, gut) were significantly different by site (Bray-Curtis, k=2, 95% confidence, adonis2 $p<0.001$; Figure 2C). Nutrient enrichment had a significant effect on the bacterial community structures in the gut samples from the northern site and the outer shell samples from both sites (Bray-Curtis, k=2, adonis2 $p<0.01$; Figure 2C). In the outer shell, this shift was driven by changes in the *Cyanobacteria*, *Bacteroidetes*, and *Actinobacteria* phyla (Figure 2B).

A PCA analysis of the bacterial phyla illustrates 46% of the variation between 16S rRNA amplicon structures (Figure 3). The presence of the orders *Tenericutes*, *Verrucomicrobia*, and *Firmicutes* was associated with gut samples of oysters collected at the northern location, while the presence of *Actinobacteria* defined gut samples of oysters from the southern location. On the other end, *Proteobacteria*, *Planctomycetes*, and *Chloroflexi* correlated with the northern outer shell samples. *Bacteroidetes* and *Epsilonbacteraeota* were associated with the inner shell samples. In order to identify

bacterial orders that may be associated with the differences in ability of oysters from the different sites and nutrient enrichment treatments to denitrify or produce nitrous oxide under high nutrient loading (as determined by gas rates measured in oysters collected from the same original buckets after transfer from the field to tanks containing higher levels of nitrogen (100 µm) and incubated at two different temperatures, Hamilton 2018), gas rate productions under those conditions were included in the PCA. Results from the PCA suggest that the relatively higher abundance of *Actinobacteria* observed in shell biofilm samples from the enriched buckets in the southern site (Figure 2) may explain differences in gas production rates (Hamilton, 2018).

When sample distances were calculated using the Manhattan similarity metric, samples clustered first by sample type, and then by location within each sample type (Figure 4A). This suggests that the physical structure of those samples (shell surfaces versus soft tissues), combined with host – microbial interactions and the specific environmental conditions microbes are exposed to within these oyster compartments, has a larger effect on bacterial community structure than the overall environmental conditions measured at each field site.

The Relative Effect of Field Site and Nutrient Enrichment on Microbial Gene Expression Differs from the Effect on Microbial Community Structure

The transcriptionally active microbes within oysters, as measured by taxonomic annotation of the metatranscriptomes, significantly differed by oyster sample type (Bray-Curtis, k=2, adonis2, $p<0.01$; Figure 4B), but not site or treatment. The overall functional activity of these microbes was also significantly different between sample

types, regardless of site or nutrient enrichment level (Bray-Curtis, k=2, adonis2, $p<0.001$; Figure 4C), a pattern of variability more similar to the one observed in the 16S rRNA amplicon clustering (Figure 4A).

A significant effect of site and enrichment, however, was detected on the gene expression patterns in the outer shell samples (Bray-Curtis, k=2, adonis2, $p<0.001$; Figure 4C; Figure S4). When samples were compared by treatment (regardless of site) within each sample type (gut, inner shell, outer shell) at the highest metabolic pathway annotation level (Level 1, SEED), the only significant difference was downregulation of genes related to metabolism of fatty acids, lipids, and isoprenoids in the gut samples (Benjamini-Hochberg adjusted $p<0.05$; Figure 5A). When samples were compared by site (regardless of treatment) for each sample type, photosynthesis and plasmid-related pathways were upregulated in the inner shell samples from oysters from the northern site, compared to oysters collected from the southern site (Benjamini-Hochberg adjusted $p<0.05$; Figure 5A). In the outer shell, secondary metabolism, respiration, regulation and cell signaling, and photosynthesis pathways were downregulated in the northern site compared to the southern site, while phage, prophages, and transposable elements were upregulated (Benjamini-Hochberg adjusted $p<0.05$; Figure 5A).

Evaluation of the effect of nutrient enrichment within each of the sites showed significant differences in gene expression in the gut tissues from oysters collected at the northern site and in outer shell tissues of oysters collected from the southern site (Figure 5B). In the gut samples of oysters collected from the northern site, nutrient enrichment resulted in significant upregulation of regulation and cell signaling, and significant downregulation of RNA metabolism, antibiotic resistance, protein

processing and modification, mono/di-/oligosaccharides, and fatty acids (Benjamini-Hochberg adjusted $p<0.01$; Figure 5B). A significant upregulation in stress responses, organic sulfur assimilation, fatty acid metabolism, and mycobacteria cell wall pathways was observed in the outer shell samples from the oysters exposed to nutrient enrichment at the southern site (Benjamini-Hochberg adjusted $p<0.01$; Figure 5B).

Effect of Sample Type on Expression of Genes involved in Nitrogen Metabolism

Further evaluation of patterns of differential expression of genes involved in nitrogen cycling using a heatmap showed clustering by sample type first, then by site, followed by nutrient enrichment treatment (Figure S5). There were different patterns in nitrogen metabolism between sample types when they were compared to each other (Figure 6A). A significant upregulation of nitrosative stress, nitrogen fixation, and ammonia assimilation pathways was observed in gut samples as compared to the other sample types (Benjamini-Hochberg adjusted $p<0.01$; Figure 6A). Expression of genes in these same pathways, as well as genes involved in denitrification, were downregulated in the outer shell samples (adjusted $p<0.05$). Conversely, genes in the amidase, urea, and nitrile hydratase pathways were upregulated in the outer shell and downregulated in the gut samples (adjusted $p<0.01$; Figure 6A). Genes involved in nitric oxide synthase were significantly upregulated in the inner shell and downregulated in the outer shell (adjusted $p<0.01$; Figure 6A). Expression of genes regulating ammonification and dissimilatory nitrite reduction processes were observed in all tissue types at relatively equivalent levels.

In addition to evaluating changes in nitrogen metabolism pathways, the differential expression of certain genes was calculated. Glutamate-ammonia-ligase

adenylyltransferase (*glnE*), a key gene involved in ammonia assimilation, was significantly upregulated in gut samples, compared to the others, and downregulated in the outer shell samples (Benjamini-Hochberg adjusted $p<0.05$; Figure 7B). Similarly, *nifA*, a gene required to activate most *nif* operons involved in nitrogen fixation was significantly upregulated in the gut samples and downregulated in the outer shell samples (adjusted $p<0.05$). Finally, *nosF*, a gene required for nitrous oxide reductase, was significantly downregulated in the gut samples (adjusted $p<0.05$). The activation of *nosF* requires *nosR* (the gene that activates nitrous-oxide reductase gene *nosZ*), which was significantly downregulated in the outer shell samples (adjusted $p<0.05$).

Field Site and Nutrient Enrichment affect Microbial Expression of Nitrogen Metabolism Genes

A significant effect of site or nutrient enrichment on nitrogen metabolism gene expression was only detected in outer shell samples (Figure 7A). When the outer shell samples were compared by site (regardless of treatment), there was a significant downregulation of nitrosative stress, nitric oxide synthase, ammonia assimilation, and amidase, urea, & nitrile hydratase pathways at the northern site, compared to the southern (Benjamini-Hochberg adjusted $p<0.05$; Figure 7A). The only significantly expressed nitrogen metabolism pathway in response to nutrient enrichment was an upregulation of cyanate hydrolysis in the outer shell samples (Benjamini-Hochberg adjusted $p<0.05$; Figure 7A).

When samples for all sample types were pooled by site (northern versus southern), no significant effect of nutrient enrichment on nitrogen metabolism was observed (Figure 7B). An effect of nutrient enrichment on differential gene expression

of nitrogen metabolism pathways was only observed in oysters collected from the southern site (Figure 7C). In these oysters, nitrosative stress was significantly downregulated in the outer shell samples exposed to nutrient enrichment (Benjamini-Hochberg adjusted $p<0.05$; Figure 7C). Nutrient enrichment also led to a significant downregulation of nitrate and nitrite ammonification and dissimilatory nitrite reductase in samples from the inner shell biofilm (Benjamini-Hochberg adjusted $p<0.01$; Figure 7C).

Discussion

Since oysters are hotspots for nitrogen metabolism, particularly denitrification, in the coastal environment, it is important to study how their microbiomes perform these processes and the effects of nutrient enrichment (Caffrey et al., 2016b; Kellogg et al., 2013). In this study, we determined the effect of nutrient enrichment on bacterial community structure and function in the gut, inner shell, and outer shell of oysters deployed at two contrasting sites within an estuary. We also characterized the effect of sample type, site, and nutrient enrichment on gene expression of selected gene pathways, such as those involved in nitrogen cycling. This study showed that oyster sample type (gut, inner shell or outer shell) had a larger impact on bacterial community composition and function than site or nutrient enrichment. Despite the fact that effects of nutrient enrichment were relatively subtle and obscured by the relatively larger effects of site and sample type on microbial composition and function, we were able to determine that nutrient enrichment significantly influenced microbial composition and function in outer shell samples. Finally, by relating the effect of

nutrient enrichment on bacterial community structure with nitrogen and nitrous oxide gas production rates measured in oysters collected from the same site and treatment when transferred to high nutrient loading conditions, we were able to determine that the presence of *Actinobacteria* may explain the effect of site and nutrient enrichment on gas production rates. These studies inform future studies characterizing the role of microbiomes on oyster responses to eutrophication.

Overall, when data from all tissue types was pooled, there was no strong effect of nutrient enrichment on microbial composition and function, despite the significant changes observed in nitrogen gas production rates observed in our companion study (Hamilton 2018). Potential reasons for not being able to observe a larger effect of nutrient enrichment on community structure and function include the experimental variability and challenges associated with our field study, lack of power due to a limited sample size, and the fact that we could not use the same oysters to determine gas production rates and microbial community structure and function. The fertilizer-induced nutrient enrichment experienced by the treated oysters was weak and highly variable per tank, with no significant effect of enrichment on NH_4^+, NO_3^-, or NO_2^- concentrations per treatment group (Hamilton, 2018). Despite the low impact of nutrient enrichment on nitrogen concentrations in the treatment group, we detected significant changes in microbial composition in the outer shell samples at the southern site, where there were lower ambient nutrient levels (and therefore more of a difference between the ambient control and the enriched treatment). The effect of nutrient enrichment on community structure in these samples was driven by a decrease in *Bacteroidetes*, which were replaced by a relative increase in *Actinobacteria*,

specifically the *Corynebacteriales* and *Microtrichales* orders. In environmental systems, *Actinobacteria* are known as gram-positive soil bacteria, pathogens, and gut commensals (Ventura et al., 2007). In previous studies of oyster microbiomes, *Actinobacteria* were frequently detected in all tissue types and increased with transplant disturbance (Wegner et al., 2013) or in oil-degrading communities (Thomas et al., 2014). This disturbance-induced increase in *Actinobacteria* is consistent with our findings of an increase in *Actinobacteria* in the outer shell samples with nutrient enrichment.

Nutrient enrichment did not have a significant impact on the total oyster nitrogen metabolism per site, likely due to the inconsistent effect of the treatment on nutrient levels per bucket (Figure 1). These results are also in agreement with previous studies of nutrient enrichment in the marine environment that observed minimal functional changes, but significant structural changes with nutrient enrichment (Bowen et al., 2011; Newsham et al., 2019; Shaver et al., 2017). By focusing on specific sample types (inner and outer shell biofilms), however, we were able to detect significant effects of nutrient enrichment on expression of certain genes in pathways involved nitrogen metabolism (downregulation of ammonification, nitrosative stress, and dissimilatory nitrite reductase pathways). These changes may be due to a direct impact of NO_3^- and NO_2^- concentrations and other environmental conditions on gene expression and/or a result of the shift in microbial composition and possible subsequent decrease in nitrogen-metabolizing microbes. Better annotation of microbial genomes will help determine the mechanisms driving changes in nutrient cycling due to nutrient enrichment.

This study also detected a large effect of site on oyster bacterial community structure, and, to a lesser extent, on bacterial community function. Besides differences in dissolved inorganic nitrogen, each site was characterized by differences in several environmental parameters, including temperature, dissolved oxygen, pH, and, to a lesser extent, salinity (Hamilton, 2018). These differences in the relative effect of site on microbial community structure versus the impact bacterial gene expression are consistent with previous results showing a pervasive effect of environmental parameters on microbial function in oysters (Chapter 2 of this dissertation), but could also be determined by differences in seawater bacterial communities (*i.e.* which microbes are available to the oysters) between sites (Chen et al., 2019; Crump et al., 2018).

The significant differences observed in taxonomic annotation of the present (16S rRNA amplicons) versus the transcriptionally active microbes (metatranscriptomes) could be attributed to various phenomena. The metatranscriptome captures the live, active microbial community, whereas the 16S rRNA amplicons capture all active, symbiotic, transient, dead, and live microbes in the sample (Yarza et al., 2014). The active taxa may be true symbionts, versus all symbiotic and transient taxa observed in the 16S rRNA amplicon method. Additionally, the annotation method and database used may have led to biases in taxonomy between the two methods (Wood and Salzberg, 2014). In this study, the only differences in the active microbial community structure was between sample types, which was likely driven by localized environmental conditions.

Finally, we have further characterized which of the gene pathways involved in nitrogen cycling are most significantly associated with the three oyster sample types collected. Each tissue type within an oyster has a specific function, microbial community structure, and set of environmental constraints, therefore promoting different microbial nitrogen processes (Arfken et al., 2017; Kellogg et al., 2014; Ray et al., 2019b). The gut microbiome of bivalves has lower oxygen and light conditions than the outer shell and is constrained by selection and amplification of certain taxa through filter feeding and host-microbe interactions (Kellogg et al., 2013; Stief et al., 2009). In these gut samples, we observed upregulation in nitrogen fixation, nitrosative stress, and ammonia assimilation pathways as compared to shell biofilm samples; these are processes commonly performed by anerobic microbes (Jetten, 2008; Moulton et al., 2016). In the outer shell biofilm, which is characterized by exposure to ambient oxygen and light conditions, facilitating a variety of more arbitrary epiphytes, a significant upregulation of genes involved in amidase, urea, and nitrile hydratase was observed. These pathways are commonly observed in *Rhodobacterales*, an order that was significantly more abundant in the outer shell samples (Komeda et al., 1996; Tauber et al., 2000). This characterization may help provide targets for gene markers associated with nitrogen cycling in oysters. See Appendix B for a summary of how overall microbial function changes in each tissue type.

In summary, the microbial community changes observed in this study highlight some of the dynamic interactions in nitrogen metabolism in the oyster microbiome. The effect of environmental conditions on microbial community structure and function in oysters depend on which sample type within oysters is evaluated. This study

suggests that bacterial communities associated with oyster outer shells may have a relatively larger role on variability of oyster responses in response to nutrient enrichment than the microbial communities associated with the gut or the inner surface of the shell. Additional research is needed to examine the role of specific environmental conditions, particularly changes in dissolved oxygen, on nitrogen cycling in oysters to better constrain these processes.

Conclusion

Due to the importance of oysters and increasing eutrophication in coastal environments, it is necessary to study how oyster-associated microbial communities contribute to nitrogen metabolism in response to nutrient enrichment. These results demonstrate that the microbial transcriptional activity of oysters is tissue-specific and differentially affected by site and nutrient enrichment (*i.e*. nutrient enrichment had the greatest influence on the outer shell microbiome structure, as compared to the effect on gut and inner shell samples). The results of this study contribute to the understanding of host-associated nitrogen cycling in coastal environments and how it may change with increased nutrient loading due to anthropogenic pressures.

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Figures

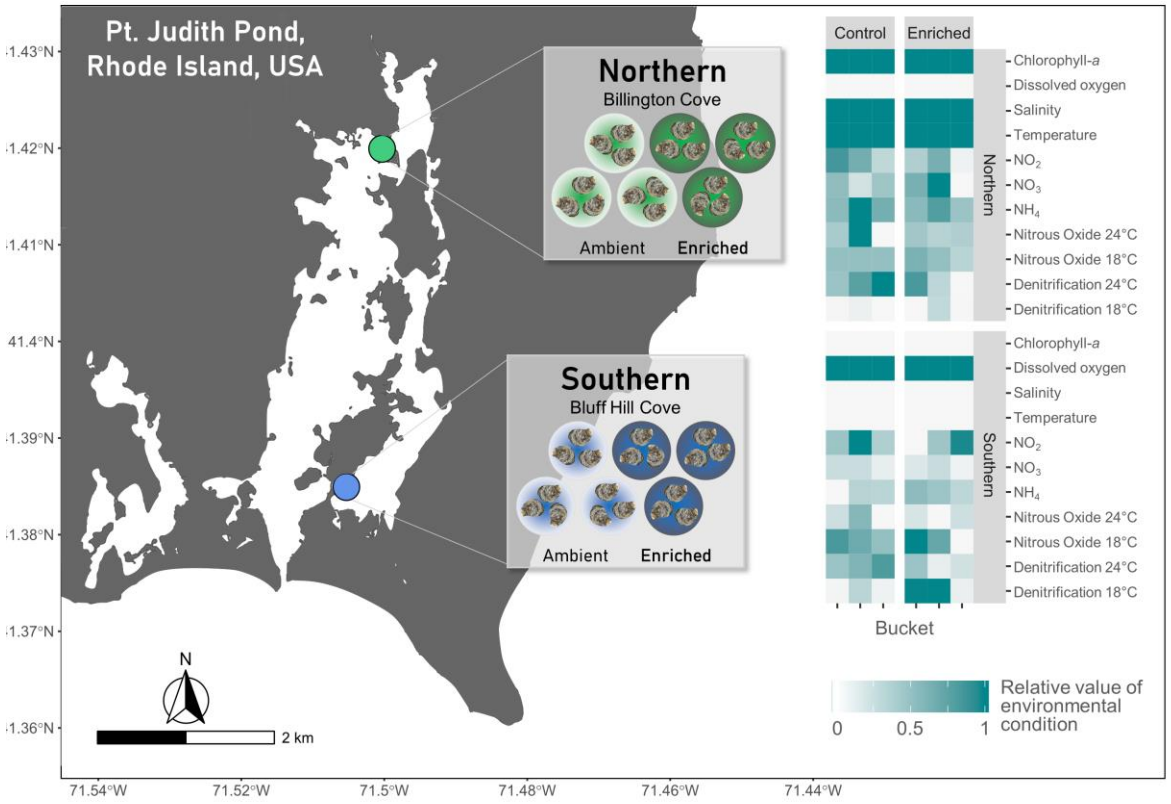


Figure III-1. Map of study area with experimental sites, setup, and environmental conditions.
The heatmap shows percentile value of averaged environmental measurements per experimental bucket (Hamilton, 2018). A dark teal tile indicates the bucket where the highest value was observed and white indicates the bucket where the lowest values was observed. Nutrient concentrations were measured from water collected in each bucket. Gas production rates at 18°C and 24°C were measured with a separate set of oysters (Hamilton, 2018). All other values are average measurements collected *in situ* during the field study.

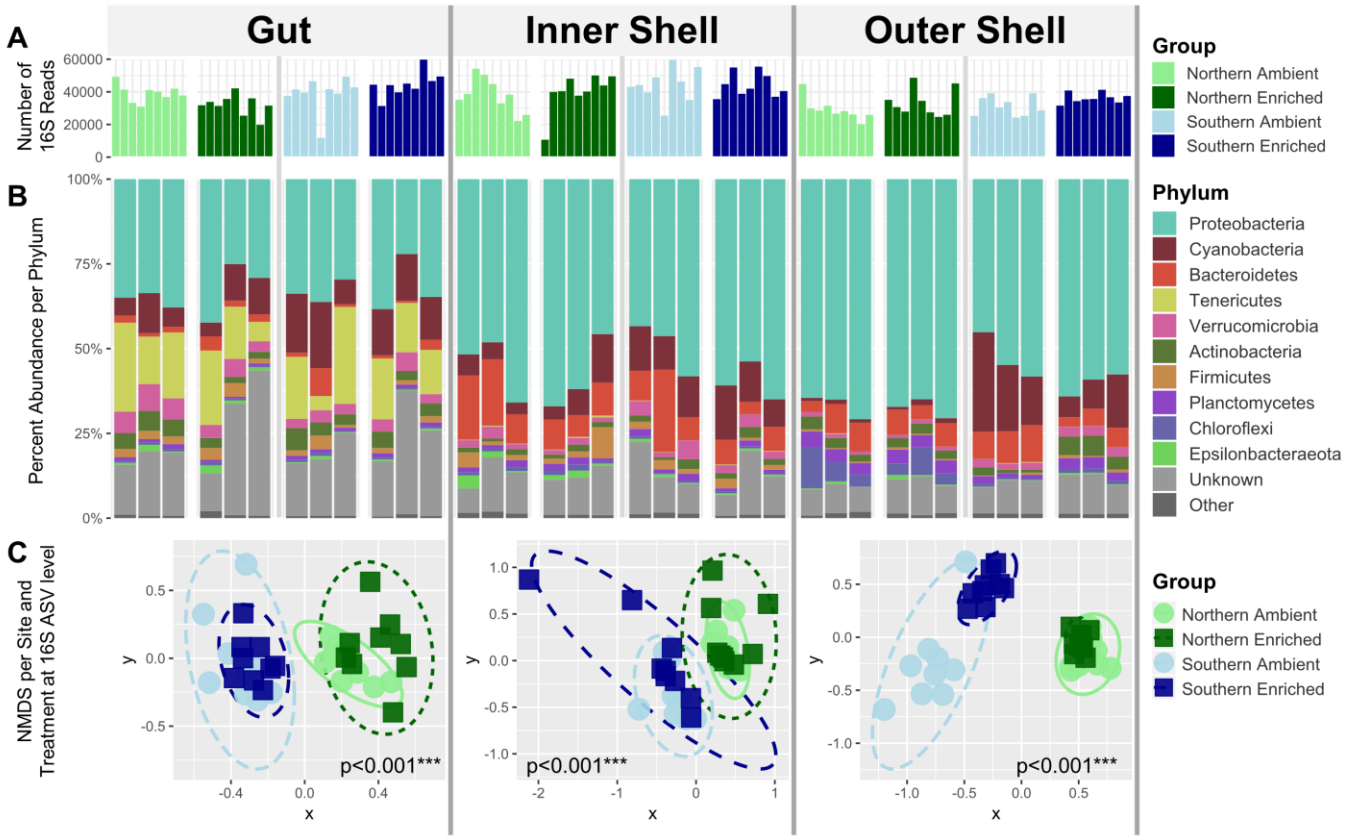


Figure III-2. Oyster gut, inner shell, and outer shell microbiome structures.
(A) Bar graph of the total abundance of quality filtered sequencing reads per oyster sample. **(B)** Averaged percent relative abundances of the 10 most abundant phyla per bucket based on 16S rRNA amplicon sequencing data (n=3). **(C)** NMDS plot visualization of Bray-Curtis beta-diversity (k = 2) for each sample type by site and treatment. The ellipse lines show the 95% confidence interval (standard deviation). p-values indicate significance of grouping with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test.

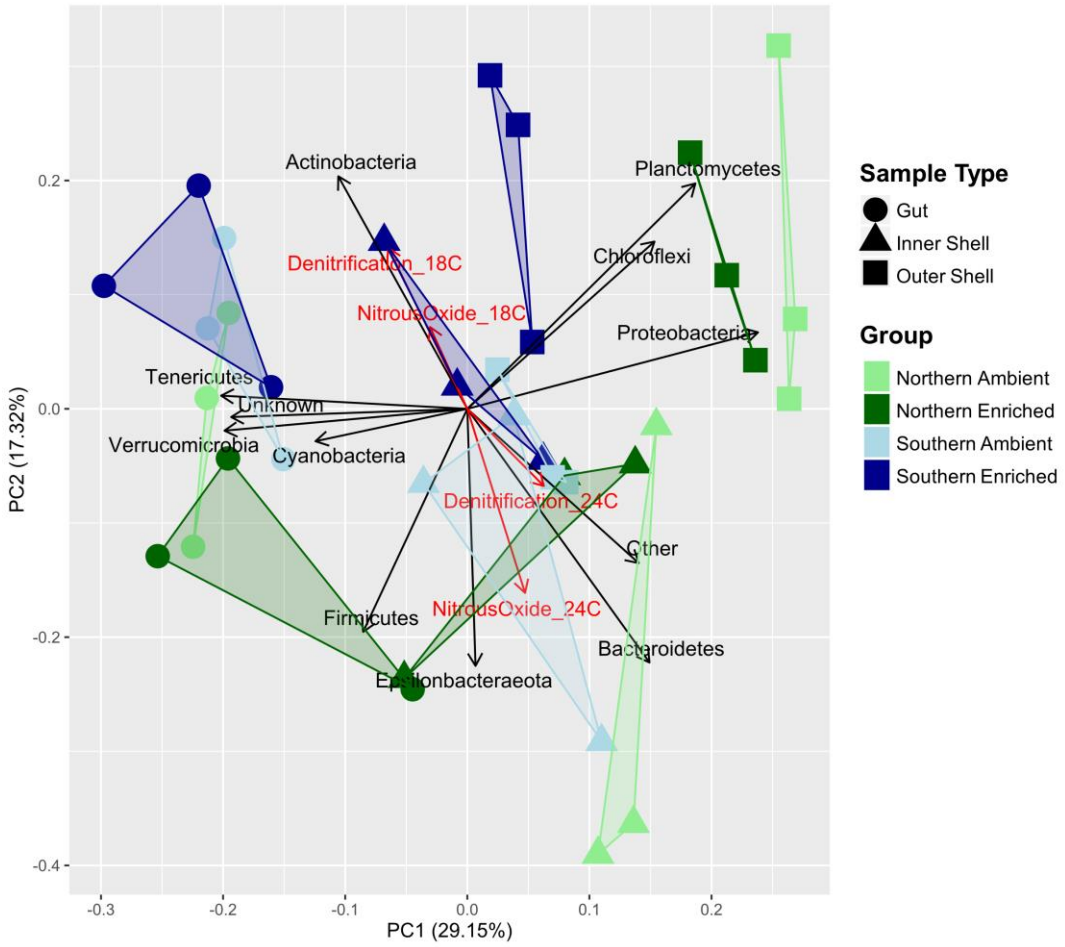


Figure III-3. PCA analysis of most abundant bacterial Phyla and their correlation with gas production rates.
Each symbol represents the averaged 16S rRNA amplicon samples per field bucket, per sample type and location/treatment (n=3). Black arrows show bacterial Phylas. Red arrows show gas production rates measured from a different set of oysters (Hamilton, 2018).

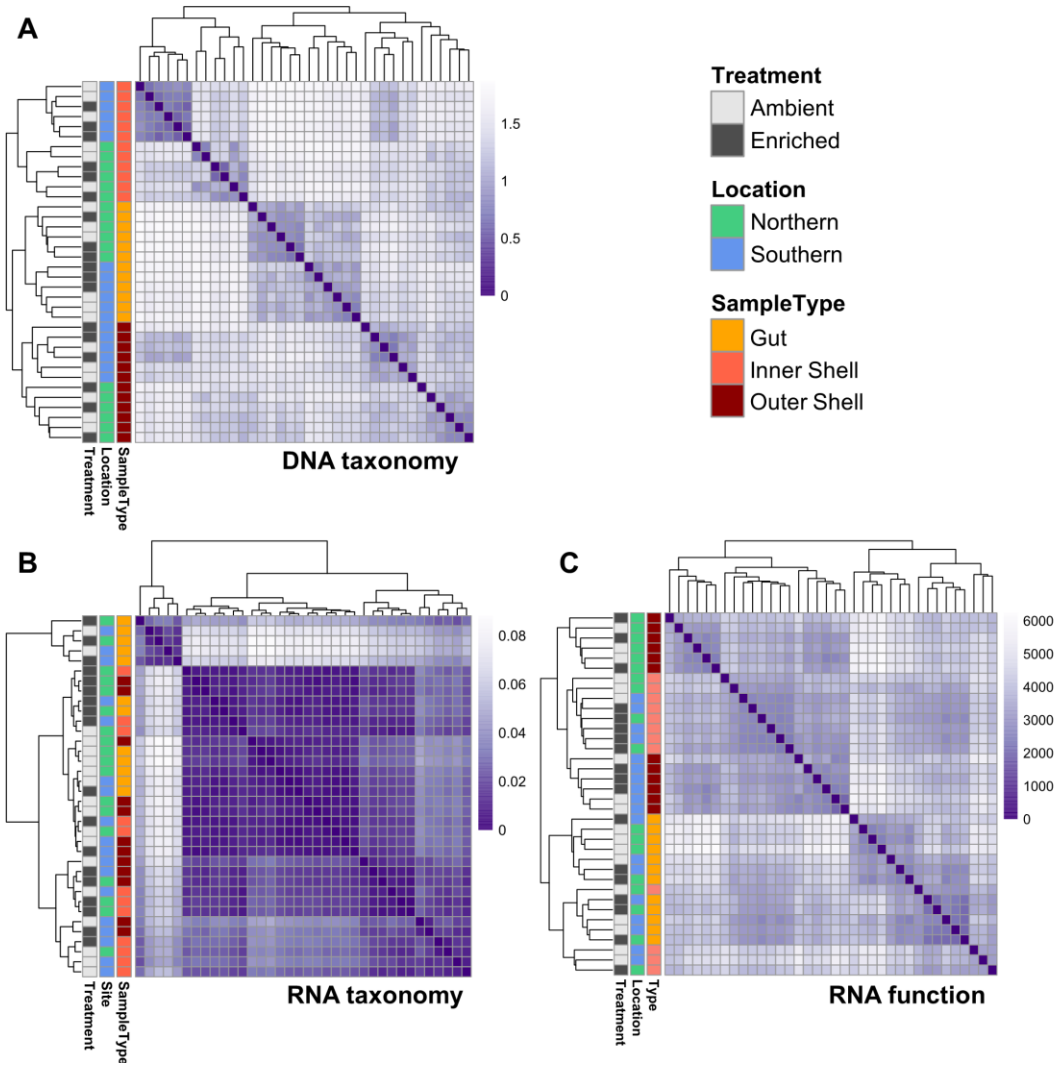


Figure III-4. Manhattan similarity between sample taxonomy (DNA and RNA) or function (RNA).
(A) Calculated using mean percent abundance data from 16S rRNA amplicons at the ASV level (n=3). **(B)** Calculated using bacteria percent abundance data from Kraken-annotated metatranscriptomes at the Species level. **(C)** Calculated using normal-transformed gene counts from RefSeq-SEED annotated metatranscriptomes.

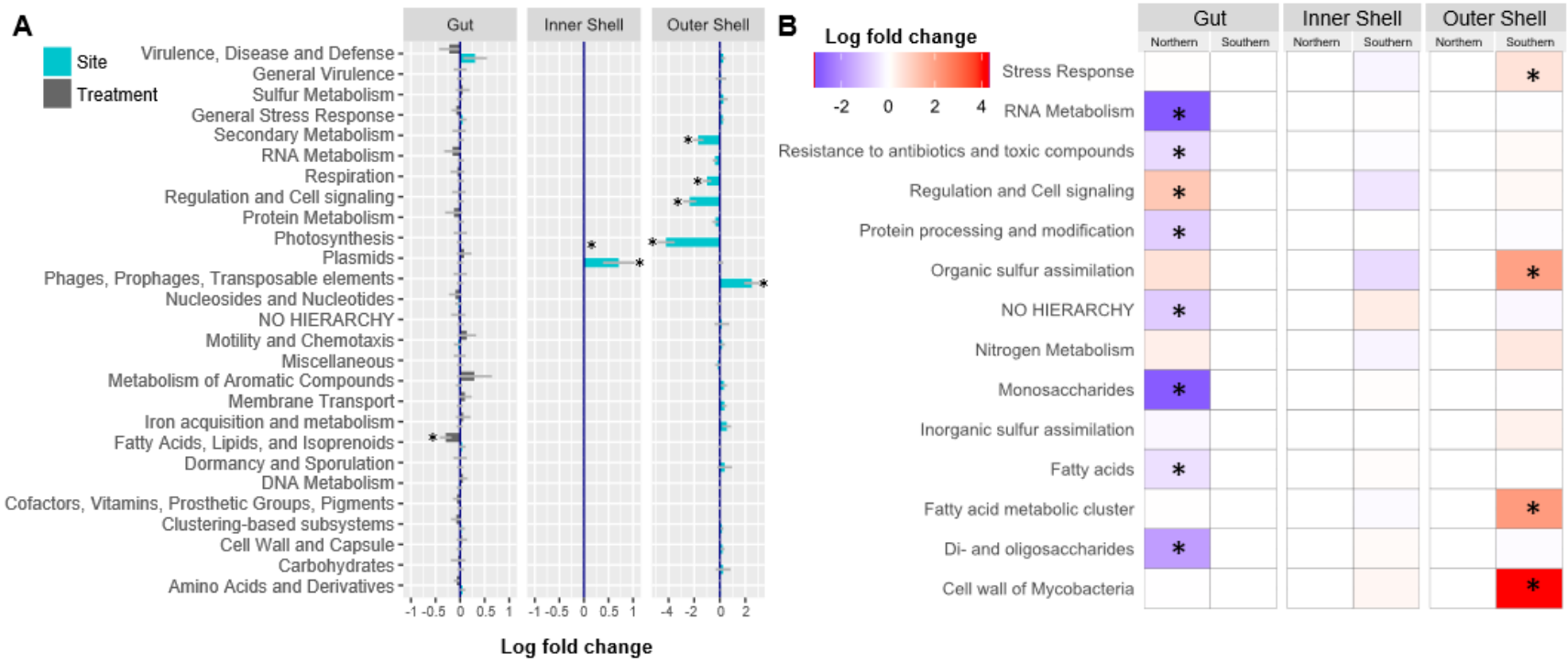


Figure III-5. Effect of site and nutrient enrichment on major pathway gene expression.
(A) Log fold change in expression of all Level-1 pathways by Site (Northern compared to Southern) and Treatment (Enriched compared to Ambient) samples. Note different scale for Outer Shell samples. **(B)** Log fold change in expression of significant Level-2 pathways in enriched samples compared to ambient samples, per sample type. Significance (padj<0.05) is indicated with a star.

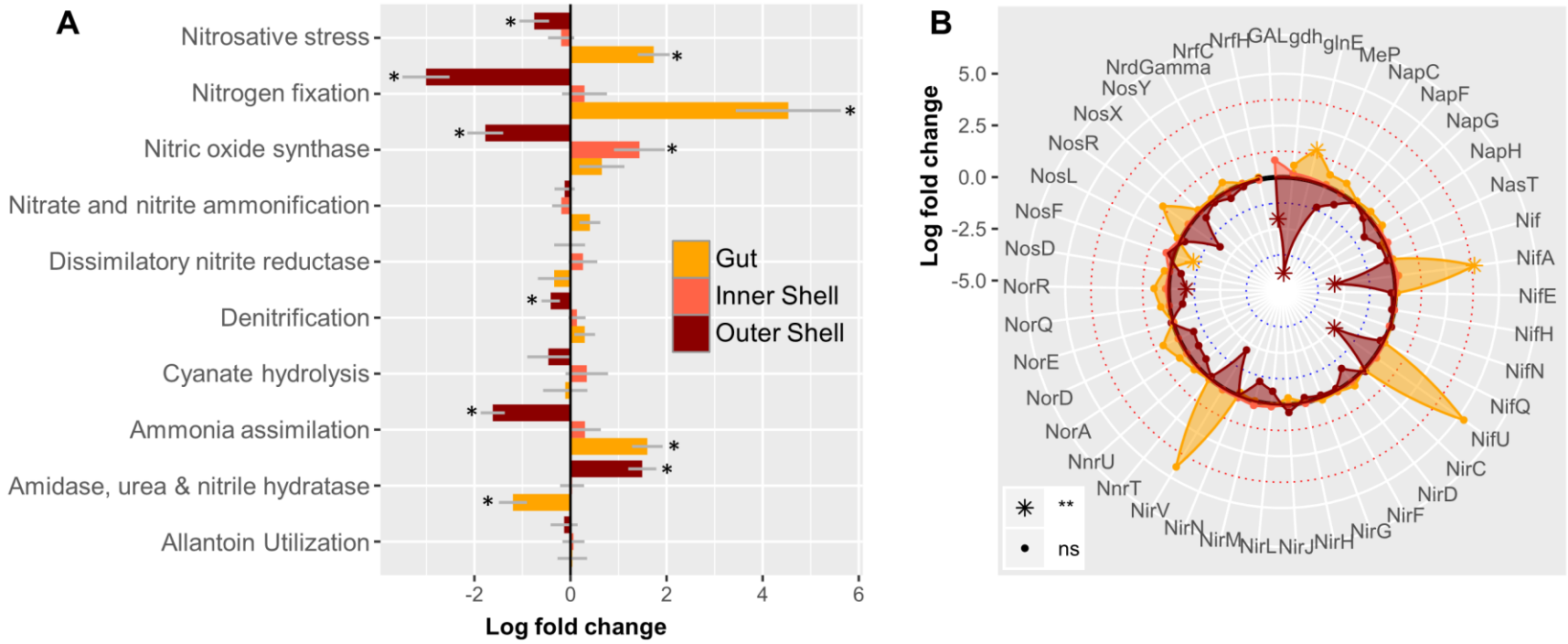


Figure III-6. Effect of sample type on Nitrogen metabolism.
Log fold change in expression of Nitrogen metabolism pathways in each sample type compared to the others. **(A)** Overall nitrogen metabolism pathways. Significance (padj<0.05) is indicated with a star. **(B)** Key nitrogen metabolism genes. Significance (padj<0.05) is indicated with a star point.

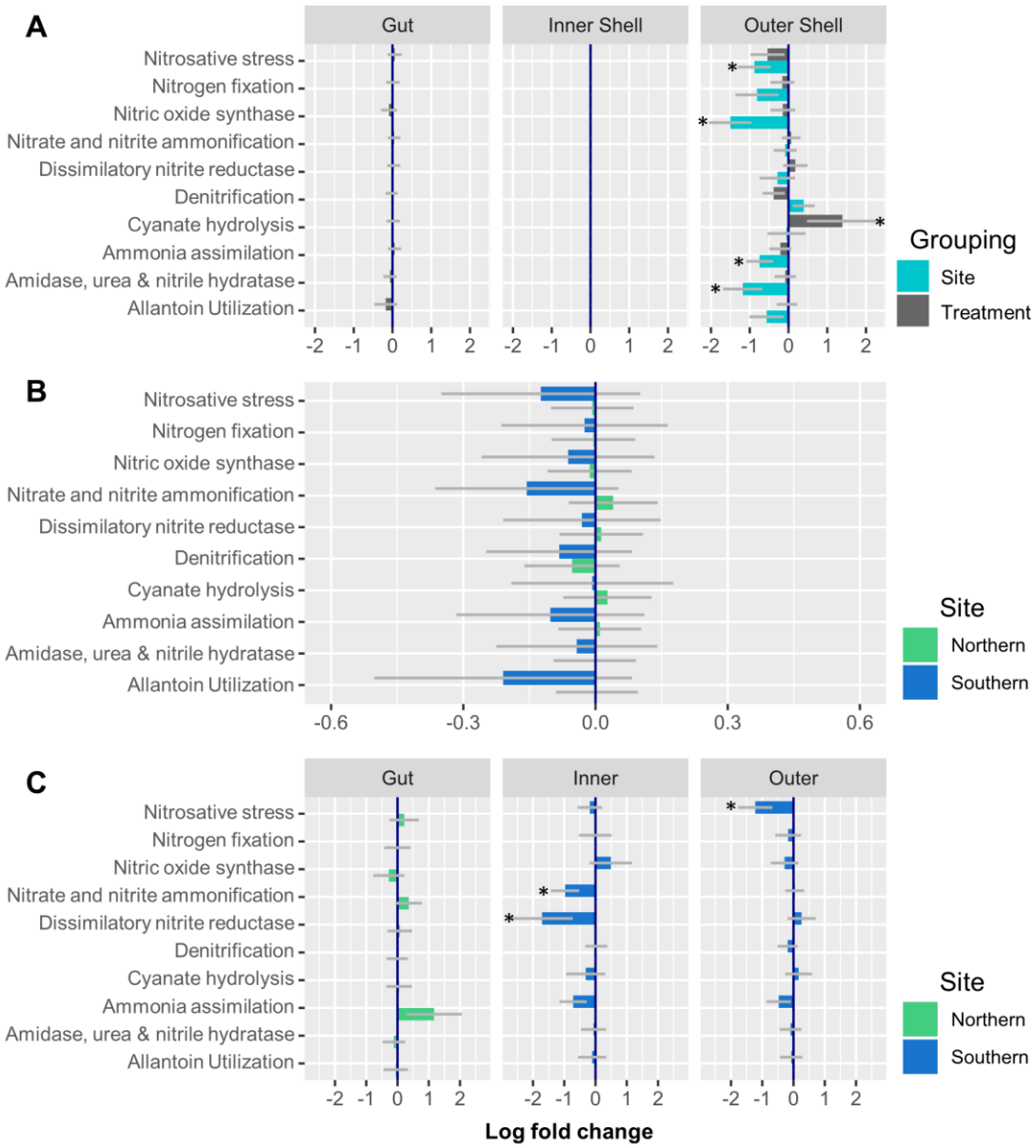
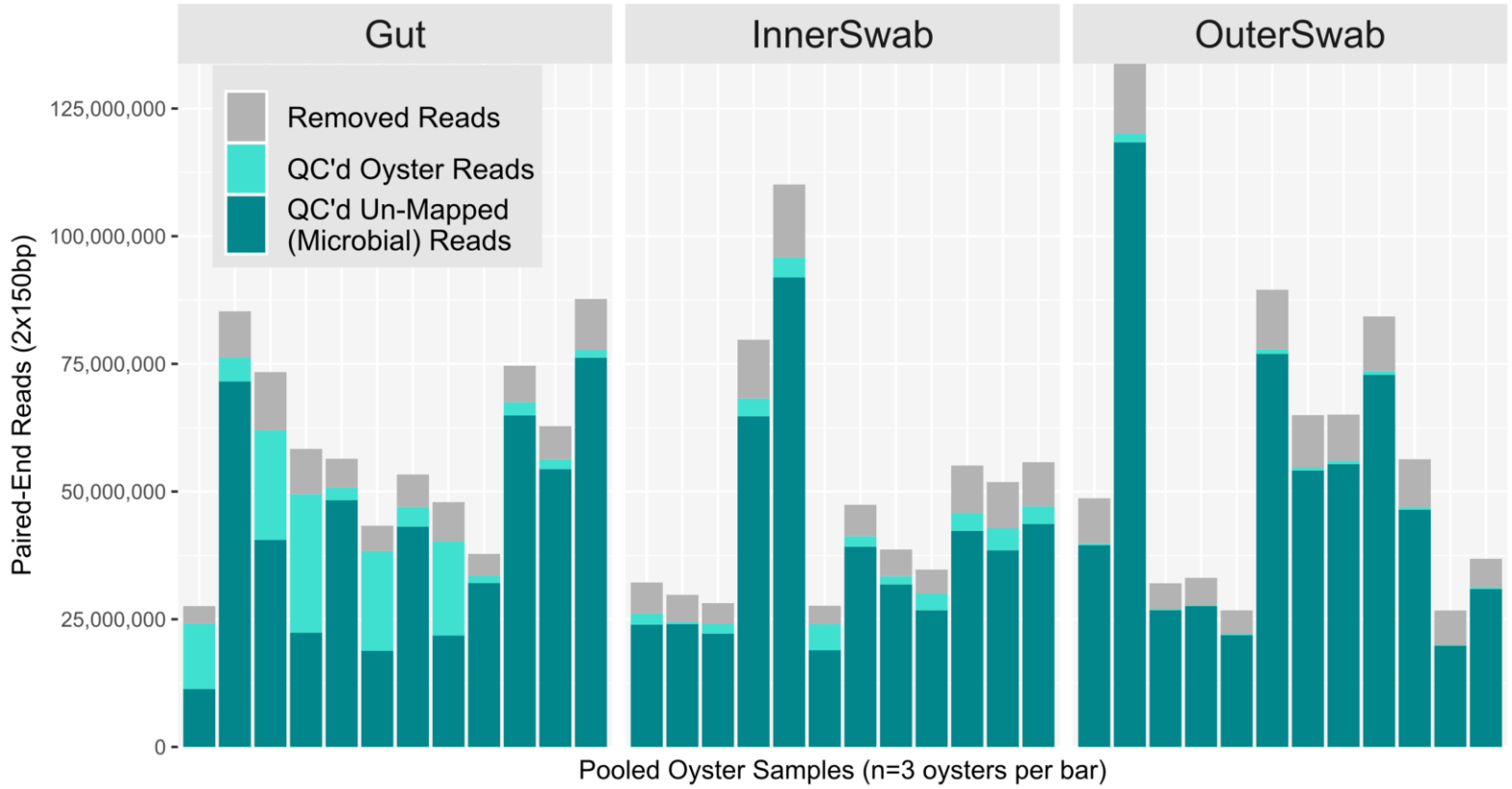
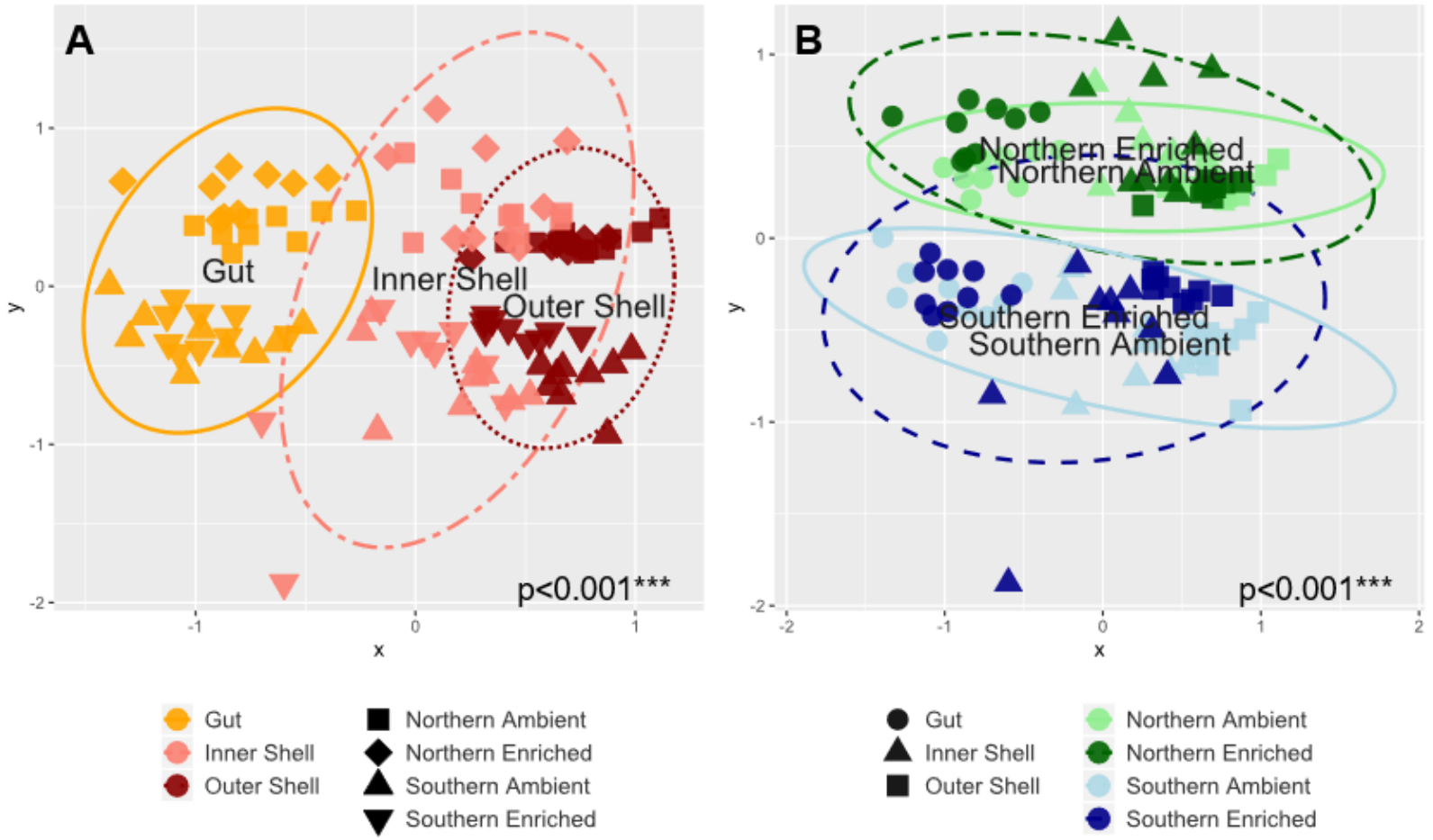


Figure III-7. Effect of site and nutrient enrichment on nitrogen metabolism. **(A)** Log fold change in expression of Nitrogen metabolism pathways by Site (Northern compared to Southern) and Treatment (Enriched compared to Ambient) samples. **(B)** Log fold change in expression of Nitrogen metabolism pathways per site in enriched samples compared to ambient samples per site. **(C)** Log fold change in expression of Nitrogen metabolism pathways per sample type and site in enriched samples, compared to ambient samples. Significance (padj<0.05) is indicated with a star. Note there was no differential expression in the Southern gut, Northern inner or outer shell samples.

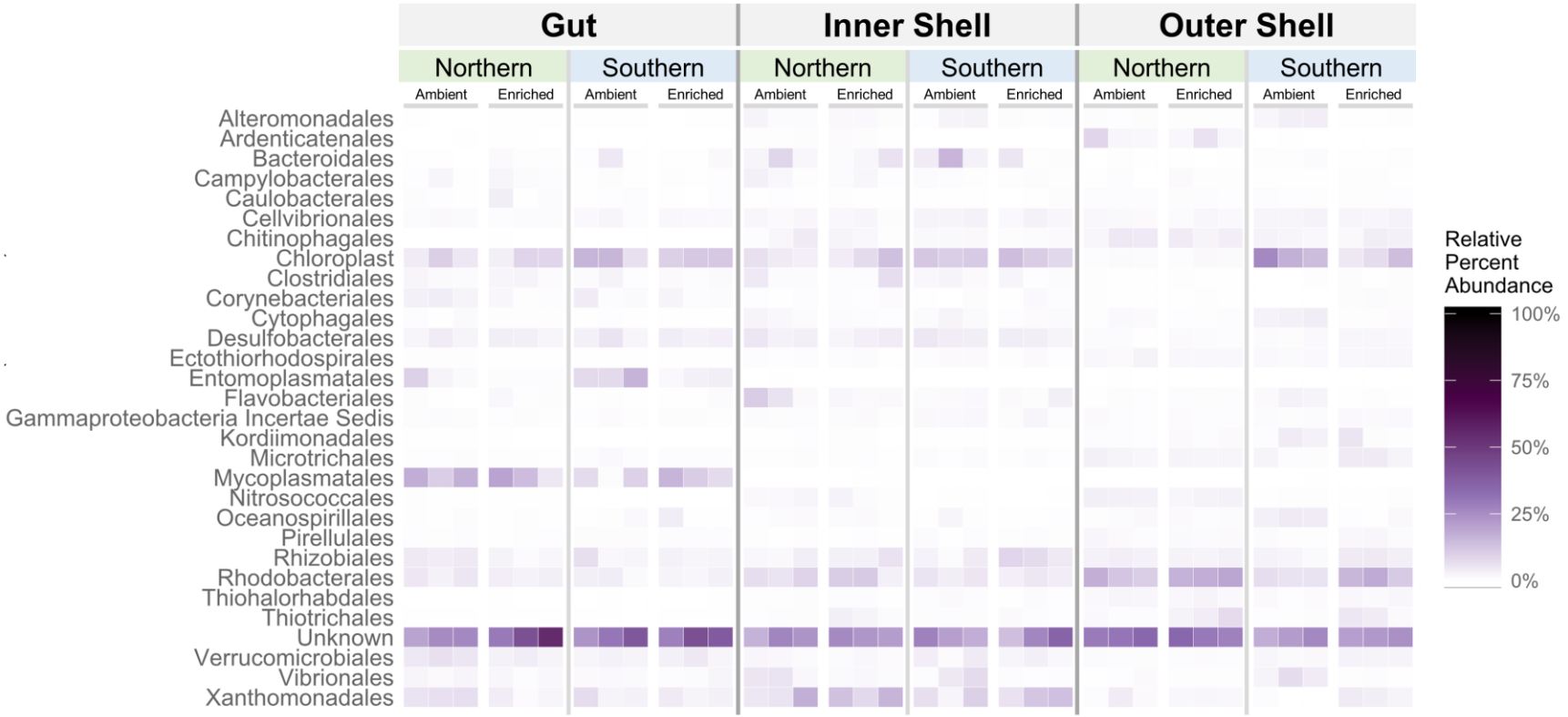
Supplementary Material



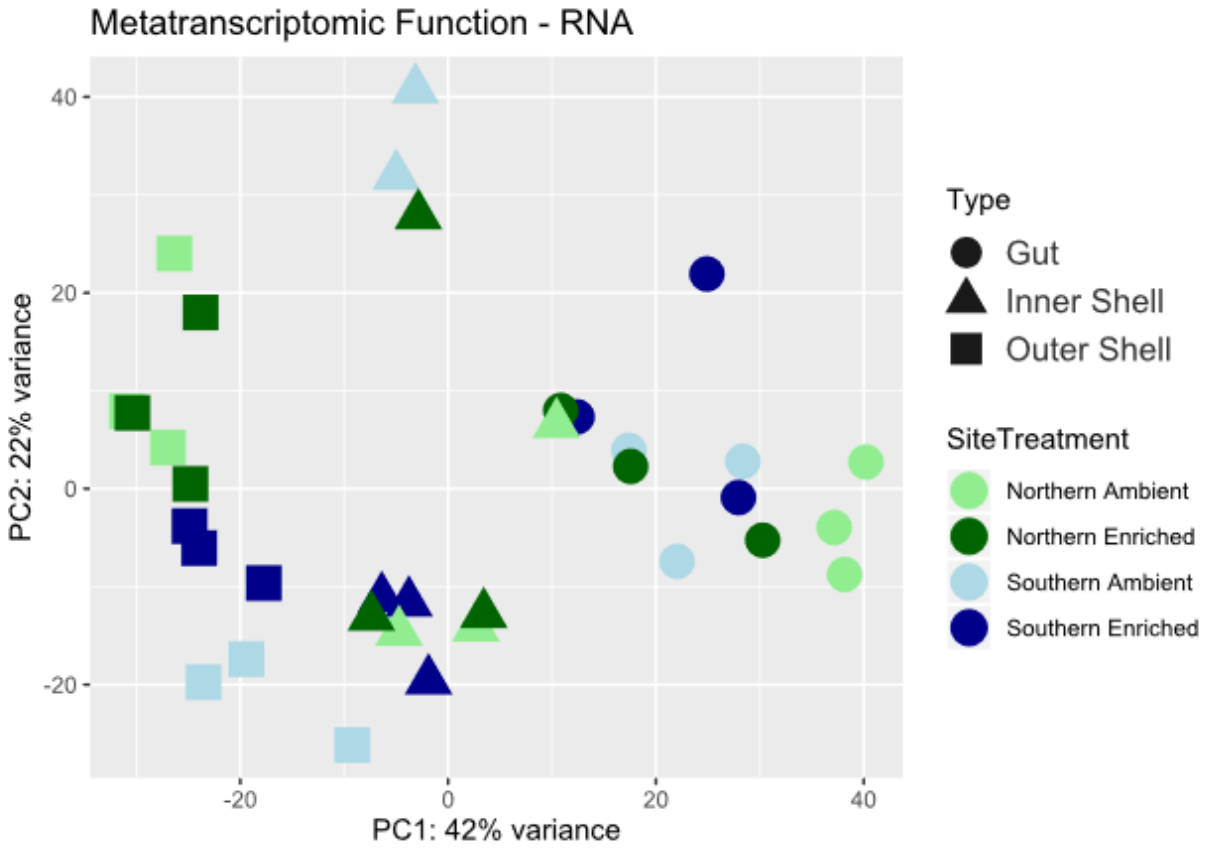
Supplementary Figure 1. Number of metatranscriptomic sequencing reads per sample. The total bar is the total number of raw reads. Grey reads were removed during quality control and light teal reads were removed by mapping the quality-controlled reads to the oyster genome. All remaining reads continued to downstream analysis.



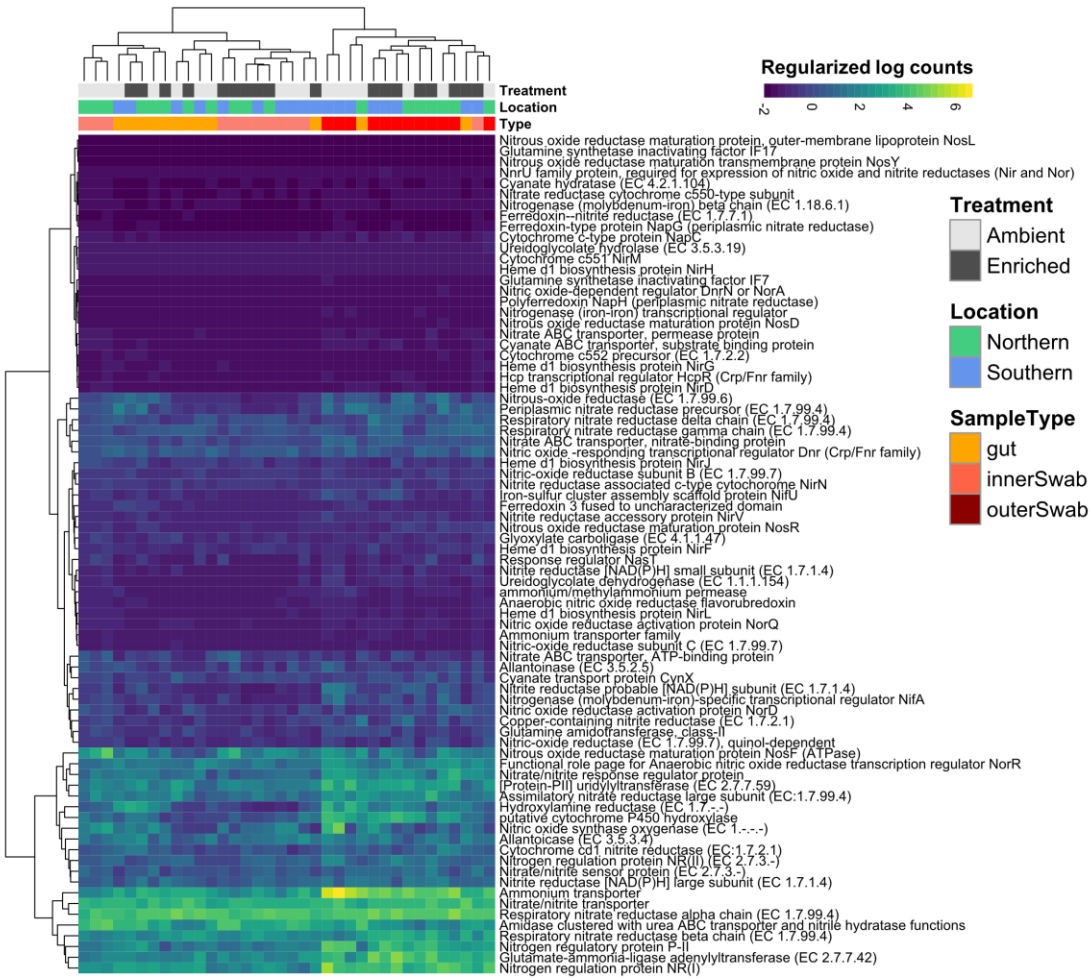
Supplementary Figure 2. NMDS plot visualization of Bray-Curtis beta-diversity (k = 2) at the ASV level for all samples by Type. The ellipse lines show the 95% confidence interval (standard deviation). p-value indicates significance of grouping with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test.



Supplementary Figure 3. Relative percent abundances of top 30 bacterial Orders in the 16S rRNA amplicon taxonomy (n=3).



Supplementary Figure 4. PCA visualization of Manhattan similarity between sample function (RNA). Distances were calculated using normal-transformed gene counts from RefSeq-SEED annotated metatranscriptomes.



Supplementary Figure 5. Expression of all Nitrogen metabolism genes. Heatmap of regularized log counts per nitrogen metabolism gene with >5 counts in each metatranscriptome sample. The type, site, and treatment of each sample is indicated by colors along the top of the heatmap.

Supplementary Table 1. Sequencing and analysis summary for metatranscriptomic samples.

Sample Number	Sample Type	Group	Raw PE reads	Percent passed QC	Percent mapped to *C. virginica*	Merged reads (%)	Merged reads	% reads assigned taxonomy	% reads assigned function
1	Gut	SouthernAmbient	27183077	87%	53.9%	97%	11043997	19%	2%
2	Gut	SouthernAmbient	56039834	90%	5.0%	94%	45481455	41%	7%
3	Gut	SouthernAmbient	42638223	88%	51.8%	97%	18310256	22%	14%
4	Gut	SouthernEnriched	52942236	88%	8.3%	93%	39937345	38%	1%
5	Gut	SouthernEnriched	47409510	84%	46.4%	97%	21266972	25%	2%
6	Gut	SouthernEnriched	37549116	89%	4.4%	93%	29872717	40%	7%
7	Gut	NorthernAmbient	74062478	90%	3.9%	92%	60029315	42%	8%
8	Gut	NorthernAmbient	62414516	90%	3.5%	94%	50889542	44%	7%
9	Gut	NorthernAmbient	87248207	89%	1.9%	96%	72876803	42%	7%
10	Gut	NorthernEnriched	84690299	89%	6.2%	93%	66750811	40%	6%
11	Gut	NorthernEnriched	72641864	84%	35.1%	97%	39317596	25%	3%
12	Gut	NorthernEnriched	57693385	85%	55.6%	98%	21925259	19%	2%
13	Outer Swab	SouthernAmbient	48544823	82%	0.8%	97%	38244607	27%	4%
14	Outer Swab	SouthernAmbient	133067463	90%	1.4%	95%	112425664	40%	7%
15	Outer Swab	SouthernAmbient	31939839	84%	0.9%	97%	25876284	35%	4%
16	Outer Swab	SouthernEnriched	33019133	84%	0.7%	96%	26593415	27%	3%
17	Outer Swab	SouthernEnriched	26648462	83%	1.1%	97%	21178985	35%	5%
18	Outer Swab	SouthernEnriched	89106653	87%	1.2%	95%	73160415	39%	6%
19	Outer Swab	NorthernAmbient	64694546	84%	1.1%	96%	51776833	41%	6%
20	Outer Swab	NorthernAmbient	64766636	86%	1.2%	94%	52291808	44%	7%
21	Outer Swab	NorthernAmbient	83933706	87%	1.1%	96%	69638736	41%	6%
22	Outer Swab	NorthernEnriched	56101462	83%	1.1%	96%	44630786	40%	6%
23	Outer Swab	NorthernEnriched	26662306	75%	0.7%	99%	19728614	36%	4%
24	Outer Swab	NorthernEnriched	36697646	85%	1.1%	95%	29516901	39%	6%

Sample Number	Sample Type	Group	Raw PE reads	Percent passed QC	Percent mapped to *C. virginica*	Merged reads (%)	Merged reads	% reads assigned taxonomy	% reads assigned function
25	Inner Swab	SouthernAmbient	32064379	81%	8.5%	96%	22945426	25%	4%
26	Inner Swab	SouthernAmbient	29675258	82%	1.7%	97%	23223666	29%	5%
27	Inner Swab	SouthernAmbient	27903283	86%	8.2%	96%	21184417	36%	5%
28	Inner Swab	SouthernEnriched	79189328	86%	5.2%	95%	61783195	38%	5%
29	Inner Swab	SouthernEnriched	109443114	87%	4.1%	94%	86631332	40%	6%
30	Inner Swab	SouthernEnriched	27103551	87%	21.7%	97%	18290238	32%	4%
31	Inner Swab	NorthernAmbient	47060589	87%	5.1%	94%	36839007	36%	6%
32	Inner Swab	NorthernAmbient	38340598	86%	4.9%	90%	28485780	39%	7%
33	Inner Swab	NorthernAmbient	34495516	87%	11.0%	96%	25602686	29%	4%
34	Inner Swab	NorthernEnriched	54852298	83%	7.6%	96%	40433990	32%	5%
35	Inner Swab	NorthernEnriched	51361820	82%	10.2%	94%	36286377	39%	6%
36	Inner Swab	NorthernEnriched	55325257	84%	7.4%	96%	41807398	34%	5%

Supplementary Table 2. Statistical testing of Bray-Curtis beta-diversity using adonis2 for groupings of 16S amplicon taxonomy at the ASV level.

Sample Type	Grouping	Df	Sum OfSqs	R^2	F-value	P-value	
All	ASVs~SampleType	2	7.96	0.25	17.68	0.001	***
All	ASVs~Site	1	3.30	0.10	12.34	0.001	***
All	ASVs~Treatment	1	0.53	0.02	1.80	0.042	*
All	ASVs~Site+Treatment	2	3.82	0.12	7.23	0.001	***
Gut	GutASVs~Site	1	1.36	0.18	7.29	0.001	***
Gut	GutASVs~Treatment	1	0.33	0.04	1.52	0.079	
Gut	GutASVs~Site+Treatment	1	1.36	0.18	7.29	0.001	***
Gut	GutASVs~NorthernTreatment	1	0.37	0.11	2.06	0.002	**
Gut	GutASVs~SouthernTreatment	1	0.24	0.08	1.33	0.131	
Inner Shell	InnerShellASVs~Site	1	1.23	0.14	5.70	0.001	***
Inner Shell	InnerShellASVs~Treatment	1	0.32	0.04	1.33	0.132	
Inner Shell	InnerShellASVs~ Site+Treatment	2	1.56	0.18	3.65	0.001	***
Inner Shell	InnerShellASVs~ NorthernTreatment	1	0.22	0.06	1.08	0.359	
Inner Shell	InnerShellASVs~ SouthernTreatment	1	0.31	0.08	1.37	0.079	
Outer Shell	OuterShellASVs~Site	1	2.62	0.36	18.82	0.001	***
Outer Shell	OuterShellASVs~Treatment	1	0.68	0.09	3.49	0.005	**
Outer Shell	OuterShellASVs~ Site+Treatment	2	3.30	0.45	13.46	0.001	***
Outer Shell	OuterShellASVs~ NorthernTreatment	1	0.21	0.14	2.65	0.003	**
Outer Shell	OuterShellASVs~ SouthernTreatment	1	0.96	0.30	6.79	0.001	***

Supplementary Table 3. Statistical testing of Bray-Curtis beta-diversity using adonis2 for groupings of metatranscriptomic taxonomy at the species level.

Sample Type	Grouping	Df	Sum of Sqs	R^2	F-value	P-value	
All	Species~SampleType	2	0.59	0.22	4.79	0.003	**
All	Species~Site	1	0.11	0.04	1.54	0.20	
All	Species~Treatment	1	0.011	0.004	0.15	0.91	
All	Species~Site+Treatment	2	0.12	0.05	0.83	0.51	
Gut	GutSpecies~Site	1	0.07	0.05	0.51	0.47	
Gut	GutSpecies~Treatment	1	0.04	0.03	0.30	0.63	
Gut	GutSpecies~Site+Treatment	2	0.12	0.08	0.38	0.66	
Gut	GutSpecies~NorthernTreatment	1	0.34	0.51	4.19	0.10	
Gut	GutSpecies~SouthernTreatment	1	0.11	0.14	0.67	0.30	
Inner Shell	InnerShellSpecies~Site	1	0.015	0.07	0.72	0.47	
Inner Shell	InnerShellSpecies~Treatment	1	0.018	0.08	0.83	0.41	
Inner Shell	InnerShellSpecies~ Site+Treatment	2	0.033	0.14	0.75	0.51	
Inner Shell	InnerShellSpecies~ NorthernTreatment	1	0.002	0.03	0.12	1.00	
Inner Shell	InnerShellSpecies~ SouthernTreatment	1	0.037	0.25	1.35	0.30	
Outer Shell	OuterShellSpecies~Site	1	0.077	0.28	3.84	0.066	
Outer Shell	OuterShellSpecies~Treatment	1	0.011	0.041	0.43	0.57	
Outer Shell	OuterShellSpecies~ Site+Treatment	2	0.088	0.32	2.10	0.14	
Outer Shell	OuterShellSpecies~ NorthernTreatment	1	0.016	0.49	3.92	0.10	
Outer Shell	OuterShellSpecies~ SouthernTreatment	1	0.008	0.05	0.20	0.80	

Supplementary Table 4. Statistical testing of Bray-Curtis beta-diversity using adonis2 for groupings of metatranscriptomic function at the gene level.

Sample Type	Grouping	Df	Sum of Sqs	R^2	F-value	P-value	
All	GeneExpression~SampleType	2	0.66	0.28	6.56	0.001	***
All	GeneExpression~Site	1	0.06	0.02	0.85	0.51	
All	GeneExpression~Treatment	1	0.06	0.03	0.95	0.41	
All	GeneExpression~Site+Treatment	2	0.12	0.05	0.90	0.53	
Gut	GutGeneExpression~Site	1	0.09	0.11	1.18	0.22	
Gut	GutGeneExpression~Treatment	1	0.11	0.14	1.59	0.09	
Gut	GutGeneExpression~Site+Treatment	2	0.20	0.24	1.44	0.12	
Gut	GutGeneExpression~NorthernTreatment	1	0.18	0.43	3.00	0.10	
Gut	GutGeneExpression~SouthernTreatment	1	0.06	0.19	0.91	0.70	
Inner Shell	InnerShellGeneExpression~Site	1	0.04	0.09	0.93	0.39	
Inner Shell	InnerShellGeneExpression~Treatment	1	0.05	0.10	1.06	0.25	
Inner Shell	InnerShellGeneExpression~ Site+Treatment	2	0.10	0.18	0.99	0.39	
Inner Shell	InnerShellGeneExpression~ NorthernTreatment	1	0.04	0.18	0.86	0.60	
Inner Shell	InnerShellGeneExpression~ SouthernTreatment	1	0.08	0.33	2.01	0.20	
Outer Shell	OuterShellGeneExpression~Site	1	0.08	0.24	3.17	0.002	**
Outer Shell	OuterShellGeneExpression~Treatment	1	0.03	0.10	1.09	0.27	
Outer Shell	OuterShellGeneExpression~ Site+Treatment	2	0.11	0.34	2.31	0.002	**
Outer Shell	OuterShellGeneExpression~ NorthernTreatment	1	0.02	0.18	0.87	0.80	
Outer Shell	OuterShellGeneExpression~ SouthernTreatment	1	0.04	0.35	2.14	0.10	

Supplementary Table 5. Wilcox Rank Sum Test for select Phyla detected in 16S rRNA amplicons by Sample Type, adjusted p-value with Benjamini-Hochberg method. The global statistics were calculated using the Kruskal-Wallis Rank Sum Test.

	Global Kruskal-Wallis			Gut vs. Inner Swab			Gut vs. Outer Swab			Inner vs. Outer Swab		
Phylum	**Pvalue**	**P adj**		**Pvalue**	**P adj**		**Pvalue**	**P adj**		**Pvalue**	**P adj**	
Actinobacteria	9.6E-7	1.3E-6	****	4.9E-8	1.5E-7	****	0.16	0.19		0.0002	0.0004	***
Bacteroidetes	7E-12	2E-11	****	5E-12	3E-11	****	2E-11	1E-10	****	0.15	0.18	
Chloroflexi	1.7E-7	3.5E-7	****	0.13	0.17		6.3E-8	1.7E-7	****	9.2E-6	1.9E-5	****
Cyanobacteria	0.0056	0.0056	**	0.24	0.26		0.0035	0.0052	**	0.015	0.02	*
Epsilonbacteraeota	7.1E-6	8.5E-6	****	0.088	0.11		0.0007	0.0011	***	1.3E-6	3.1E-6	****
Firmicutes	4E-12	2E-11	****	0.39	0.4		4E-14	7E-13	****	2E-10	7E-10	****
Planctomycetes	6E-10	1.4E-9	****	0.011	0.015	*	3E-11	2E-10	****	1.3E-6	3.1E-6	****
Proteobacteria	5E-15	3E-14	****	9E-14	1E-12	****	2E-16	9E-16	****	0.0058	0.0084	**
Tenericutes	2E-16	2E-16	****	3E-13	2E-12	****	3E-13	2E-12	****	0.0002	0.0004	***
Unknown	3.2E-7	4.8E-7	****	0.0001	2.0E-4	***	1.1E-8	3.7E-8	****	0.18	0.2	
Verrucomicrobia	2.1E-7	3.5E-7	****	4.8E-5	9.5E-5	****	5.7E-9	2.0E-8	****	0.33	0.35	
Other	5.4E-5	5.9E-5	****	0.0012	0.0018	**	8.7E-6	1.9E-5	****	0.39	0.4	

Supplementary Table 6. Wilcox Rank Sum Test for select Phyla detected in 16S rRNA amplicons by Field Site and Nutrient Enrichment, adjusted p-value with Benjamini-Hochberg method.

	Northern vs. Southern			Control vs. Enriched	
Order	**P value**	**P adj**		**P value**	**P adj**
Actinobacteria	0.41909	0.56		0.218	0.5
Bacteroidetes	0.46654	0.56		0.052	0.33
Chloroflexi	3.60E-06	2.10E-05	****	0.587	0.78
Cyanobacteria	6.00E-10	7.20E-09	****	0.794	0.87
Epsilonbacteraeota	0.00092	0.0037	***	0.055	0.33
Firmicutes	0.17351	0.35		0.289	0.5
Planctomycetes	0.37792	0.56		0.724	0.87
Proteobacteria	0.0381	0.091		0.265	0.5
Tenericutes	0.63766	0.64		0.415	0.62
Unknown	0.59934	0.64		0.162	0.49
Verrucomicrobia	0.01588	0.048	*	0.953	0.95
Other	0.46654	0.56		0.088	0.35

Supplementary Table 7. Wilcox Rank Sum Test for select Orders detected in 16S rRNA amplicons by Sample Type, adjusted p-value with Benjamini-Hochberg method. The global statistics were calculated using the Kruskal-Wallis Rank Sum Test.

	Global Kruskal-Wallis			Gut vs. Inner Swab			Gut vs. Outer Swab			Inner vs. Outer Swab		
Order	**Pvalue**	**P adj**		**Pvalue**	**P adj**		**Pvalue**	**P adj**		**Pvalue**	**P adj**	
Alteromonadales	5.7E-6	1.9E-5	****	7.4E-7	5.1E-6	****	1.5E-6	9.5E-6	****	0.13	0.17	
Ardenticatenales	0.040	0.045	*	0.068	0.099		0.033	0.052		0.18	0.23	
Bacteroidales	8.3E-5	0.0002	****	0.0005	0.0013	***	0.44	0.49		3.0E-6	1.5E-5	****
Campylobacterales	0.009	0.011	**	0.29	0.34		0.045	0.068		0.0023	0.0053	**
Caulobacterales	0.0053	0.0073	**	0.20	0.25		0.22	0.26		0.0001	0.0004	***
Cellvibrionales	0.0092	0.011	**	0.0083	0.014	**	0.0068	0.012	**	0.71	0.74	
Chitinophagales	1.3E-6	1.2E-5	****	7.4E-7	5.1E-6	****	7.4E-7	5.1E-6	****	0.0029	0.0061	**
Chloroplast	0.40	0.42		0.93	0.95		0.22	0.26		0.32	0.37	
Clostridiales	1.6E-5	4.4E-5	****	0.9774	0.98		3.0E-6	1.5E-5	****	3.0E-6	1.5E-5	****
Corynebacteriales	0.0002	0.0003	***	0.0029	0.0061	**	3.3E-5	0.0001	****	0.059	0.088	
Cytophagales	0.0011	0.0017	**	0.0002	0.0006	***	0.0045	0.0085	**	0.51	0.56	
Desulfobacterales	6.3E-6	1.9E-5	****	0.27	0.31		7.4E-7	5.1E-6	****	7.4E-7	5.1E-6	****
Ectothiorhodospirales	4.9E-7	7.8E-6	****	5.0E-5	0.0002	****	7.4E-7	5.1E-6	****	3.0E-6	1.5E-5	****
Entomoplasmatales	5.2E-6	1.9E-5	****	3.2E-5	0.0001	****	3.2E-5	0.0001	****	0.69	0.72	
Flavobacteriales	0.0005	0.0009	***	0.0001	0.0003	***	0.11	0.16		0.0068	0.012	**

	Global Kruskal-Wallis			Gut vs. Inner Swab			Gut vs. Outer Swab			Inner vs. Outer Swab		
Order	**Pvalue**	**P adj**		**Pvalue**	**P adj**		**Pvalue**	**P adj**		**Pvalue**	**P adj**	
Gammaproteobacteria Incertae Sedis	0.0045	0.0064	**	0.0045	0.0085	**	0.0036	0.0073	**	0.48	0.52	
Kordiimonadales	1.6E-6	1.2E-5	****	0.0018	0.0043	**	7.4E-7	5.1E-6	****	5.2E-6	2.3E-5	****
Microtrichales	0.0001	0.0002	***	0.63	0.67		0.0001	0.0003	***	3.3E-5	0.0001	****
Mycoplasmatales	2.5E-6	1.5E-5	****	7.4E-7	5.1E-6	***	3.4E-5	0.0001	****	0.023	0.039	*
Nitrosococcales	0.0002	0.0004	***	0.033	0.052		5.2E-6	2.3E-5	****	0.033	0.052	
Oceanospirillales	0.013	0.016	*	0.22	0.26		0.0045	0.0085	**	0.060	0.088	
Pirellulales	0.0011	0.0017	**	0.18	0.23		0.0036	0.0073	**	0.0007	0.0016	***
Rhizobiales	0.56	0.56		0.41	0.46		0.59	0.63		0.41	0.46	
Rhodobacterales	5.0E-6	1.9E-5	****	0.0011	0.0027	**	7.4E-7	5.1E-6	****	0.0005	0.0013	***
Thiohalorhabdales	5.2E-7	7.8E-6	****	7.4E-7	5.1E-6	***	7.4E-7	5.1E-6	****	0.0001	0.0004	***
Thiotrichales	5.5E-6	1.9E-5	****	7.4E-7	5.1E-6	***	7.4E-7	5.1E-6	****	0.18	0.23	
Unknown	0.021	0.025	*	0.0068	0.012	**	0.13	0.17		0.18	0.23	
Verrucomicrobiales	0.0004	0.0008	***	0.0029	0.0061	**	0.0001	0.0003	***	0.18	0.23	
Vibrionales	0.067	0.072		0.98	0.98		0.078	0.11		0.028	0.047	*
Xanthomonadales	4.4E-5	0.0001	****	0.0029	0.0061	**	0.010	0.017	*	9.7E-5	0.0003	****

Supplementary Table 8. Wilcox Rank Sum Test for select Orders detected in 16S rRNA amplicons for all sample types combined by Field Site and Nutrient Enrichment, adjusted p-value with Benjamini-Hochberg method.

	Northern vs. Southern			Control vs. Enriched	
Order	**P value**	**P adj**		**P value**	**P adj**
Alteromonadales	0.8636	1		0.1916	0.84
Ardenticatenales	9.90E-09	3.00E-07	****	0.9875	0.99
Bacteroidales	0.4064	0.75		0.696	0.99
Campylobacterales	0.0129	0.077		0.2142	0.84
Caulobacterales	0.0591	0.27		0.5841	0.99
Cellvibrionales	0.0013	0.0096	**	0.355	0.97
Chitinophagales	0.181	0.54		0.8882	0.99
Chloroplast	6.00E-06	9.10E-05	****	0.6503	0.99
Clostridiales	0.8636	1		0.9378	0.99
Corynebacteriales	0.9626	1		0.2788	0.84
Cytophagales	0.8636	1		0.0071	0.11
Desulfobacterales	0.4245	0.75		0.9875	0.99
Ectothiorhodospirales	0.9378	1		0.7905	0.99
Entomoplasmatales	0.5402	0.85		0.2592	0.84
Flavobacteriales	0.5212	0.85		0.7666	0.99
Gammaproteobacteria Incertae Sedis	0.2027	0.55		0.7428	0.99
Kordiimonadales	0.2262	0.55		0.5841	0.99
Microtrichales	0.0637	0.27		0.7193	0.99
Mycoplasmatales	0.6457	0.97		0.7875	0.99
Nitrosococcales	2.40E-05	0.00024	****	0.5418	0.99
Oceanospirillales	1	1		0.1108	0.83
Pirellulales	0.2387	0.55		0.5841	0.99
Rhizobiales	0.8636	1		0.2788	0.84
Rhodobacterales	0.1108	0.37		0.9875	0.99
Thiohalorhabdales	1	1		0.8391	0.99
Thiotrichales	0.2788	0.6		0.0736	0.74
Unknown	0.4245	0.75		0.1708	0.84
Verrucomicrobiales	0.0736	0.28		0.9875	0.99
Vibrionales	0.9626	1		0.0021	0.063
Xanthomonadales	0.788	1		0.6693	0.99

Supplementary Table 9. Wilcox Rank Sum Test for select Orders detected in 16S rRNA amplicons for Gut samples by Field Site and Nutrient Enrichment, adjusted p-value with Benjamini-Hochberg method.

	Gut samples				
	Northern vs. Southern			Control vs. Enriched	
Order	**P value**	**P adj**		**P value**	**P adj**
Alteromonadales	0.8182	0.85		0.18	0.77
Ardenticatenales	0.0022	0.016	*	1	1
Bacteroidales	0.5887	0.77		0.699	1
Campylobacterales	0.0649	0.19		0.31	0.77
Caulobacterales	0.2403	0.45		1	1
Cellvibrionales	0.132	0.28		0.485	0.86
Chitinophagales	0.0411	0.15		0.24	0.77
Chloroplast	0.026	0.13		0.937	1
Clostridiales	0.4848	0.69		0.937	1
Corynebacteriales	0.8182	0.85		0.041	0.41
Cytophagales	0.0411	0.15		0.485	0.86
Desulfobacterales	0.6991	0.84		0.818	1
Ectothiorhodospirales	0.3095	0.46		0.394	0.79
Entomoplasmatales	0.0649	0.19		0.041	0.41
Flavobacteriales	0.3095	0.46		0.093	0.56
Gammaproteobacteria Incertae Sedis	0.5887	0.77		0.31	0.77
Kordiimonadales	0.0022	0.016	*	1	1
Microtrichales	0.0022	0.016	*	0.394	0.79
Mycoplasmatales	0.132	0.28		0.699	1
Nitrosococcales	0.0022	0.016	*	0.589	0.98
Oceanospirillales	0.8182	0.85		0.31	0.77
Pirellulales	0.026	0.13		0.937	1
Rhizobiales	0.8182	0.85		0.132	0.66
Rhodobacterales	0.1797	0.36		0.24	0.77
Thiohalorhabdales	0.3095	0.46		0.818	1
Thiotrichales	0.132	0.28		0.818	1
Unknown	0.6991	0.84		0.041	0.41
Verrucomicrobiales	0.132	0.28		0.699	1
Vibrionales	0.3095	0.46		0.394	0.79
Xanthomonadales	0.9372	0.94		0.093	0.56

Supplementary Table 10. Wilcox Rank Sum Test for select Orders detected in 16S rRNA amplicons for Inner Shell samples by Field Site and Nutrient Enrichment, adjusted p-value with Benjamini-Hochberg method.

	Inner shell samples				
	Northern vs. Southern			Control vs. Enriched	
Order	**P value**	**P adj**		**P value**	**P adj**
Alteromonadales	0.4848	0.66		0.1797	0.67
Ardenticatenales	0.0022	0.032	*	0.6991	0.84
Bacteroidales	1	1		0.1797	0.67
Campylobacterales	0.132	0.33		0.9372	0.94
Caulobacterales	0.3939	0.59		0.9372	0.94
Cellvibrionales	0.0931	0.33		0.3095	0.71
Chitinophagales	0.3095	0.55		0.3095	0.71
Chloroplast	0.0411	0.33		0.4848	0.81
Clostridiales	1	1		0.2403	0.71
Corynebacteriales	0.6991	0.81		0.0931	0.56
Cytophagales	0.9372	1		0.0087	0.26
Desulfobacterales	0.5887	0.71		0.3939	0.74
Ectothiorhodospirales	0.4848	0.66		0.2403	0.71
Entomoplasmatales	0.1999	0.43		0.8643	0.93
Flavobacteriales	0.1797	0.41		0.5887	0.84
Gammaproteobacteria Incertae Sedis	0.132	0.33		0.4848	0.81
Kordiimonadales	0.132	0.33		0.3095	0.71
Microtrichales	0.132	0.33		0.3939	0.74
Mycoplasmatales	0.3939	0.59		0.5887	0.84
Nitrosococcales	0.0022	0.032	*	0.6991	0.84
Oceanospirillales	0.132	0.33		0.3939	0.74
Pirellulales	0.0649	0.33		0.8182	0.91
Rhizobiales	0.3939	0.59		0.026	0.31
Rhodobacterales	0.0931	0.33		0.5887	0.84
Thiohalorhabdales	0.2403	0.45		0.6991	0.84
Thiotrichales	0.5887	0.71		0.0411	0.31
Unknown	0.9372	1		0.8182	0.91
Verrucomicrobiales	0.0649	0.33		0.6991	0.84
Vibrionales	0.2403	0.45		0.0411	0.31
Xanthomonadales	0.5887	0.71		0.1797	0.67

Supplementary Table 11. Wilcox Rank Sum Test for select Orders detected in 16S rRNA amplicons Outer Shell samples by Field Site and Nutrient Enrichment, adjusted p-value with Benjamini-Hochberg method.

	Outer shell samples					
	Northern vs. Southern			Control vs. Enriched		
Order	**P value**	**P adj**		**P value**	**P adj**	
Alteromonadales	0.9372	1		0.0022	0.065	
Ardenticatenales	0.0022	0.013	*	0.9372	0.94	
Bacteroidales	0.0931	0.25		0.3939	0.51	
Campylobacterales	0.3095	0.55		0.0649	0.22	
Caulobacterales	0.0152	0.057		0.132	0.34	
Cellvibrionales	0.0043	0.022	*	0.9372	0.94	
Chitinophagales	0.0931	0.25		0.9372	0.94	
Chloroplast	0.0022	0.013	*	0.6991	0.84	
Clostridiales	1	1		0.1797	0.34	
Corynebacteriales	1	1		0.026	0.19	
Cytophagales	0.3939	0.59		0.0411	0.22	
Desulfobacterales	0.132	0.26		0.0649	0.22	
Ectothiorhodospirales	0.9372	1		0.2403	0.4	
Entomoplasmatales	0.124	0.26		0.1463	0.34	
Flavobacteriales	0.9372	1		0.1797	0.34	
Gammaproteobacteria Incertae Sedis	0.132	0.26		0.3095	0.44	
Kordiimonadales	0.4848	0.69		0.9372	0.94	
Microtrichales	0.6991	0.91		0.2403	0.4	
Mycoplasmatales	0.0062	0.026	*	0.3611	0.49	
Nitrosococcales	0.0022	0.013	*	0.3095	0.44	
Oceanospirillales	0.3939	0.59		0.132	0.34	
Pirellulales	0.5887	0.8		0.1797	0.34	
Rhizobiales	1	1		0.1797	0.34	
Rhodobacterales	0.132	0.26		0.026	0.19	
Thiohalorhabdales	0.0649	0.22		0.3095	0.44	
Thiotrichales	0.3939	0.59		0.0043	0.065	
Unknown	0.0022	0.013	*	0.9372	0.94	
Verrucomicrobiales	0.0022	0.013	*	0.4848	0.61	
Vibrionales	0.2403	0.45		0.0649	0.22	
Xanthomonadales	0.8099	1		0.045	0.22	

CHAPTER IV: Bacterial Community Dynamics in an Oyster Hatchery in Response to Probiotic Treatment

By

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Abstract

Larval oysters in hatcheries are susceptible to diseases caused by bacterial pathogens, including *Vibrio* spp. Previous studies have shown that daily addition of the probiotic *Bacillus pumilus* RI06-95 to water in rearing tanks increases larval survival when challenged with the pathogen *Vibrio coralliilyticus*. We propose that the presence of probiotics causes shifts in bacterial community structure in rearing tanks, leading to a net decrease in the relative abundance of potential pathogens. During three trials spanning the 2012-2015 hatchery seasons, larvae, tank biofilm, and rearing water samples were collected from control and probiotic-treated tanks in an oyster hatchery over a 12-day period after spawning. Samples were analyzed by 16S rRNA sequencing of the V4 or V6 regions followed by taxonomic classification, in order to determine bacterial community structures. There were significant differences in bacterial composition over time and between sample types, but no major effect of probiotics on the structure and diversity of bacterial communities (phylum level, Bray-Curtis k=2, 95% confidence). Probiotic treatment, however, led to a higher relative percent abundance of *Oceanospirillales* and *Bacillus* spp. in water and oyster larvae. In the water, an increase in *Vibrio* spp. diversity in the absence of a net increase in relative read abundance suggests a likely decrease in the abundance of specific pathogenic *Vibrio* spp., and therefore lower chances of a disease outbreak. Co-occurrence network analysis also suggests that probiotic treatment had a systemic effect on targeted members of the bacterial community, leading to a net decrease in potentially pathogenic species.

Introduction

Diseases caused by bacterial pathogens result in losses in aquaculture and wild populations of commercially important shellfish and finfish (Groner et al., 2016; Lafferty et al., 2015; Pérez-Sánchez et al., 2018). World aquaculture production is valued at $243.5 billion USD, and disease is a primary limiting factor on its growth and economic worth (FAO, 2018; Stentiford et al., 2012). Larval oysters are especially susceptible to disease, often by etiological agents from the genus *Vibrio* (Beaz-Hidalgo et al., 2010a; Dubert et al., 2017; King et al., 2018; Le Roux et al., 2016; Richards et al., 2015). Pathogenic *Vibrio* spp. are naturally occurring microbes in coastal waters, which makes them difficult to avoid. In an effort to maintain a healthy environment, hatcheries work towards optimum water quality by controlling larval culture density and the use of water treatment systems (Mckindsey et al., 2007; Pérez-Sánchez et al., 2018).

An alternative method for the management of disease in aquaculture involves the use of probiotics, microorganisms that provide health benefits to the host, including protection against bacterial pathogens. Probiotics exert their beneficial effects through a variety of mechanisms, including direct pathogen inhibition, competition for nutrients, secretion of antibacterial substances, and improvement of water quality (Kesarcodi-Watson et al., 2008, 2012; Prado et al., 2010). Previous studies have shown that treatment of larval oysters in the laboratory or the hatchery with the probiotic bacterium *Bacillus pumilus* RI06-95 significantly increases their survival when challenged with the pathogen *Vibrio coralliilyticus* (Karim et al., 2013; Sohn et al., 2016a). Additionally, administration of this probiotic in a hatchery setting results

in reductions in total *Vibrio* abundance in tank water and surfaces, compared to the control tanks (Sohn et al., 2016a).

However, there is a lack of knowledge regarding the effects of probiotics on the systems in which they are used. There are concerns about using probiotic bacteria to combat disease in open aquaculture systems, as they will eventually disperse into the environment and may thus affect bacterial diversity in these systems (Newaj-Fyzul et al., 2014). Improper selection of probiotics may result in bacterial dysbiosis, which could ultimately impact host health (Verschuere et al., 2000). As filter feeders that process large volumes of seawater daily, bivalves are especially susceptible to changes in bacterial community composition in the water (Burge et al., 2016). Moreover, bacteria both contribute to and serve as indicators of oyster health and function of the microbial community (Le Roux et al., 2016) and likely mediate the effect(s) of probiotics on the host. Therefore, it is important to assess the effects of probiotics not only on the health and protection of the host, but also on the bacterial communities in the systems in which oysters are grown.

Previous studies of microbiomes in adult oysters have shown differences in microbiota according to tissue type, geographic location, season, and environmental conditions (Chauhan et al., 2014; King et al., 2012; Lokmer et al., 2016b; Lokmer and Wegner, 2015; Pierce et al., 2016; Pierce and Ward, 2018). Additionally, the oyster microbiomes are distinct from those of the surrounding water and are often dominated by *Proteobacteria*, *Cyanobacteria*, and *Firmicutes* (Lokmer et al., 2016a). Three independent microbiome studies of larval cultures of the Pacific oyster, *Crassostrea gigas* found that, even though the microbiome in the rearing water changes throughout

the year, there is little effect from direct manipulation of rearing conditions themselves, including salinity and temperature (Asmani et al., 2016; Powell et al., 2013; Trabal Fernández et al., 2014). Microbiome studies of juvenile Kumamoto oysters treated with *Streptomyces* N7 and RL8 showed an increase in species diversity and changes in the relative abundances of taxa, compared to control oysters (García Bernal et al., 2017). However, the effect of probiotics on bacterial communities in an oyster hatchery has not yet been determined.

In this study, we analyzed the structure and diversity of bacterial communities in larval oysters, their rearing water, and in tank biofilms over a 12-day period following treatment with the probiotic *Bacillus pumilus* RI06-95. We hypothesized that probiotic treatment has a cascading effect on the bacterial community structure that alters the microbiomes of the rearing water, tank biofilms, and larvae, leading to a net decrease in potentially pathogenic species.

Materials and Methods

Bacterial Strain and Culture Conditions

The probiotic strain *Bacillus pumilus* RI06-95, previously isolated from a marine sponge from the Pettaquamscutt River in Rhode Island (Karim et al., 2013), was cultured in yeast peptone with 3% salt (mYP30) media (5 g L^{-1} of peptone, 1 g L^{-1} of yeast extract, 30 g L^{-1} of ocean salt (Red Sea Salt, Ohio, USA)) at 28 °C with shaking at 170 rpm. The bacterial cell concentration was estimated by OD_{550} measurements using a spectrophotometer (Synergy HT, BioTek, USA) and confirmed using serial

dilution and spot plating on mYP30 agar plates to determine colony forming units (CFU).

Experimental Design and Sample Collection

Samples for microbiome analysis were collected during 3 hatchery trials performed at the Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA) (Table 1). Eastern oysters (*Crassostrea virginica*) were spawned following standard procedures (Helm and Bourne, 2004). Spawning day is referred to as Day 0 throughout the manuscript. Larvae (1-day old) were distributed and maintained in static conditions in triplicate 120 L conical tanks for each treatment containing filtered and UV sterilized seawater at 21 - 23 °C and a salinity of 28 psu. Tanks were randomly assigned to treatments including no probiotics (control) and probiotic treatment with probiotic *B. pumilus* RI06-95. The probiotic was administered daily at 10^4 CFU/mL, regardless of the length of the trial, to treatment tanks after being mixed with the microalgal feed. The microalgae strains used for feeding included *Chaetoceros muelleri* (CCMP1316), *Isochrysis galbana* (CCMP1323), *Tisochrysis lutea* (CCMP1324), *Pavlova lutheri* (CCMP1325), *Tetraselmis* sp. (CCMP892), and *Thalassiosira weisflogii* (CCMP1336). Experimental tanks were drained every other day to perform larval counts and grading. Tanks were washed thoroughly with a diluted bleach solution, rinsed, and replenished with filtered and UV-treated water prior to restocking the larvae. Sampling timepoints and trial lengths varied according to the hatchery-scheduled drain down days, so that extensive larval counts would coincide with sampling days.

Rearing water (volumes in Table 1) was collected from each of the triplicate tanks during drain-down and filtered through a 0.22 μm Sterivex filter (Millipore, Milford, MA, USA). The Sterivex filters were immediately frozen and stored at -80 °C until DNA extraction. Biofilm swab samples were collected from the surface inside of each tank after drain-down of the water by swabbing a line of approximately 144 cm in length with sterile cotton swabs. The cotton tips of the swabs were stored in RNAlater (Ambion, Inc., Foster City, CA, USA). Oyster larvae were collected on a 55 μm sieve after drain-down of tank water and resuspended in 5 L of seawater. 10 mL of oyster larvae (from each tank, about 150 – 1500 larvae) were then placed into a sterile tube. In the laboratory, oyster larvae were collected on a 40 μm nylon membrane and rinsed with filtered sterile seawater (FSSW) to reduce loosely attached environmental bacteria. Swab and larvae samples were flash frozen in liquid nitrogen and stored at -80 °C until DNA extraction. Extracts from swab, larvae, and water samples were cultured on selective media to perform culturable *Vibrio* counts following methods in Sohn et al., 2016b. All sample types were collected during Trials 1 and 2, but only water samples were collected during Trial 3 (Table 1). In Trial 3, water (1 – 2 L) was also collected from the inflow (water piped directly from the environment) and outflow (water collected after filtration and UV-treatment prior to reaching the hatchery tanks) and processed as described above for tank water.

DNA Extraction, Amplification, and Sequencing

Total DNA from water samples was extracted from the filters using the PowerWater Sterivex DNA Isolation Kit (MoBio Laboratories, USA) according to manufacturer recommendations (Trials 1 and 2) or Gentra Puregene Reagents (Qiagen,

Hilden, Germany) with an added proteinase K-lytic enzyme digestion step (Sinigalliano et al., 2007; Trial 3). In addition, total bacterial DNA from the tank biofilm swabs and oyster larvae were extracted using the PowerSoil DNA Isolation Kit (MoBio) with slight modifications detailed below. In brief, frozen pooled oyster larvae were ground in a mortar with a sterile pestle and then placed into bead tubes for extraction (Qiagen). The RNAlater samples containing the cotton tops of the swabs were placed directly into bead tubes. Bead tubes were incubated at 65 °C for 10 min and then shaken horizontally at maximum speed for 10 min using the MoBio vortex adaptor. Following extraction, DNA concentration was quantified with both a Nanodrop 2000 instrument and a Qubit Fluorometer (ThermoFisher Scientific, Wilmington, DE, USA). The performance and quality of DNA extractions was comparable between Trials and sample types.

16S rRNA gene amplicon analysis was performed using 515F/806R primers to amplify the V4 region (Trials 1 and 2) or 967F/1064R primers to amplify the V6 region (Trial 3). The V4 region was used in Trials 1 and 2 for better taxonomic resolution of all sample types and the V6 region was used in Trial 3 for independent confirmation with greater sequencing depth. A two-step PCR reaction following Illumina's 16S Metagenomic Sequencing Library Preparation Protocol was performed on the samples from Trials 1 and 2 (Illumina Inc., San Diego, CA, USA). The PCR products were then analyzed with 250 bp paired-end sequencing to obtain fully overlapping reads on an Illumina MiSeq at the Genomics and Sequencing Center at the University of Rhode Island. The samples from Trial 3 were prepared with a 2-step fusion primer PCR amplification according to the protocols from the Keck Sequencing

Center at the Marine Biological Laboratory (MBL). Paired-end sequencing was performed at the MBL on an Illumina HiSeq 2500 to generate 100 bp double strand reads with full overlap of the V6 region.

Processing of Sequencing Data

Sequences from Trials 1 and 2 were demultiplexed using FastQC v0.11.4 (Andrews, 2010), then merged and trimmed using Trimmomatic v0.32 (Bolger et al., 2014). All sequences shorter than 200 bp were removed from the dataset. Sequences from Trial 3 were demultiplexed and quality filtered following standard protocols at the MBL Bay Paul Center that remove reads where forward and reverse sequences do not match perfectly (Eren et al., 2013b). All sequences were uploaded to VAMPS (Visualization and Analysis of Microbial Population Structure) and classified directly using the GAST pipeline with the SILVA database, in order to compare between the three trials (Huse et al., 2014). The taxonomy data from each trial were separately normalized to the total reads of each sample to provide relative abundance of each taxa in percentage, and then exported as a matrix or BIOM file for analysis in R (Version 3.3.1). *Vibrio* spp. sequences in water samples from Trial 3 were processed through the oligotyping pipeline described in Eren et al. (2013a) as implemented in VAMPS, and annotated using SILVA.

Statistical and Network Analysis

All descriptive and statistical analyses were performed in the R statistical computing environment with the *vegan* and *phyloseq* packages (Dixon, 2003; McMurdie and Holmes, 2013). Simpson's diversity values were calculated for each sample at the order level using the *vegan* package Version 2.4-1 and analyzed and

analyzed using the non-parametric Kruskal-Wallis rank sum test in R. Non-metric dimensional analysis (NMDS) was used to determine the influence of time, probiotic treatment, or sample type on the bacterial community composition, based on methods by Torondel et al. (2016) and implemented using *vegan*. The Bray-Curtis dissimilarity metric was calculated with k=2 for max 50 iterations and 95% confidence intervals (standard deviation) were plotted. Statistical testing of the beta-diversity was done using the adonis2 test implemented in *vegan* (method="bray", k=2) (Mcardle and Anderson, 2010; Warton et al., 2012). Additionally, relative percent abundances of specific taxa were extracted and plotted according to treatment and time, and analyzed using the Kruskal-Wallis test in R.

A co-occurrence network was generated with normalized taxa counts at the Order level from water samples in Trial 3 (n=18) to determine hypothetical relationships resulting from each treatment. The make_network() command from the *phyloseq* package was used with the Bray-Curtis dissimilarity metric, max distance=0.5. The mean resulting relationship table including 123 taxa (nodes) and 670 relationships (edges) was exported to Cytoscape Version 3.6.0 for visualization and analysis (Shannon et al., 2003). Nodes were assigned continuous size attributes based on the number of total reads in all samples per taxa (2 to 2,720,021), and discrete shape and continuous color according to whether the taxa were more abundant in the control or probiotic-treated samples (0 to 3.6 times).

Results

Bacterial Structure and Diversity Over Time

In order to determine the effect of probiotics on the microbial community dynamics in an oyster hatchery, we needed to first characterize bacterial structure and diversity in different environmental niches within the hatchery (water, tank surfaces, and larvae) over time. A total of 18,103,647 quality-controlled 16S rRNA gene amplicon sequences were analyzed from 42 rearing water samples, 24 tank biofilm swabs, and 21 pooled larvae samples from three hatchery trials. There was an average of 208,087 reads for each of the 87 samples, ranging between 961-1,117,380 depending on the sequencing method and sample type (Figure 1, top). Direct taxonomical classification resulted in the detection of a total of 168 Orders across 29 Phyla in all samples. Overall, bacterial communities for each trial and sample type shared many of the most dominant phyla, although differences in relative abundance were seen between trials, time points, and sample types (Figure 1, bottom left). The most dominant phyla in the water community, averaged from all samples, were *Proteobacteria* (53 ± 6%), *Bacteroidetes* (26 ± 10%), *Cyanobacteria* (12 ± 10%), *Actinobacteria* (5 ± 5%), and *Planctomycetes* (2 ± 1%) (Figure 1, bottom right). The larval samples were dominated by *Proteobacteria* (87 ± 12%) and the swab samples by *Proteobacteria* (68 ±17%), *Cyanobacteria* (19 ± 16%), and *Bacteroidetes* (8 ± 4%) (Figure 1, bottom left). Percent abundance of *Cyanobacteria* was significantly higher in swab than in water samples ($p<0.001$, Table S1). Larval and swab samples showed a significantly higher proportion of *Proteobacteria*, and lower percent abundance of *Bacteroidetes*, as compared to water samples ($p<0.001$, Table S1). No significant

effect of probiotic treatment was observed on the relative abundance of dominant phyla ($p>0.38$).

Overall, the bacterial communities in rearing water were significantly more diverse than the communities in oyster larvae and tank biofilm swab samples (Simpson's Diversity Index, $p<0.001$, Figure 2, Table S2), reflecting an enrichment in specific community members in larvae and tank surfaces from the more diverse rearing water community (Figure 1). Simpson's Diversity Index indicated significantly higher diversity in rearing water samples from Trial 3 (0.66±0.04), than from Trials 1 (0.59±0.3) and 2 (0.53±0.5) ($p<0.001$, Figure 2, Table S2), most probably due to the greater sequencing depth and different target 16S variable region in Trial 3 (Figure S1), but potentially also due to seasonal and yearly differences in bacterial composition of the rearing water source (Table 1). There was also high variability among replicate samples from each timepoint and treatment, especially in oyster larvae samples (Figure 2, Figure S2). Significant increases in bacterial diversity over time were detected in the oyster larvae and biofilm swabs in Trial 1 ($p<0.01$, Table S3), and in the rearing water in Trials 2 and 3 ($p<0.01$, Figure 2, Tables S4, S5). No significant differences in Simpson's Diversity Index were detected between control and treated samples at any timepoints for any of the sample types ($p=0.52$).

The bacterial community structures of the water and oyster larvae samples were significantly different (Bray-Curtis, k=2, 95% confidence, adonis2 $p=0.001$) in both Trial 1 and Trial 2 (Figure 3A, Table S6). The community structure of microbiomes in tank biofilms (swab samples) was not significantly different from the structure of either the water or oyster larvae samples, suggesting an intermediate microbiome

stage. Bacterial communities in the rearing water were significantly different between sampling timepoints (Bray-Curtis, k=2, 95% confidence, adonis2 $p<0.02$) in all three trials (Figure 3B, Table S6). Moreover, the bacterial community in samples of inflow and outflow seawater, which were collected on days 5, 8, and 12 during Trial 3, was distinct from that of the water in rearing tanks (Figure S3, adonis2 $p=0.001$, Table S6). These results suggest that hatchery tanks containing oyster larvae have dynamically developing microbiomes, despite the fact that they are all receiving the same inflow seawater. There was no significant effect of treatment on the beta-diversity in water samples from all time points (Figure 3C, Table S6).

Effects of the Probiotic on the Selected Members of the Bacterial Community

Although control and probiotic-treated tanks showed no significant differences in diversity and structure of bacterial communities overall (Figure 3C), significant differences in the relative read abundance of several specific taxa were detected. In all trials, *Bacillales* reads in the probiotic-treated water samples increased through time, and were significantly more abundant in samples from treated tanks than in the control tanks by the final sampling day in all trials ($p<0.05$, Figure 4A, Table S7). These consistent results suggest that the relative increase in reads corresponded to the added probiotic. The relative percent of *Oceanospirillales* reads was also significantly higher by 20-34% at all but one time point in probiotic-treated rearing water as compared to control water in all trials ($p<0.05$, Figure 4B, Table S8). The relative percent abundance of *Oceanospirillales* reads in the water significantly decreased over time by 41-62% (depending on the trial) ($p<0.05$, Figure 4B, Table S8). No significant changes in relative percent read abundance of these two selected members of the

bacterial community were detected in larval oysters or swabs, but percent abundance was low in these sample types (Trials 1 and 2; not shown).

Vibrio is a taxon that comprises a significant number of larval oyster pathogens (Elston et al., 1981, 2008; Le Roux et al., 2016; Richards et al., 2015), therefore we evaluated the effect of probiotic treatment on changes in *Vibrio* spp. diversity, relative abundance, and culturable colonies on selective media, over time during each of the hatchery trials (Figures 5, S4, S5). Probiotic treatment led to a significant increase in *Vibrio* diversity (as measured using the Simpson's Index of diversity) in water samples collected on day 12 in Trial 1 ($p < 0.05$; Figure 5A, Table S9). No significant differences in relative percent abundance of *Vibrio* spp. between control and probiotic-treated tanks were detected for any of the sample types (Figure 5B, Table S10). Colony counts of culturable *Vibrios*, however, were significantly lower in probiotic-treated tanks, relative to control tanks ($p<0.05$, Figure 5C, Table S11). When considering the effect of sample type, *Vibrio* relative abundance and culturable *Vibrios* were significantly lower in water samples than in swabs or oysters (all time points) and in swabs than in oysters (Day 12 only; $p<0.05$, Figures 5B and 5C, Tables S10 and S11). When considering data from all timepoints together, the diversity of *Vibrio* spp. as detected using 16S rRNA gene sequencing was significantly higher in swab and oyster samples than in water samples ($p<0.05$, Figure 5A, Table S9). An evaluation of the effect of time on *Vibrio* relative abundance and diversity showed a significant increase in the diversity of *Vibrio* spp. in swab and water samples (Trial 1, $p<0.005$, Figure 5A, Table S9), and a significant decrease in relative abundance in all sample types (Trial 1, $p<0.005$, Figure 5B, Table S10). This decrease is abundance is

further seen in colony counts of culturable *Vibrios* in the water samples (Trial 1, $p<0.05$, Figure 5C, Table S11).

Since the V6 region of the 16S rRNA gene was deeply sequenced in Trial 3, we were able to perform an oligotyping analysis - a method that detects genetic variants within a taxon - of the 1,727 *Vibrio* reads in the 18 water samples. Changes in the overall composition of the *Vibrio* community over time and by treatment were observed by oligotyping (Figure 6). On Day 5, while the *Vibrio* community in control tanks was dominated by an oligotype most closely related to *V. alginolyticus* WW1 (64 ± 6%), probiotic tanks showed a mix of *V. alginolyticus* WW1 (31 ± 3%) and *Halovibrio* sp. 5F5 (31 ± 3%). By Day 12, the *Vibrio* composition in water in control tanks was dominated by *V. celticus* 5OM18 (75 ± 3%), while a mix of *V. orientalis* LK2HaP4 (51 ± 10%) and *V. celticus* 5OM18 (35 ± 8%) was detected in probiotic tanks.

Bacterial Relationships with Co-Occurrence Analysis

A co-occurrence analysis of members of the bacterial community (Figure 7) in the 18 water samples from Trial 3 was performed to illustrate: a) how abundance of each Order changed relative to others (edge connections); b) which Orders were relatively most abundant in the system (node size); and c) how probiotic treatment affected their relative abundances (node color and shape). The most abundant taxa (*Rhodobacterales, Micrococcales, Sphingobacteriales, Flavobacteriales*, *Deferribacterales,* and *Oceanospirillales*) changed in similar fashion, but had different occurrence ratios between control and treatment samples. Orders that were more abundant in the treatment samples than in control samples included *Oceanospirillales*,

Caulobacterales, *Lentispherales*, *Acidithiobacillales*, *Chrococcales*, and *Bacillales*. These nodes were scattered throughout the network and did not share direct edges, but were within 3-5 edges of each other.

Bacillales, the Order to which the probiotic used in these experiments belongs, was shown to be most directly associated in the network with four other Orders that changed in relative abundance between control and treatment samples: *Chromatiales*, *Xanthomonadales*, *Cytophagia* Order II, and *Vibrionales*. This direct connection between *Bacillales* and *Vibrionales* in the network indicated that the probiotic may have directly affected members of *Vibrionales*. *Oceanospirillales* was placed in the network 5 edges away from *Bacillales*, sharing an edge with the treatment-abundant *Flavobacteriales*, a common environmental bacteria taxon (Bernardet et al., 2015). This network suggests that the probiotic did not directly alter the overall bacterial community in the rearing water in an oyster hatchery, but targeted specific members of the community.

Discussion

A better understanding of bacterial community dynamics in aquaculture systems is critical for optimizing disease management strategies such as probiotic treatment. This study characterized: a) changes in microbial communities in an oyster hatchery through the rearing process; and b) the effect of probiotic treatment on those communities. To our knowledge, this is the first study to characterize the effects of probiotics on microbiomes in a bivalve hatchery. Despite the high spatial (by sample type and replicate tank) and temporal variability in bacterial composition at the

hatchery detected in this research, results support the hypothesis that probiotic treatment leads to shifts in the microbial community in the hatchery from a state promoting the growth of potential pathogens to one that inhibits it.

Our results showed high variability in bacterial composition between replicate samples within trials and between trials, especially among the bacterial communities of oyster larvae. Variability between the 3 trials, conducted in July, January, and June in different years, is consistent with natural seasonal variation in microbial communities in Narragansett Bay (Staroscik and Smith, 2004). High variability in microbial communities in oysters from a single location is consistent with past studies, and is most probably driven by genetic and environmental effects on host-microbe interactions (King et al., 2012; Wegner et al., 2013). Moreover, variability between replicates (tanks within the hatchery) and between trials, may have been due to inevitable variance in husbandry and handling techniques at the hatchery (Elston et al., 1981, 2008).

Despite the high variability observed in these trials, our study observed clear differences in diversity and bacterial community structure between the rearing water, the biofilms on tank surfaces (swabs), and the oyster larvae. In particular, oyster larvae microbiomes were a subset of taxa present in the water and in biofilms, including *Firmicutes* and *Proteobacteria*, while tank biofilms showed a diversity and composition state that was intermediate between water and larvae. Lower diversity indices in the larvae and tank biofilms (swabs) than the water indicates niche selection of larval and biofilm colonizers, particularly *Cyanobacteria* in tank biofilms and *Proteobacteria* in oyster larvae. The dominance of *Proteobacteria* in the system, the

most abundant phylum in all samples (up to 87% in larvae), is consistent with previous studies where it was shown to make up the largest and most diverse phylum in oyster-associated microbiota (Dittmann et al., 2018; Hernández-Zárate and Olmos-Soto, 2006; Trabal Fernández et al., 2014). Bacteria are an essential component of aquaculture nutrition, as a source of both nutrients and growth factors for the microalgae, and as food for the larvae (Kamiyama, 2004; Natrah et al., 2014; Nevejan et al., 2016). Factors such as size, nutrient availability, metabolites, and accompanying bacteria lead to differential ingestion of algae and associated microbes in eastern oysters (Baldwin, 1995; Nevejan et al., 2016; Newell and Jordan, 1983; Pales Espinosa et al., 2009). Interestingly, strong temporal changes were seen in the structure of microbial communities of oyster larvae, tank surface biofilms, and/or rearing water in each of the trials. Considering the short duration of the trials (less than 15 days), this indicates that temporal changes in microbial communities in the tanks may be driven by developmental and health changes in the oyster larvae, since it is unlikely that these major changes are due to transient changes in the microbial composition of incoming water (as observed in Trial 3). More research is needed to evaluate the role of oyster-microbial interactions on the dynamics of microbial communities in rearing tanks in hatcheries.

There was no effect on bacterial community diversity or structure in any of the sample types, suggesting that the primary probiotic effect of *B. pumilus* RI06-95 is exerted directly on larval health (e.g. by modulation of the immune system) and/or that it is mediated by subtle, targeted changes in the oyster microbiomes that are obscured by larger temporal effects and/or by homogenization of large pools of larvae from

each tank. The presence of the probiotic was confirmed with higher relative abundance of *Bacillales* in the probiotic-treated water and increased relative abundance throughout the duration of each trial, suggesting that the probiotic accumulates in the larvae through time (tanks were scrubbed and water changed every other day). Previous studies of the impact of probiotics on microbiota in humans and fish also showed subtle changes of certain taxa, but no consistent effect on the diversity of the host's bacterial community (Boutin et al., 2013; Laursen et al., 2017; Merrifield and Carnevali, 2014; Schmidt et al., 2017; Standen et al., 2015). However, other studies report dramatic changes in fish intestinal microbiomes as a result of prebiotic treatment (Geraylou et al., 2013; Gonçalves and Gallardo-Escárate, 2017).

In addition to *Bacillales*, significant amplification of taxa was observed in probiotic-treated water samples compared to the control samples, most notably in the *Oceanospirillales* order. *Oceanospirillales* are heterotrophs commonly associated with mollusks and are found in the gills of many bivalves (Beinart et al., 2014; Costa et al., 2012; Jensen et al., 2010; Zurel et al., 2011). Additionally, they are recognized for their ability to degrade organic compounds in the environment and their abundance in oil plume microbial communities (Dubinsky et al., 2013; Hazen et al., 2010). These observations indicate that *Oceanospirillales* may confer a beneficial effect to the oyster host and contribute to the mechanism of oyster larval protection by the *B. pumilus* RI06-95 probiotic. Additionally, this suggests that the presence of *B. pumilus* RI06-95 has targeted effects on specific members of the microbial community in larval tanks in the hatchery.

Previous research showed that probiotic treatment with *B. pumilus* RI06-95 decreases levels of *Vibrio* spp. in the hatchery (Sohn et al., 2016a). This may be due to the production of antimicrobial secondary metabolites produced by *B. pumilus* RI06-95, as well as other *Bacillus* spp., that inhibit the growth of vibrios (Karim et al., 2013; Sohn et al., 2016a; Vaseeharan and Ramasamy, 2003). In the current study, a similar trend (as determined by a reduction in relative abundance, with overall trends confirmed using *Vibrio* spp. colony counts on selective media) was observed in treated tanks, but high variability and small sample sizes may have hindered detecting statistically significant differences. Moreover, failure to detect a significant decrease in *Vibrio* reads in Trial 2 (performed in January) was most probably due to the low abundance of *Vibrio* spp. in this trial, which is consistent with low levels of these species in coastal waters of the North Atlantic during winter (Staroscik and Smith, 2004). Interestingly, our research indicates that probiotic treatment leads to increased *Vibrio* diversity in rearing water through time. This increase in diversity in the absence of a net increase in relative abundance signifies a likely decrease in the relative abundance of specific pathogenic *Vibrio* spp., and therefore lower chances of a disease outbreak. Moreover, rRNA oligotyping of the *Vibrio* species in the water samples revealed a transition in the *Vibrio* community in probiotic-treated tanks from a predominance of potentially pathogenic species (*Vibrio alginolyticus*, a virulent pathogen originally isolated from amphioxius (Zou et al., 2016) and *Vibrio celticus*, a virulent anaerobic clam pathogen (Beaz-Hidalgo et al., 2010b)) to a predominance of a likely non-pathogenic species (*Vibrio orientalis*, a species that has been associated with adaptive functions (Mukhta et al., 2016; Tangl, 1983). This trend further

confirms that addition of *B. pumilus* RI06-95 causes targeted changes in certain taxa, especially *Vibrios*, which is highly relevant for decreasing infective doses and, consequently, disease dynamics (Chauhan and Singh, 2018).

This interpretation is consistent with results from the co-occurrence network analysis, a tool used to identify associations, patterns, roles, and inform hypotheses from 16S abundance data (Barberán et al., 2012). This analysis suggests a negative association between *Bacillales* with *Vibrionales* in the trials performed in summer months (Trials 1 and 3), when *Vibrionales* are more abundant in the environment and oysters. Previous research and sequencing of the genome of *B. pumilus* RI06-95 show that potential mechanisms of probiotic action can include direct competition with other species and biofilm formation (Hamblin et al., 2015; Karim et al., 2013). Competition between *B. pumilus* RI06-95 and other bacteria (including *Vibrionales*) could open niches in the oyster microbiome for potentially beneficial microbes.

In summary, the bacterial community dynamics observed in this study indicate a variety of interactions between larval oysters and specific members of the microbiome, such as *Vibrio* spp. and the *Bacillus* probiotic. First, *Vibrio* spp., as well as other Proteobacteria, appear to be particularly capable of colonizing and surviving within oyster larvae (Romalde et al., 2014). As seen in other probiotic species, these opportunistic *Vibrios* may be outcompeted by pre-colonization of other bacteria in the system, leading to a decrease in *Vibrio* abundance and/or an increase in diversity over time (Beaz-Hidalgo et al., 2010a; Zhao et al., 2016, 2018). We hypothesize that inhibition of *Vibrio* spp. by probiotic *B. pumilus* RI06-95 may allow for potentially beneficial *Oceanospirillales* to become more abundant in the system. Additional

research is needed to examine the specific interactions between *Oceanospirillales* symbionts, the *Bacillus* probiotic, *Vibrio* pathogens, and the oyster host. Elucidating such interactions will require more targeted 16S rRNA and functional metagenomic analyses to track specific species over time, as well as functional studies using *in vitro* and *in vivo* competition experiments.

Conclusion

This study investigated the effects of time and probiotic treatment on bacterial communities in an oyster hatchery. Understanding how probiotic treatment affect microbiota in aquaculture systems may help in optimizing their benefits and preventing undesirable side-effects (Kesarcodi-Watson et al., 2008). Our results show that there is a strong effect of time on the microbiomes within oyster larvae, on tank walls and in the rearing water, and that probiotic treatment leads to subtle changes in certain bacterial taxa, including an increase in the relative abundance of *Oceanospirillales* in the rearing water and changes in the *Vibrio* community. These results inform how probiotics may influence bacterial communities in an oyster hatchery over temporal and spatial scales, leading to an overall improvement in larval health.

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Data Availability

The raw sequences generated for this study can be found in the NCBI Short Read Archive under BioProject no. PRJNA518081. All processed 16S rRNA amplicon percent abundances at the Phylum and Order level and the R-markdown file to reproduce the figures in the manuscript can be found on Zenodo (https://doi.org/10.5281/zenodo.2658685). In addition, further public analysis and exploration of Trial 3 data are possible on the VAMPS website (vamps.mbl.edu) using software under the project name AFP_RWU1_Bv6.

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Figures and Tables

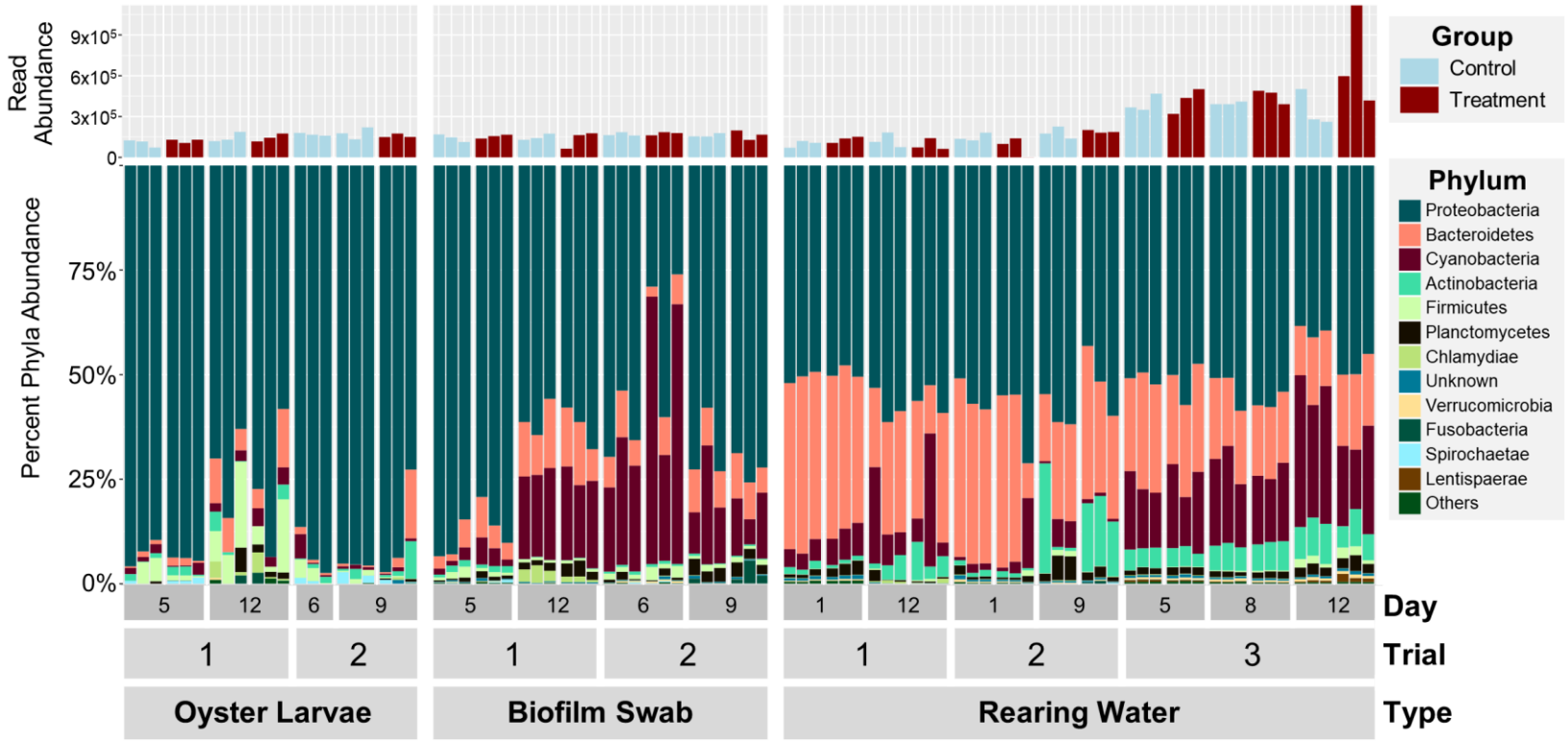


Figure IV-1. Sequencing read abundances and percent abundance by Phyla.
Percent abundances of the 12 most abundant phyla in oyster larvae, biofilm swab, and rearing water samples from all 3 Trials based on 16S rRNA amplicon sequencing data (bottom). The total abundance of quality filtered sequencing reads is shown in the bar graph (top). The 12 dominant phyla include Actinobacteria, Bacteroidetes, Cyanobacteria, Deferribacteres, Firmicutes, Fusobacteria, Lentisphaerae, Planctomycetes, Proteobacteria, Spirochaetae, Verrucomicrobia, and Unknown. Note: there are no treated oyster larvae samples from Trial 2, Day 6.

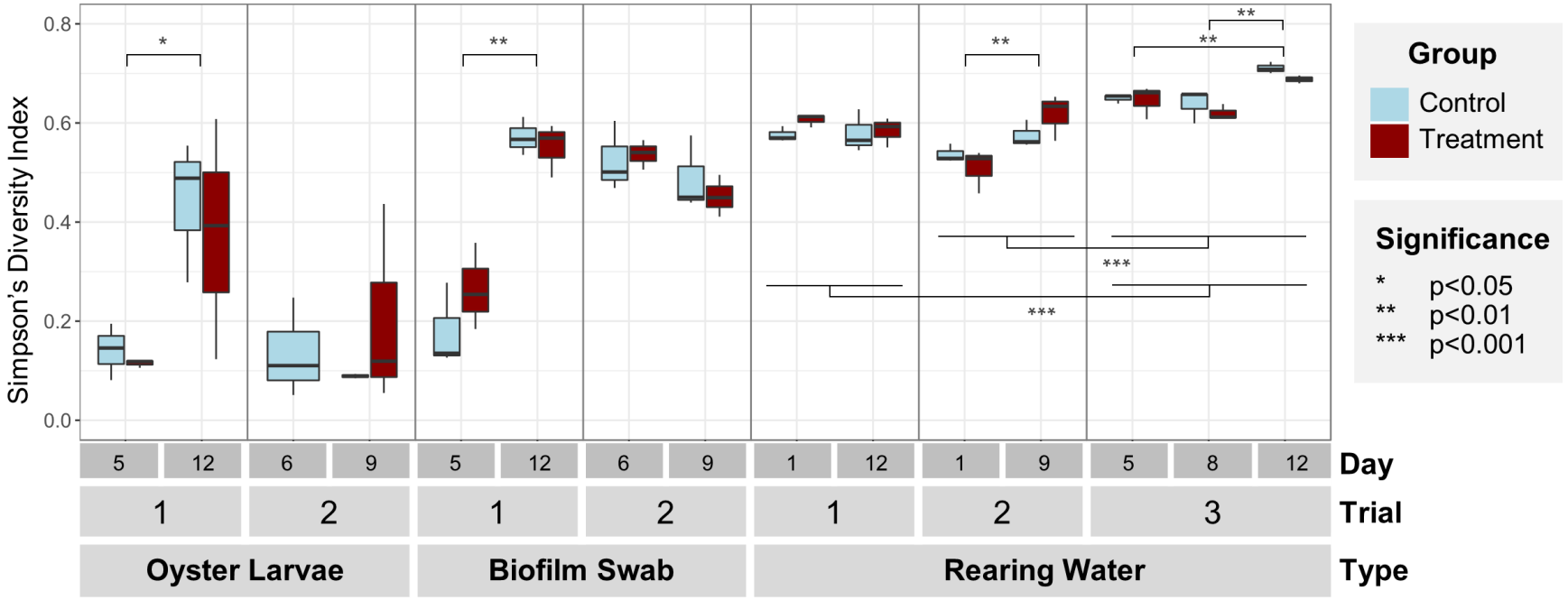


Figure IV-2. Simpson's index of diversity of bacterial communities by sample (larvae, swab, water) and trial (n=3 tanks). No significant differences in diversity were found between control (light blue) and treatment (dark red) within each sample type and trial. Bacterial community diversity significantly increased over time in larvae, swab, and water samples from Trial 1, and water samples from Trial 3. Diversity in water was significantly higher in Trial 3 than Trials 1 and 2. Note: there are no treated oyster larvae samples from Trial 2, Day 6.

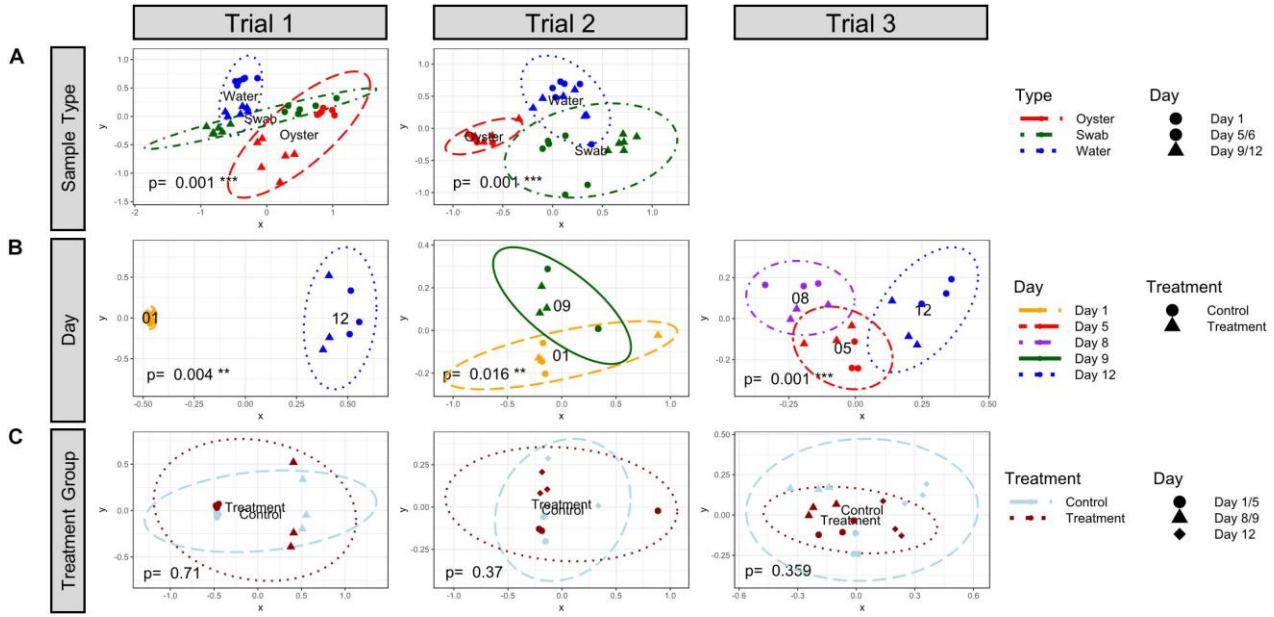


Figure IV-3. NMDS plot visualization of Bray-Curtis beta-diversity (k=2) at the Order level by (A) sample type, (B) sampling day, and (C) treatment.

The ellipse lines show the 95% confidence interval. p-values indicate significance of grouping with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test. (A) The different types of samples are indicated by colors (Oyster=dashed red, Swab=dashdot green, Water=dotted blue) and the days are indicated by symbols (Timepoint 1=circle, Timepoint 2=triangle). The water and swab communities were significantly distinct from each other in both trials. (B) The sampling timepoints are indicated by colors (1=longdash yellow, 5=shortdash red, 8=dashdot purple, 9=solid green, 12=dotted blue) and the treatment group is indicated by symbols (control=circle, probiotic treatment=triangle). The water community was significantly different between timepoints. (C) The treatment group is indicated by colors (control=light blue dashed, probiotic treatment=dark red dotted) and sampling timepoints are indicated by symbols. No significant differences in community structure in water from control and probiotic-treated tanks was detected when samples from all time points were analyzed together.

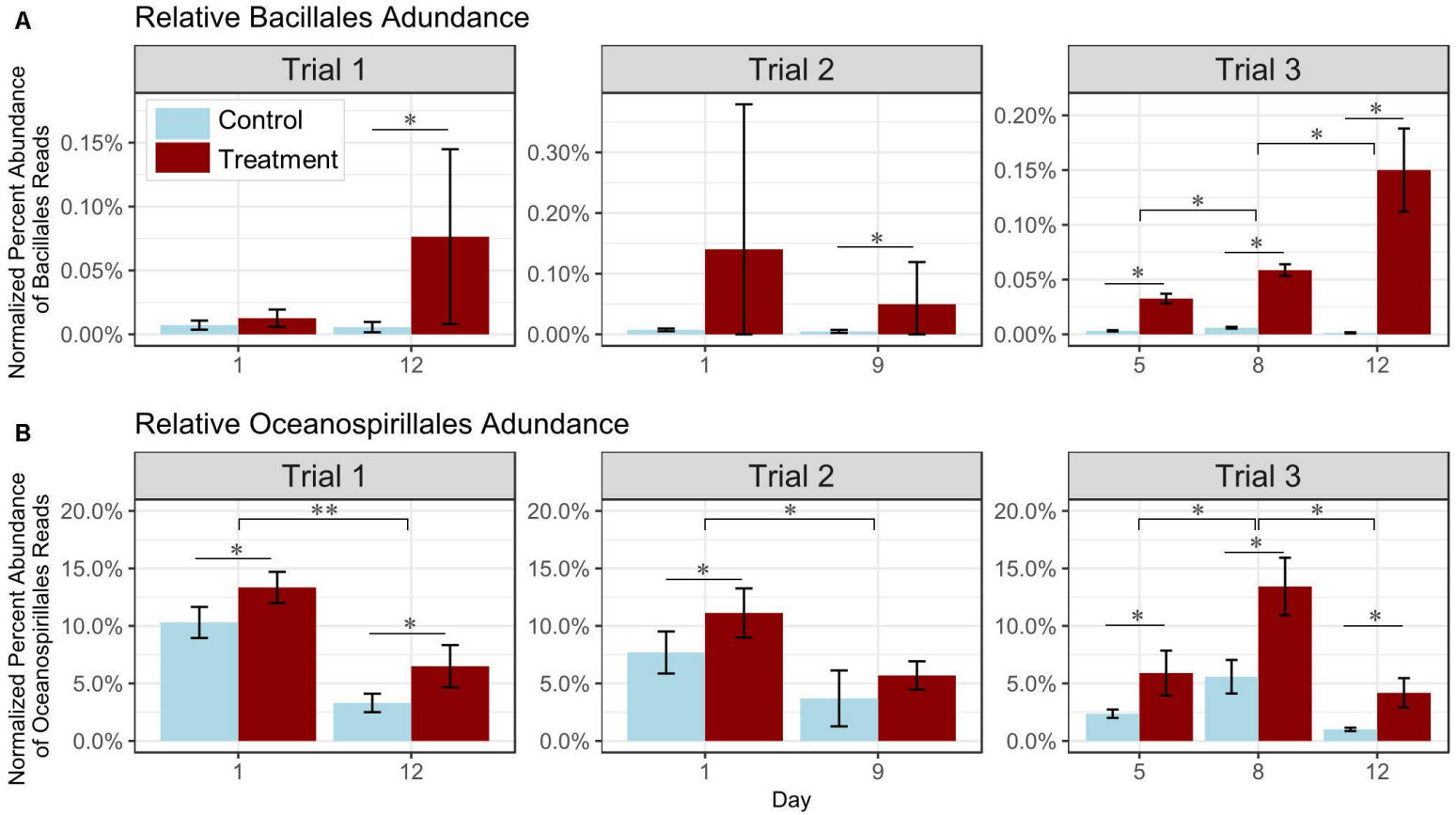


Figure IV-4. Probiotic treatment effect on relative percent read abundance of (A) *Bacillales* and (B) *Oceanospirillales* in water. Number of reads in treated (dark red) and control (light blue) samples (n=3 tanks per treatment) are represented for each sampling day and trial. (A) *Bacillales* was relatively significantly higher in the treated than the control water after 5 days of treatment, and (B) *Oceanospirillales* were consistently more abundant in probiotic-treated tank rearing water, and decreased with time.
Significance: *p<0.05, **p<0.01, ***p<0.001

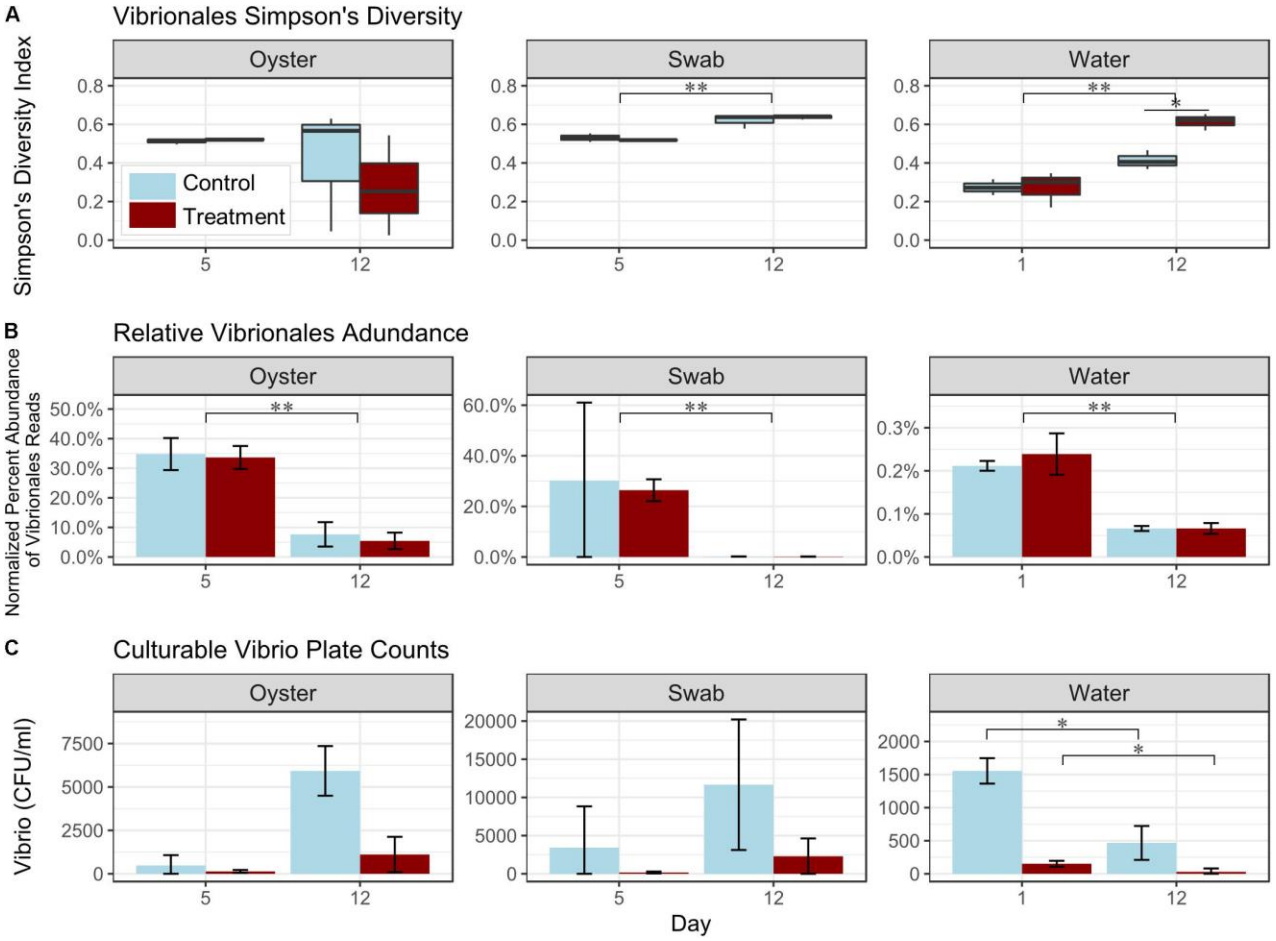


Figure IV-5. Probiotic treatment effect on *Vibrionales* abundance and diversity.
Effect of treatment, time, and sample type on Simpson's Index of Diversity for *Vibrionales* (A, boxplots), total *Vibrionales* relative percent read abundance (B, bar graph), and culturable *Vibrio* plate counts (C, bar graph). Representative data from Trial 1 (n=3 tanks per treatment). Note different scales for (B) and (C). **Significance**: *p<0.05, **p<0.01, ***p<0.001

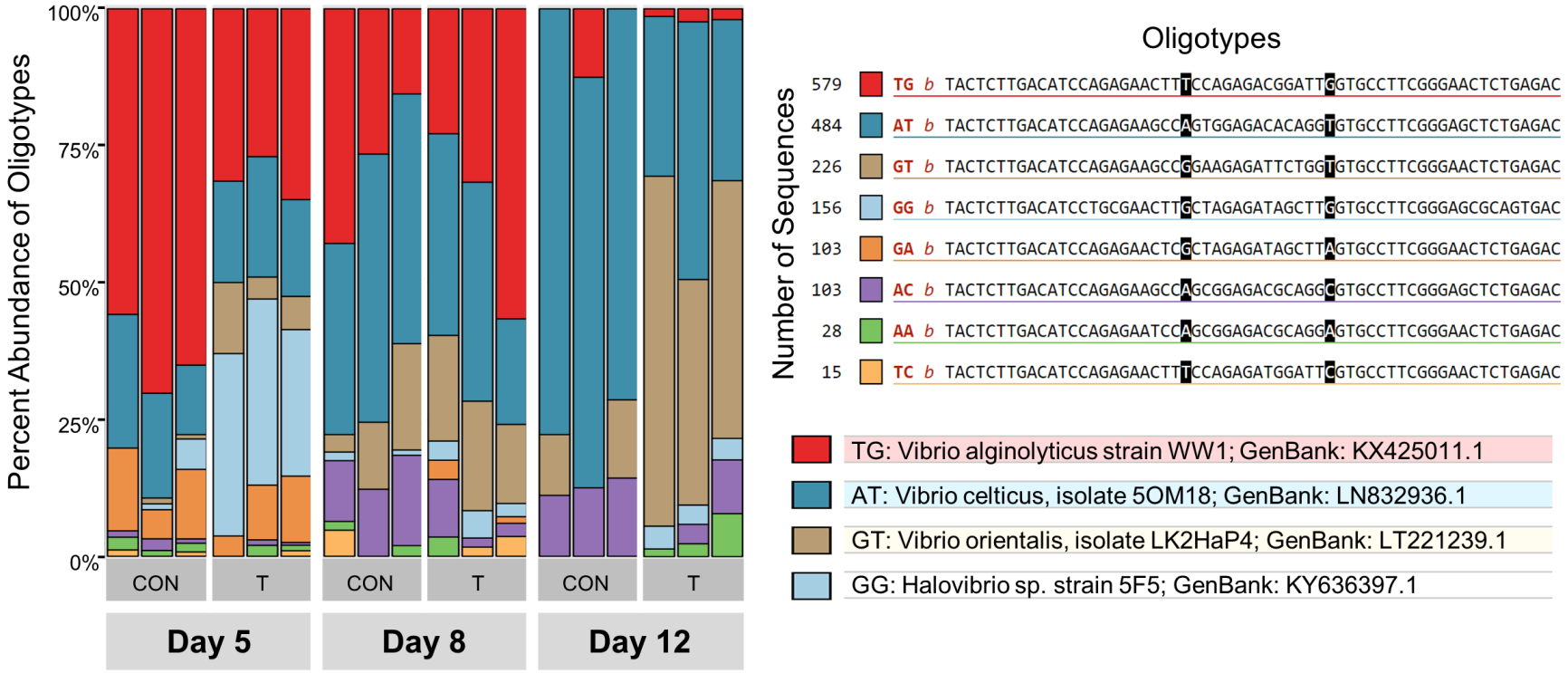


Figure IV-6. Vibrio spp. oligotypes in Control (CON) and Treatment (T) water samples on Days 5, 8, and 12 from Trial 3. *Vibrio* spp. oligotypes in Control (CON) and Treatment (T) water samples on Days 5, 8, and 12 from Trial 3. These 8 oligotypes were generated from changes in positions 23 and 37 in a total of 1727 sequences, represented with the 2 letter abbreviations in the legend. The taxonomy of the 4 most abundant oligotypes is shown. *Vibrio* oligotypes showed differences in succession of species over time between control and treatment rearing water.

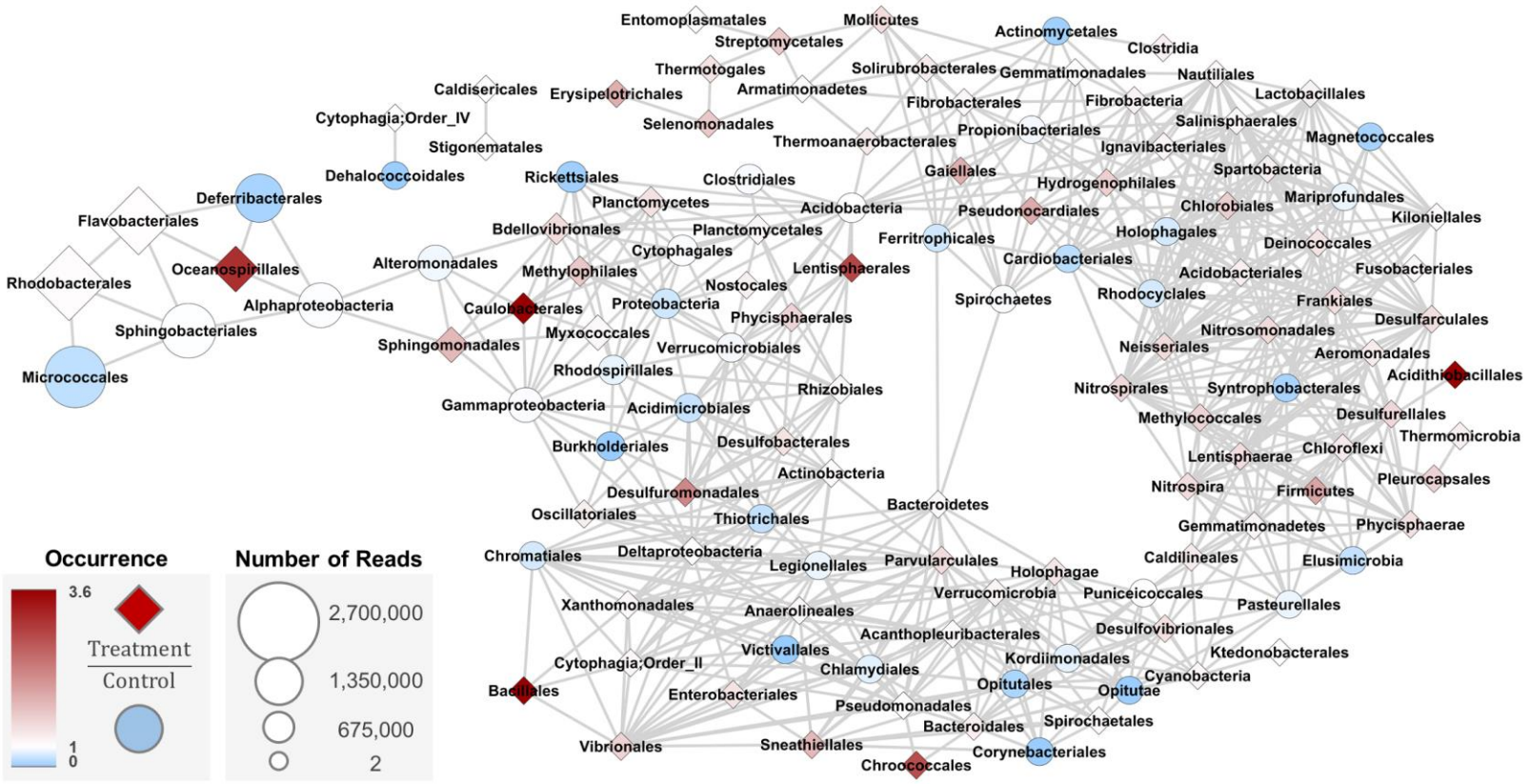


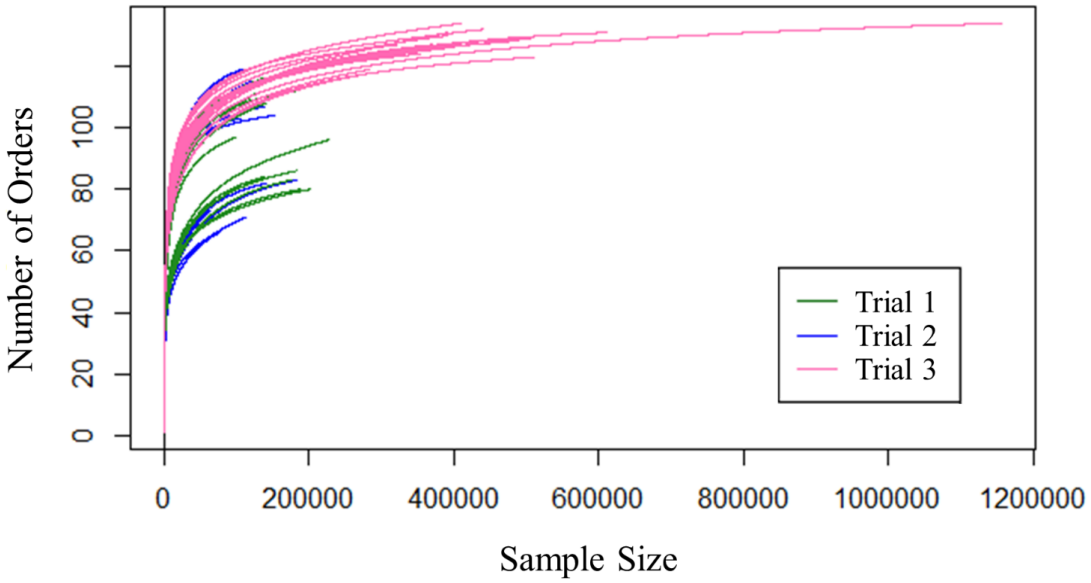
Figure IV-7. Co-occurrence network analysis based on Bray-Curtis dissimilarity metric (max distance =0.5, Order level) for water samples from Trial 3 (n=3 tanks per treatment and day, total of 18).

Taxa that change in the same way share an edge; nodes that have edges occur in the same proportions and in the same samples. Darker blue circle nodes indicate taxa that occur in the Control significantly more than Treated water samples. White nodes have equal occurrence in treated and control water samples. Darker red diamond nodes indicated taxa that occurs in the Treated significantly more than Control water samples.

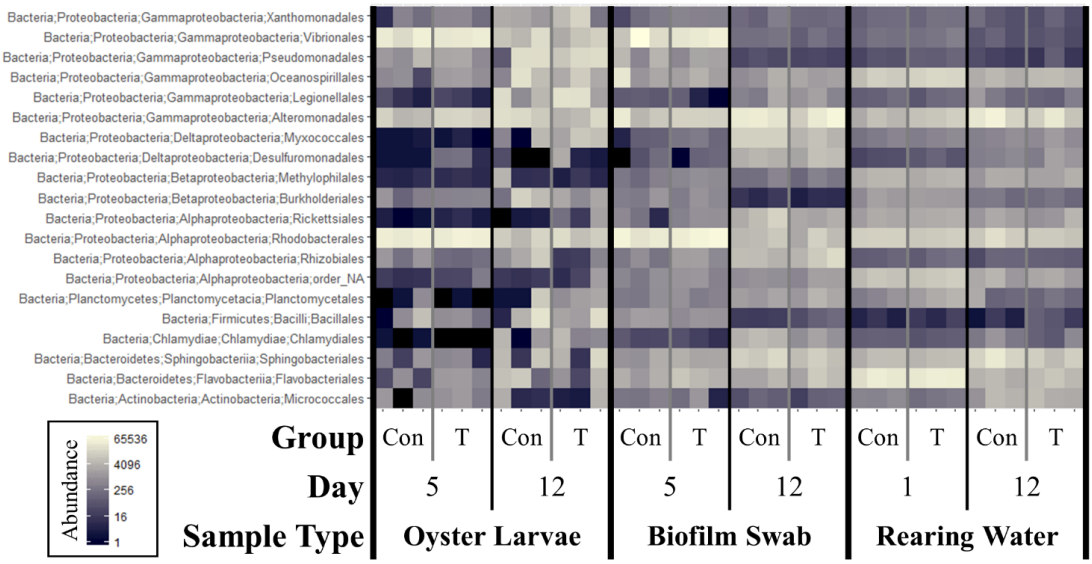
Table IV-1. Summary of probiotic trial information and sequencing data.

	Trial 1	Trial 2	Trial 3
Sample Types	Water, Swabs, Oysters	Water, Swabs, Oysters	Water
Sampling Days (0=spawn)	Water: 1,12 Oysters, Swabs: 5,12	Water: 1,9 Oysters, Swabs: 6,9	Water: 5,8,12
Volume water Filtered	410-750 mL	7-10 mL	1300-1700 mL
Trial Dates	July 11-23, 2012	Jan 9-18, 2013	June 3-15, 2016
Bacterial reads from 12 water samples	1.3 million	1.8 million	5.7 million
Methods	MoBio extraction MiSeq, 2x250 PE	MoBio extraction MiSeq, 2x250 PE	Puregene extraction HiSeq, 2x100 PE
16S region	V4	V4	V6

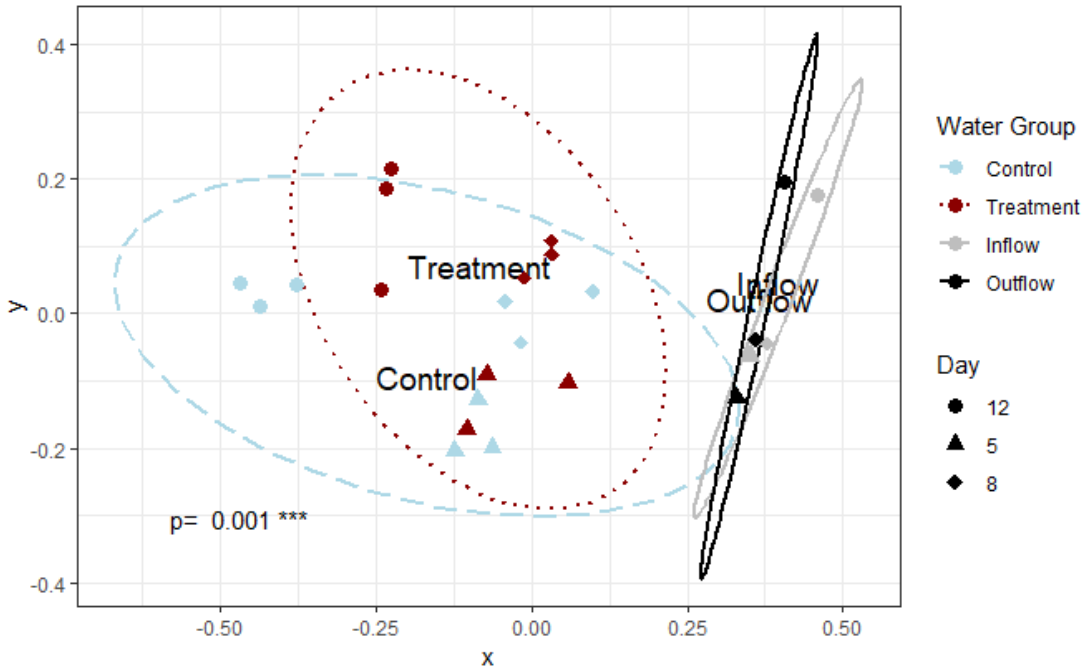
Supplementary Material



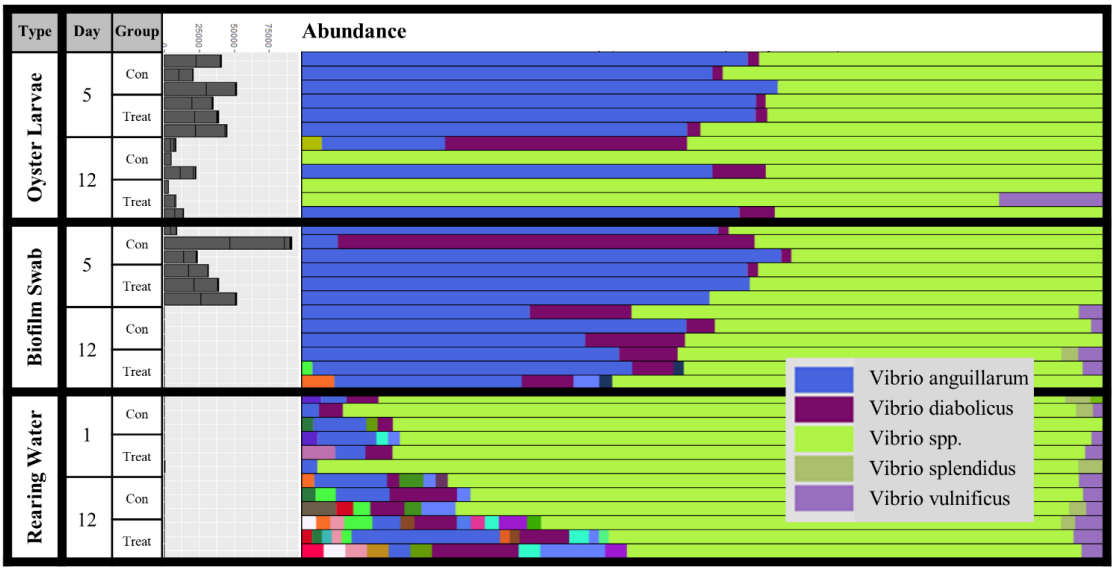
Supplementary Figure 1. Rarefaction curve from all water samples from all three Trials based on taxonomic classification at the order level.



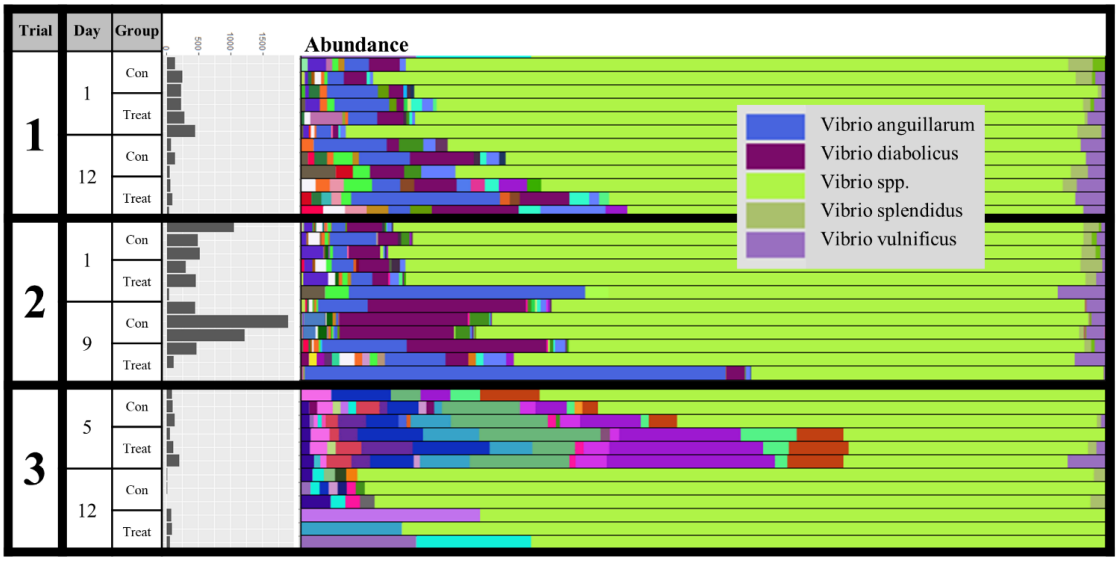
Supplementary Figure 2. The relative abundances of the 20 most abundant orders in oyster, swab, and water samples from Trial 1.



Supplementary Figure 3. NMDS plot visualization of Bray-Curtis beta-diversity (k=2) at the Order level by Treatment or Water Source of water samples from Trial 3. The ellipse lines show the 95% confidence interval. The water group is indicated by colors (control=light blue dashed, probiotic treatment=dark red dotted, inflow=grey solid, outflow=black solid) and sampling timepoints are indicated by symbols. The inflow water (water piped directly from the environment into the hatchery) and outflow water (inflow water UV-treated and sterilized) are significantly distinct groups, separate from the experimental samples. p-value indicates significance of groupings with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test.



Supplementary Figure 4. Percent abundances of *Vibrio* species in all sample types in Trial 1. The total abundance of sequencing reads is shown in the bar graph. The structure of total *Vibrios* is different based on the sample type and time point.



Supplementary Figure 5. Percent abundances of *Vibrio* species in rearing water samples from all 3 Trials. The total abundance of sequencing reads is shown in the bar graph. The structure of total *Vibrio* counts in the rearing water is different between Trials and changes over time.

Supplementary Table 1. Kruskal-Wallis Rank Sum Test for percent abundances of *Proteobacteria*, *Cyanobacteria*, and *Bacteroidetes* by Sample Type from all trials.

<All Trials – *Proteobacteria* >				
	DF	**Chi-Squared**	**P value**	
Sample Type	2	52.745	3.521e-12	***
Treatment	1	0.75065	0.3863	
< All Trials – *Cyanobacteria* >				
	DF	**Chi-Squared**	**P value**	
Sample Type	2	33.113	6.451e-08	***
Treatment	1	0.093506	0.7598	
< All Trials – *Bacteroidetes* >				
	DF	**Chi-Squared**	**P value**	
Sample Type	2	63.422	1.691e-14	***
Treatment	1	0.23442	0.6283	

Supplementary Table 2. Kruskal-Wallis Rank Sum Test for the Simpson's Index of Diversity values by Trial, Sample Type, Day, and/or Treatment.

< All Trials – Simpson's Index of Diversity >				
	DF	**Chi-Squared**	**P value**	
Trial	2	38.553	4.25e-09	***
Trial – water only	2	24.809	4.099e-06	***
Type	2	51.932	5.285e-12	***
Day	2	9.1136	0.0105	*
Treatment	1	0.32388	0.5693	

Supplementary Table 3. Kruskal-Wallis Rank Sum Test for the Simpson's Index of Diversity values by Day and Treatment in Trial 1.

< Trial 1 - Water >				
	DF	**Chi-Squared**	**P value**	
Day	1	0.41026	0.5218	
Treatment	1	0.92308	0.3367	
< Trial 1 – Biofilm Swab >				
	DF	**Chi-Squared**	**P value**	
Day	1	8.3077	0.003948	**
Treatment	1	0.10256	0.7488	
< Trial 1 – Oyster Larvae >				
	DF	**Chi-Squared**	**P value**	
Day	1	6.5641	0.01041	*
Treatment	1	0.41026	0.5218	

Supplementary Table 4. Kruskal-Wallis Rank Sum Test for the Simpson's Index of Diversity values by Day and Treatment in Trial 2.

< Trial 2 - Water >				
	DF	**Chi-Squared**	**P value**	
Day	1	7.4103	0.006485	**
Treatment	1	0.10256	0.7488	
< Trial 2 – Biofilm Swab >				
	DF	**Chi-Squared**	**P value**	
Day	1	3.6923	0.05466	
Treatment	1	0.10256	0.7488	
< Trial 2 – Oyster Larvae >				
	DF	**Chi-Squared**	**P value**	
Day	1	0	1	
Treatment	1	0.6	0.4386	

Supplementary Table 5. Kruskal-Wallis Rank Sum Test for the Simpson's Index of Diversity values by Day and Treatment in Trial 3.

< Trial 3 - Water >				
	DF	**Chi-Squared**	**P value**	
Day	2	11.942	0.002552	**
Treatment	1	0.32943	0.566	

Supplementary Table 6. Permutational Multivariate Analysis of Variance Using Distance Matrices (adonis2) for Bray-Curtis beta-diversity (k=2) in each Trial by Sample Type, Day, and Treatment.

< Trial 1 – Bray-Curtis beta-diversity >							
		DF	**Sum of Squares**	**R^2**	**F**	**Pr(>F)**	
Type		2	2.0175	0.3053	7.2513	0.001	***
	Residual	33	4.5907	0.6947			
Day (Water only)		1	0.71911	0.7375	28.095	0.006	**
	Residual	10	0.25596	0.2625			
Treatment (Water only)		1	0.02426	0.02488	0.2551	0.719	
	Residual	10	0.9508	0.97512			
< Trial 2 – Bray-Curtis beta-diversity >							
		DF	**Sum of Squares**	**R^2**	**F**	**Pr(>F)**	
Type		2	2.7762	0.57915	20.642	0.001	***
	Residual	30	2.0173	0.42085			
Day (Water only)		1	0.18331	0.24214	3.195	0.013	*
	Residual	10	0.57375	0.75786			
Treatment (Water only)		1	0.07318	0.09666	1.07	0.316	
	Residual	10	0.68389	0.90334			
< Trial 3 – Bray-Curtis beta-diversity >							
		DF	**Sum of Squares**	**R^2**	**F**	**Pr(>F)**	
Day		1	0.14732	0.32893	7.8424	0.002	**
	Residual	16	0.30056	0.67107			
Treatment		1	0.02889	0.06451	1.1033	0.337	
	Residual	16	0.41898	0.93549			
Water Source Group (Figure S3)		3	0.37087	0.57173	8.9	0.001	***
	Residual	20	0.27781	0.42827			

Supplementary Table 7. Kruskal-Wallis Rank Sum Test for relative percent abundance of *Bacillales* reads in water samples per Trial by Day and Treatment Group.

< Trial 1 - *Bacillales* >				
	DF	**Chi-Squared**	**P value**	
Day	1	0.64103	0.4233	
Treatment: Day 1	1	1.1905	0.2752	
Treatment: Day 12	1	3.8571	0.04953	*
< Trial 2 - *Bacillales* >				
	DF	**Chi-Squared**	**P value**	
Day	1	0.23077	0.631	
Treatment: Day 1	1	0.42857	0.5127	
Treatment: Day 9	1	3.8571	0.04953	*
< Trial 3 - *Bacillales* >				
	DF	**Chi-Squared**	**P value**	
Day	2	0.94737	0.6227	
Treatment: All	1	12.789	0.0003486	***
Treatment: Day 5	1	3.8571	0.04953	*
Treatment: Day 8	1	3.8571	0.04953	*
Treatment: Day 12	1	3.8571	0.04953	*
< Trial 3 - *Bacillales* Days 5 and 8 >				
	DF	**Chi-Squared**	**P value**	
Day	1	3.8571	0.04953	*
< Trial 3 - *Bacillales* Days 5 and 12 >				
	DF	**Chi-Squared**	**P value**	
Day	1	3.8571	0.04953	*
< Trial 3 - *Bacillales* Days 8 and 12 >				
	DF	**Chi-Squared**	**P value**	
Day	1	3.8571	0.04953	*

Supplementary Table 8. Kruskal-Wallis Rank Sum Test for relative percent abundance of *Oceanospirillales* reads in water samples per Trial by Day and Treatment Group.

< Trial 1 - *Oceanospirillales* >				
	DF	**Chi-Squared**	**P value**	
Day	1	8.3077	0.003948	**
Treatment: Day 1	1	3.8571	0.04953	*
Treatment: Day 12	1	3.8571	0.04953	*
< Trial 2 - *Oceanospirillales* >				
	DF	**Chi-Squared**	**P value**	
Day	1	5.7692	0.01631	*
Treatment: Day 1	1	3.8571	0.04953	*
Treatment: Day 9	1	1.1905	0.2752	
< Trial 3 - *Oceanospirillales* >				
	DF	**Chi-Squared**	**P value**	
Day	2	8.2222	0.01639	*
Treatment: Day 5	1	3.8571	0.04953	*
Treatment: Day 8	1	3.8571	0.04953	*
Treatment: Day 12	1	3.8571	0.04953	*
< Trial 3 - *Oceanospirillales* Days 5 and 8 >				
	DF	**Chi-Squared**	**P value**	
Day	1	4.3333	0.03737	*
< Trial 3 - *Oceanospirillales* Days 5 and 12 >				
	DF	**Chi-Squared**	**P value**	
Day	1	1.2564	0.2623	
< Trial 3 - *Oceanospirillales* Days 8 and 12 >				
	DF	**Chi-Squared**	**P value**	
Day	1	6.5641	0.01041	*

Supplementary Table 9. Kruskal-Wallis Rank Sum Test for Simpson's Index of Diversity of *Vibrionales* relative percent reads in Trial 1 per Sample Type by Day and Treatment Group.

< Trial 1 All Samples – *Vibrio* diversity >				
	DF	**Chi-Squared**	**P value**	
Type	2	8.4324	0.01475	*
Day	2	10.89	0.004318	**
Treatment	1	0.25626	0.6127	
< Trial 1 Oyster Larvae – *Vibrio* diversity >				
	DF	**Chi-Squared**	**P value**	
Day	1	0	1	
Treatment	1	0.41026	0.5218	
< Trial 1 Biofilm swab – *Vibrio* diversity >				
	DF	**Chi-Squared**	**P value**	
Day	1	8.3077	0.003948	**
Treatment	1	0.025641	0.8728	
< Trial 1 Water – *Vibrio* diversity >				
	DF	**Chi-Squared**	**P value**	
Day	1	8.3077	0.003948	**
Treatment	1	0.64193	0.4233	
Treatment – Day 12	1	3.8571	0.04953	*

Supplementary Table 10. Kruskal-Wallis Rank Sum Test for relative percent abundance of *Vibrionales* reads in Trial 1 per Sample Type by Day and Treatment Group.

< Trial 1 All Samples – *Vibrio* percent abundance >				
	DF	**Chi-Squared**	**P value**	
Type	2	16.722	0.0002338	***
Day	2	22.651	1.206e-05	***
Treatment	1	0.0009009	0.9244	
< Trial 1 Oyster Larvae – *Vibrio* percent abundance >				
	DF	**Chi-Squared**	**P value**	
Day	1	8.3077	0.003948	**
Treatment	1	0.10256	0.7488	
< Trial 1 Biofilm swab – *Vibrio* percent abundance >				
	DF	**Chi-Squared**	**P value**	
Day	1	8.3077	0.003948	**
Treatment	1	0	1	
< Trial 1 Water – *Vibrio* percent abundance >				
	DF	**Chi-Squared**	**P value**	
Day	1	8.3077	0.003948	**
Treatment	1	0.025641	0.8728	

Supplementary Table 11. Kruskal-Wallis Rank Sum Test for culturable *Vibrio* colony counts in Trial 1 per Sample Type by Day and Treatment Group.

< Trial 1 All Samples – *Vibrio* colony counts >				
	DF	**Chi-Squared**	**P value**	
Type	2	2.4254	0.2974	
Day	2	2.4406	0.2951	
Treatment	1	10.234	0.001379	**
< Trial 1 Oyster Larvae – *Vibrio* colony counts >				
	DF	**Chi-Squared**	**P value**	
Day	1	3.7053	0.05424	
Treatment	1	1.8591	0.1727	
< Trial 1 Biofilm swab – *Vibrio* colony counts >				
	DF	**Chi-Squared**	**P value**	
Day	1	2.0769	0.1495	
Treatment	1	3.1026	0.07817	
< Trial 1 Water – *Vibrio* colony counts >				
	DF	**Chi-Squared**	**P value**	
Day	1	2.0989	0.1474	
Control by Day	1	3.9706	0.0463	*
Treated by Day	1	4.0909	0.04311	*
Treatment	1	8.3958	0.003761	**

CHAPTER V: Summary of Results

Contributions of this Dissertation

This dissertation explored the microbial community dynamics in the eastern oyster (*Crassostrea virginica*) in response to environmental perturbations: an estuarine gradient, nutrient enrichment, and probiotic treatment. Larval, adult wild, and adult farmed oysters were evaluated, providing important contributions to aquaculture, fisheries, and conservation efforts. The overarching questions answered in this dissertation are as follows:

1. What is the microbial community of larval and adult oysters in Narragansett Bay, RI?
2. How does this community composition change over time and spatial scales?
3. What functions are performed by the oyster microbiome, as determined by metatranscriptomic activity?
4. How do these communities and functions change with environmental perturbation?

In the introductory chapter, we proposed a model for how the environment may influence the tightly coupled host-associated microbial community structure and function (Figure I-2). Previous studies of host-associated microbiomes, especially in oysters, have detected changes in composition or function, without connecting these observations to determine ecological relevance (King et al., 2019; Pierce and Ward, 2018). Given these knowledge gaps, it was expected that the microbial function will directly reflect the environment and microbial composition (Chapter 1 of this dissertation). However, the results of this dissertation indicate that the environment

influences the oyster-associated microbial composition differently than how it affects the microbial function, perhaps due to host mediation.

We determined that oyster microbiomes are highly dynamic, and primarily change according to life stage, sample type, and ambient environmental conditions. When these large-scale effects are removed, then we detect subtle changes in microbial community composition and function due to probiotic treatment or nutrient enrichment. Metatranscriptomic analysis of the oyster-associated microbiomes showed that transcriptional activity reflects local environmental conditions more than the microbial composition. This suggests the presence of a functionally redundant and diverse microbiome that allows for plasticity according to the environmental conditions. Additionally, this pattern implies that the microbial composition is driven by factors different from those that shape the functional response. In other words, the microbial community structure and function are responding to different influences (Louca et al., 2018). Furthermore, high levels of variability within oyster microbiomes at a site or in a rearing tank were observed, confirming there are other important factors in determining the composition or function of these microbiomes (*i.e.* host genetics, host physiology, host health status, microbial intra-community dynamics).

The observations presented in this dissertation are the first use of metatranscriptomics to describe how oyster microbiomes respond to an estuarine gradient and nutrient enrichment. In addition, we presented the first known characterization of the bacterial community dynamics in a bivalve hatchery in response to probiotics. These comparisons of microbial community structure and

function inform how the oyster microbiome responds to environmental conditions and eventually, may show resilience to climate change scenarios.

Changes in Microbial Community Structure

Environmental Conditions and Perturbation

Previously published studies of microbial communities in oysters are limited to descriptive studies at ambient conditions, without considering the influence of environmental conditions. All sites surveyed in this study are dispersed throughout Narragansett Bay, Rhode Island, a temperature estuary with a prevalent eutrophic gradient and shellfish culture (Figure 1) (Oviatt, 2008; Wallace et al., 2014). The variation in environmental conditions ranged dramatically throughout the bay at the time of sampling: pH 6.9-8.2, salinity 18-32 psu, dissolved oxygen 5.5-9.5 mg/L, and chlorophyll-*a* 3.8-24 μg/L (Figure 2 and Table 1). This variety in environmental conditions provides an opportunity to synthesize how environmental conditions and perturbations affect the microbial community composition of oysters. When 16S rRNA amplicon data from select oyster gut samples in Chapter 2 and the ambient gut samples in Chapter 3 are analyzed together, we can determine general trends in oyster microbial composition throughout Narragansett Bay. *Proteobacteria, Cyanobacteria, Bacteroidetes,* and *Tenericutes* were the most abundant phyla in oyster gut microbiomes (Figure 3). Overall, the oyster gut microbiome was highly variable, especially within each site, suggesting additional selection by the host (Figure 4). A principal component analysis (PCA) of these samples showed correlations between

sites, their gut microbial phyla, and environmental conditions (Figure 5). The samples separated by site, with the least variation shown at 4.NAR and 7.NIN.

If we consider which bacterial phyla are affected by different perturbations, we can identify the possible key taxa responsible to microbial acclimation and change. In the oyster gut samples from Narragansett Bay, phyla *Chlamydiae* and *Dependentiae* correlated with salinity, *Lentisphaerae* correlated with chlorophyll-*a*, *Verrucomicrobia* and *Actinobacteria* correlated with ammonium, and *Fusobacteria* correlated with dissolved oxygen (Figure 5). *Oceanospirillales*, *Vibrionales*, *Caulobacterales*, and *Lentisphaerales* were associated with probiotic treatment (Figure IV-7) and *Actinobacteria* and *Bacteroidetes* were affected by nutrient enrichment (Figure III-2). The phyla *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* and *Lentisphaerae* make up the "PVC superphylum," a monophyletic group of phylum that often live in close association with eukaryotic hosts (Cho et al., 2004; Wagner and Horn, 2006). Interestingly, these are phyla that we saw changing in response to environmental perturbation, which indicates that these changes in microbial community structure may be host-dependent and limited to certain taxa.

Developmental Stage and Tissue Type

In addition to the range of environmental conditions studied, this study also considered changes in oyster microbiomes at different developmental stages and tissues. When all 16S rRNA amplicon data from this dissertation was compared at the Phyla level, there was high variability within microbiomes from the same sample type (Figure 6). Adult wild oysters at different sites in Narragansett Bay have significantly different microbial community structures that are selected from the water microbiome

at each site (Figure 6). Regardless of probiotic treatment, nutrient enrichment, or site, the oyster microbiome showed a transition from *Proteobacteria* and *Bacteroidetes* dominated seawater and larval bacterial communities to an adult microbiome abundant in *Cyanobacteria*, *Tenericutes*, and *Unknown* taxa (Figure 7). These same averaged samples were compared using a PCA analysis to determine which taxa are driving the changes between the sample types (Figure 8). All of the seawater samples (regardless of site or hatchery trial) clustered with the larval hatchery samples, and correlated with *Proteobacteria*, *Bacteroidetes*, *Deferribacteres*, and *Chlamydiae* (Figure 8A). At the Phylum level, larval oysters in a hatchery are similar to water throughout Narragansett Bay, despite seasonal and site differences. All adult oyster gut samples clustered together, and correlated with *Cyanobacteria*, *Firmicutes*, and *Tenericutes*. The adult oyster inner and outer shell samples were the most diverse and variable sample types (Figures 7 and 8).

The experiments described in this dissertation confirm that the oyster microbiome composition varies by developmental stage and sample type (larvae, adult gut, inner shell, outer shell). As the oyster grows, it will have different filtering mechanisms and nutritional requirements, promoting and maintaining a changing microbiome (Hoellein et al., 2015; Pierce and Ward, 2018). For example, the adult gut microbiome is dependent upon filtration and selection by the oyster host. Surprisingly, we observed that sample type and developmental stage have a greater effect on the oyster microbiome composition than seasonality, site and environmental conditions, and sequencing method. This finding is especially important to the study of host-microbiome interactions, since many studies will focus on just one tissue in an

organism. Our results indicate that one tissue may not be representative of all the changes occurring in a host's microbiome, and multiple tissues should be considered in experiments.

Changes in Microbial Community Function

Increased nutrient loading in coastal environments threatens oysters and is likely to alter microbially-driven ecosystem functions, particularly nitrogen and phosphorus cycling (Carmichael et al., 2012; Oviatt, 2008). Oyster-associated microbiomes play an important role in nitrogen cycling by performing coupled nitrification-denitrification processes, but knowledge of the responsible microbes, their abundances, and their roles is lacking. Despite differences in the microbial composition, the transcriptionally active taxa are similar throughout the bay, but performing different functions depending on the environmental conditions. The oysters from sites with high nutrients and anoxia show significant upregulation of genes associated with stress response and phosphorus metabolism. Conversely, the oysters from sites with low nutrients and higher DO show upregulation of genes associated with nitrogen metabolism and downregulation of stress response genes.

The model we developed in Chapter 2 describes how environmental conditions determine nitrogen and phosphorus metabolism in oyster gut microbiomes (Figure II-6). At sites with lower dissolved oxygen and pH, and higher nutrient availability, there will be downregulation of nitrogen metabolism genes. This model also holds true for nitrogen metabolism in the gut metatranscriptomes analyzed in Chapter 3. When the field sites are compared, there is significant downregulation of nitrogen pathways in

the Northern site, where there are high nutrient levels and lower dissolved oxygen (Figure III-7A). This will result in decreased denitrification provided by the oysters, and a reduction in the value of their ecosystem services.

Technical Issues and Limitations

The results presented in this dissertation are limited by a variety of technical issues and biases that are discussed in each chapter. In particular, the methods and programs used to analyze high throughput sequencing data are constantly improving, and each comes with a set of limitations (Jovel et al., 2016; Tremblay et al., 2015). One of the primary limitations is the small scope of databases for taxonomy and functional annotation of environmental samples (Kim et al., 2013). In the 16S rRNA amplicon data, there were many *Unknown* bacterial reads that lack the support of database annotation (Antczak et al., 2019). The metatranscriptomic results presented are based on annotation of only 5-40% of the total metatranscriptomic reads – the rest were uncharacterized and discarded. Similarities in the metatranscriptome annotation may have been a partial result of this database bias. However, the changes we observed in functional and taxonomic diversity within sample sets were obtained using the same methods and databases, removing any bias between samples in a dataset. In order to improve the accuracy of taxonomic and functional studies, improved database and protein characterization are needed.

A large percentage (5-40%) of the metatranscriptomic reads matched to the eastern oyster genome and were thus removed from the microbial study, leaving fewer reads for downstream analysis. Metagenomes sequenced from the same oyster gut

samples were made of 70-90% oyster gene sequences, making them unusable for microbial analysis at the current sequencing depth (unpublished data). Improved methods to obtain host-depleted metagenomes and metatranscriptomes are needed, so that we can better study host-associated microbiomes at reasonable costs (Pereira-Marques et al., 2019).

Relevance and Future Directions

In total, this dissertation significantly contributes to the knowledge of marine host-associated microbiomes and their roles in coastal ecosystems. Oyster-associated microbiomes may not have different functions from the water microbial community, but likely amplify certain taxa and functions due to substrate and metabolite provision (Apprill, 2020). The functions of oyster microbiomes are dependent upon their environment; this may limit the ecosystem services provided, but contributes to the plasticity of the system. Oysters, and especially their diverse microbiomes, are resilient and will likely acclimate to changing environmental conditions.

The results of this dissertation prompt a multitude of questions that should be addressed in future studies to further understand oyster-associated microbial community dynamics. Many hypotheses were generated from Chapter 4, where 16S rRNA sequencing was used to determine the effect of probiotics on oyster hatchery microbiomes. We observed changes in the interactions between the *Bacillus pumilus* RI06-95 probiotic, *Vibrio* spp. pathogens, and *Oceanospirillales* symbionts. Targeted *in vitro* metagenomic and metatranscriptomic studies of this system would help to further elucidate these relationships and describe the probiotic effect on larval oysters.

Studies of *Vibrio* infections have shown that a population of multiple *Vibrio* species/strains are responsible for oyster infections (Lemire et al., 2015). Functional analysis of this system, especially sampled through a disease outbreak, would help to characterize the etiology of these infections.

We are just beginning to understand how host-associated microbiomes respond to environmental conditions and how these functions may impact coastal ecosystem services. The results of Chapters 2 and 3 provide a basis for how oyster-associated microbiomes exhibit plastic responses to environmental conditions. Future host-depleted metagenomic studies or targeted qPCR of relevant genes would determine if functional redundancy is driving this plasticity. Furthermore, mesocosm experiments where pH, dissolved oxygen, and nutrient concentrations are manipulated will confirm the model proposed in Chapter 2. Throughout this dissertation, we discuss whether the observed taxa in the oyster microbiome are transient food filtered by the oyster or symbionts that are thriving in the oyster. This distinction would determine the role and importance of each taxa in the oyster microbiome. Increased observations of *Cyanobacteria* in 16S rRNA amplicon data are common in oyster microbiome studies, but are often discounted as "transient food" or artifacts deriving from microalgal feed. Depuration of oysters or controlled experiments with a known microalgal feed microbiome would allow us to determine which microbes are transient, versus those that are symbionts (Lee et al., 2008).

The scope of this dissertation was limited to one oyster species in a single estuary at discrete timepoints. Future studies should confirm our observations in additional oyster species, bivalves, and other coastal keystone organisms. There is inherent

variability in environmental studies that we are not yet able to explain, especially in studies of microbial communities. We believe that this variability is systematic, and will be elucidated in the future by new discoveries and more accurate methods. Increased sample sizes over broader spatial scales and timepoints would help constrain the dynamics of oyster-associated microbiomes, and enable understanding of their contributions to coastal ecosystems.

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Figures and Tables

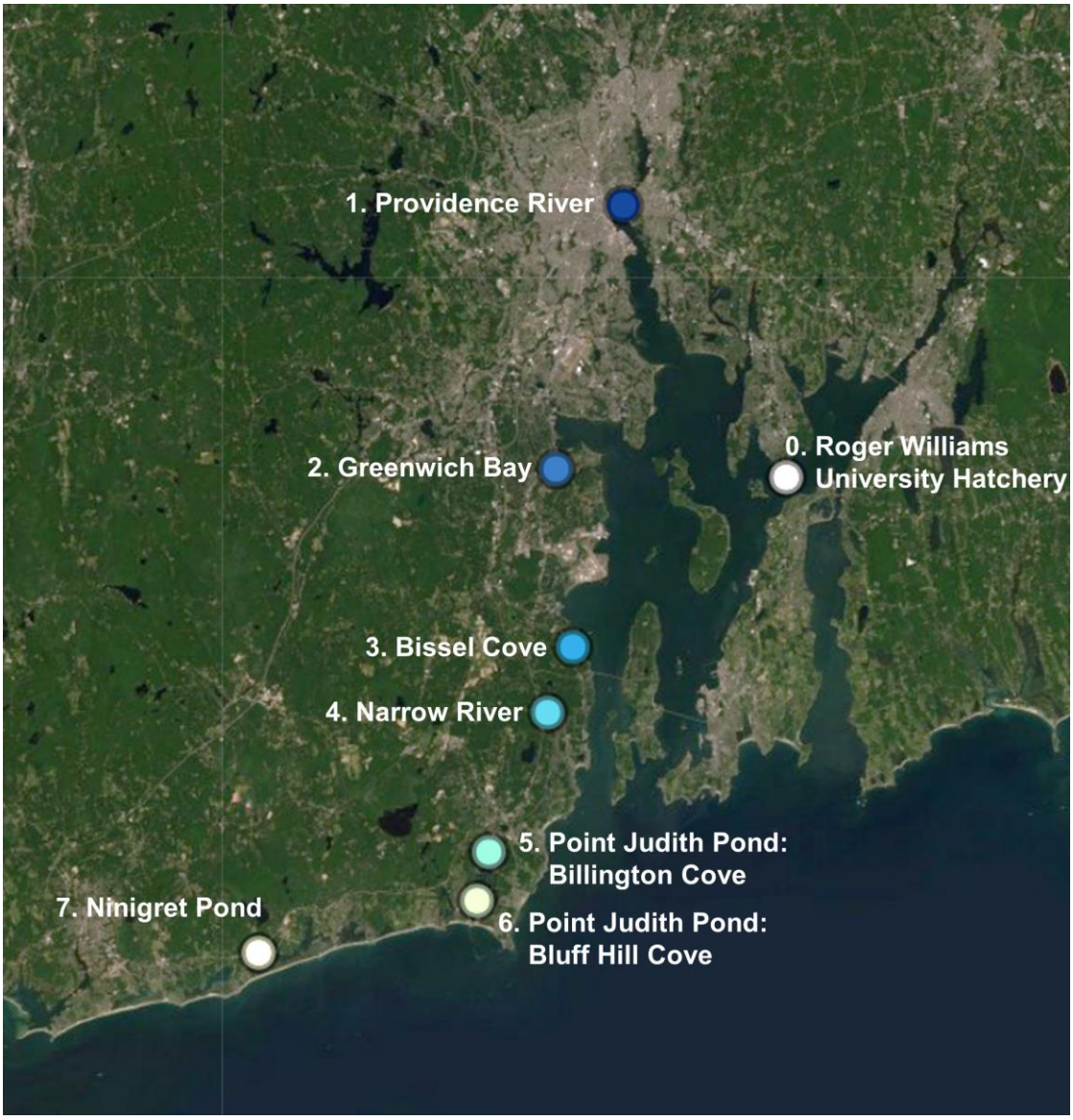

Figure V-1. Map of dissertation study area with experimental sites.

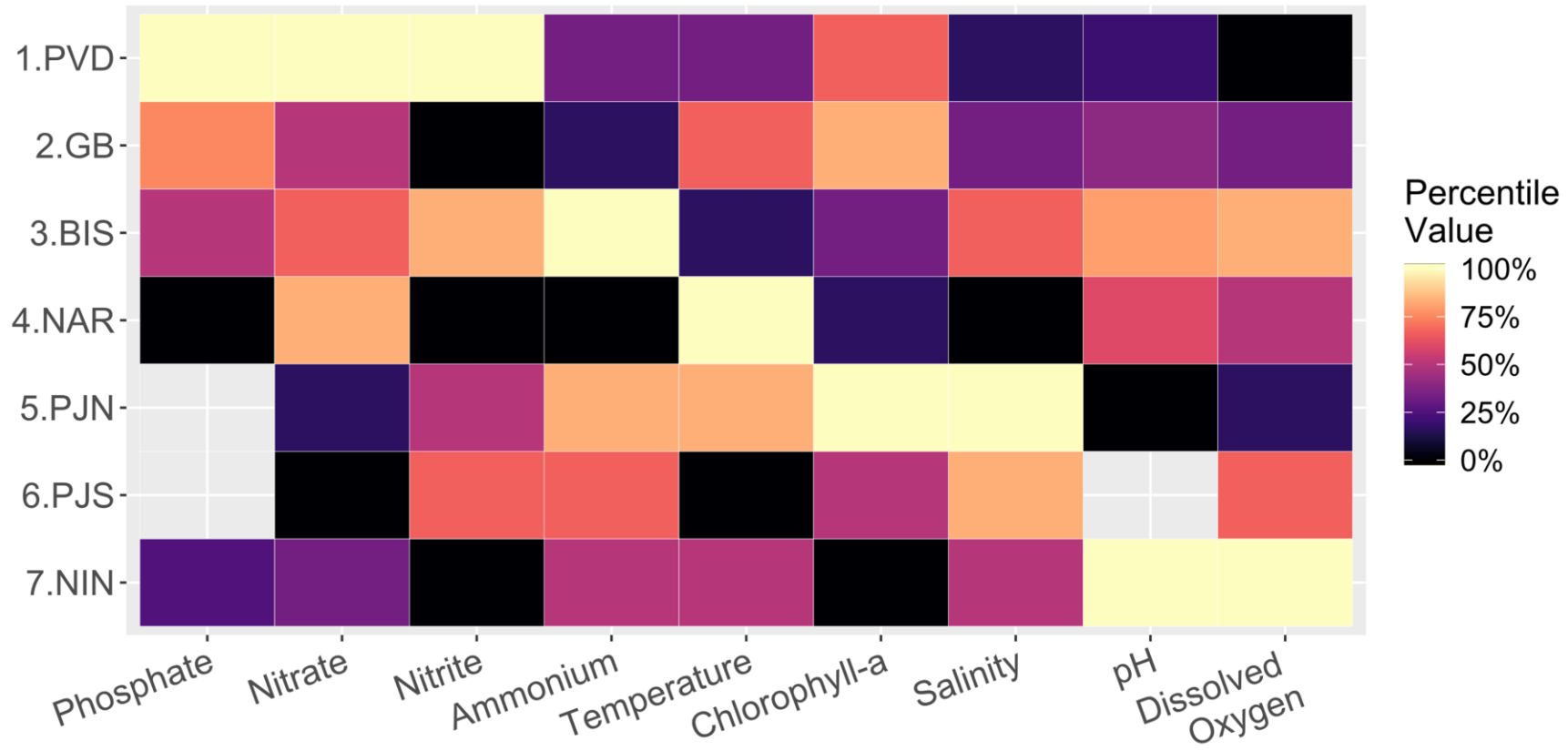

Figure V-2. Heatmap showing percentile value of environmental parameters at each site.
A yellow tile indicates the site where the highest value was observed and black indicates the site where the lowest values was observed. Nutrient concentrations were measured from filtered water collected at each site. All other values were input as average measurements collected *in situ* during sampling.

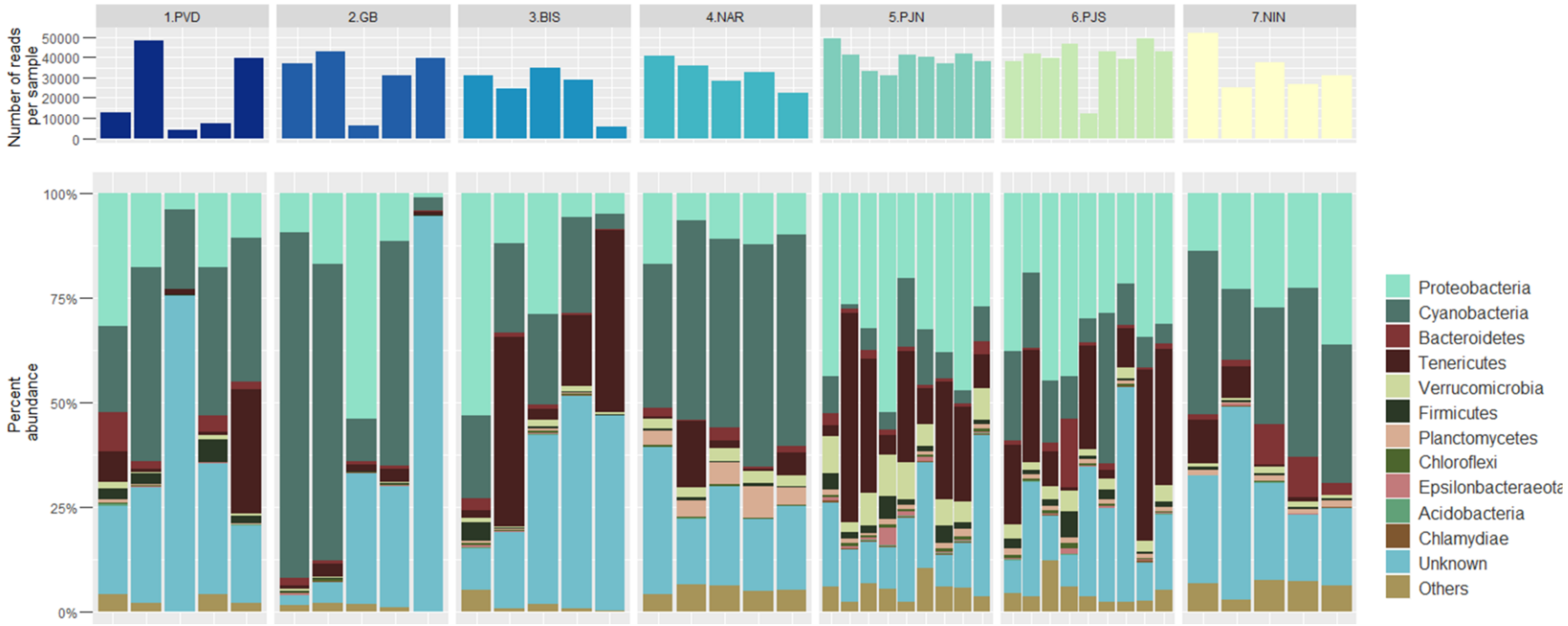

Figure V-3. Percent abundance of the top 20 most abundant phyla per oyster gut sample at each site using 16S rRNA amplicon data.

The total number of quality-controlled sequencing reads is shown in the top bar plot.

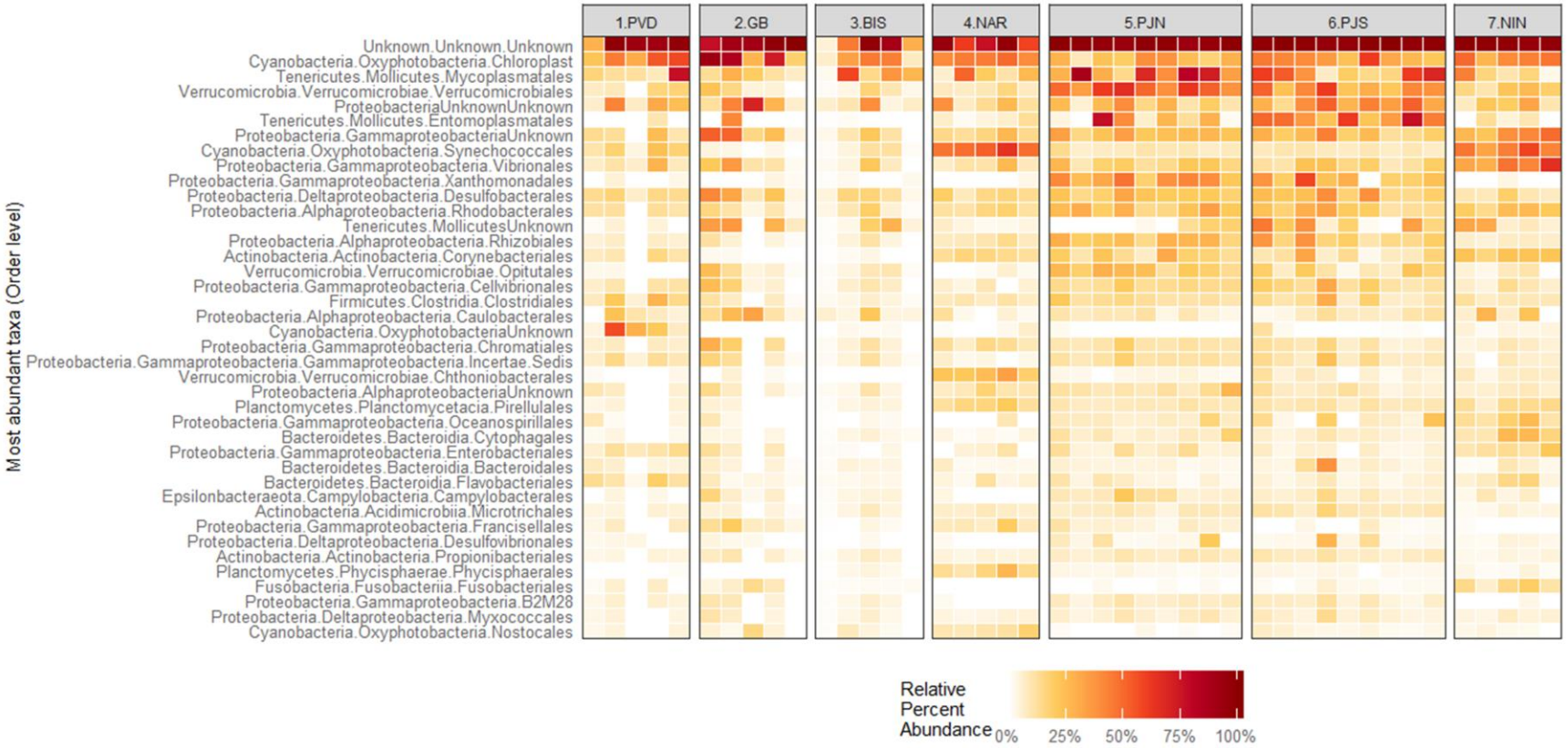

Figure V-4. Percent abundance of the top 40 most abundant orders per oyster gut sample at each site using 16S rRNA amplicon data.

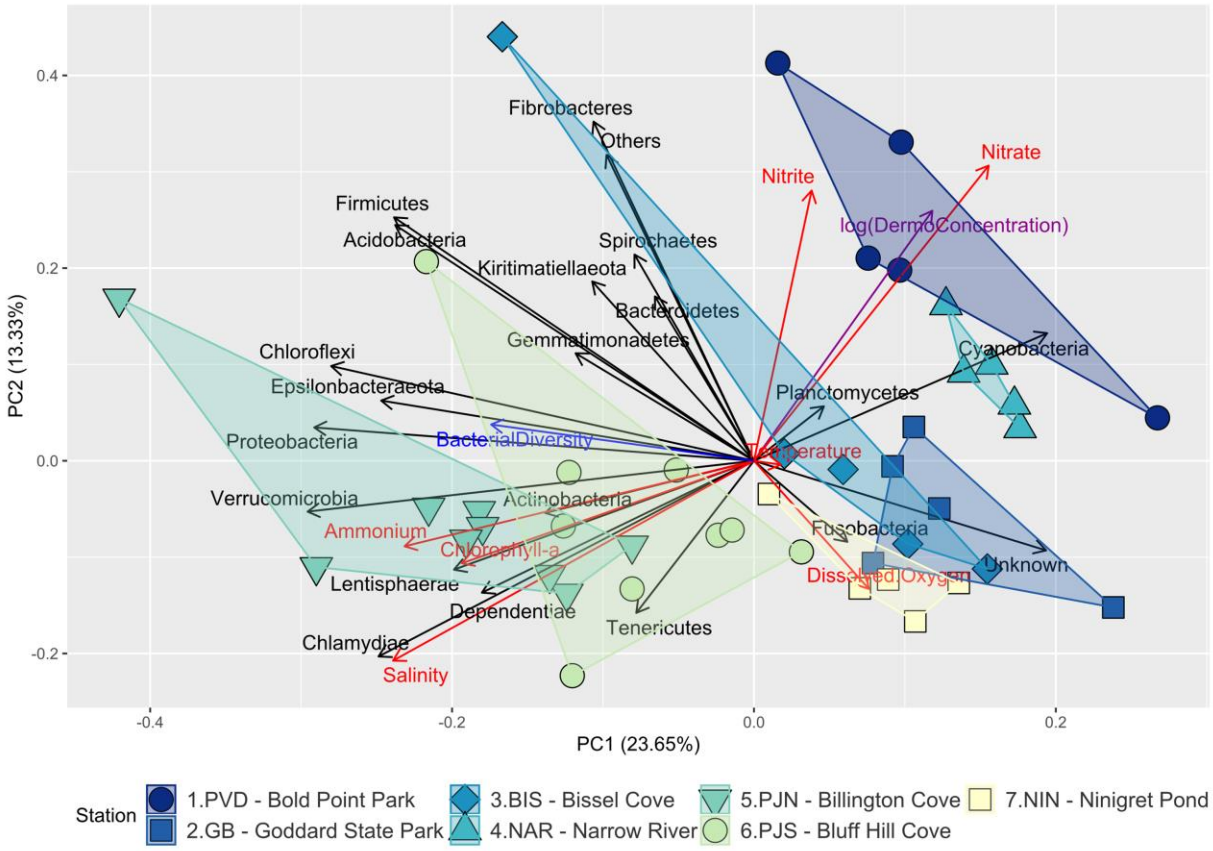

Figure V-5. PCA plot showing correlations between environmental parameters, qPCR Dermo levels, bacterial diversity and 16S rRNA amplicon data by Phylum across sites.

Each oyster (n=43) is represented by a symbol. Environmental parameters are shown with red arrows, qPCR Dermo concentration data is shown with a purple arrow, 16S phylas are shown with black arrows, and bacterial diversity (Simpson's Index) is shown with a blue arrow.

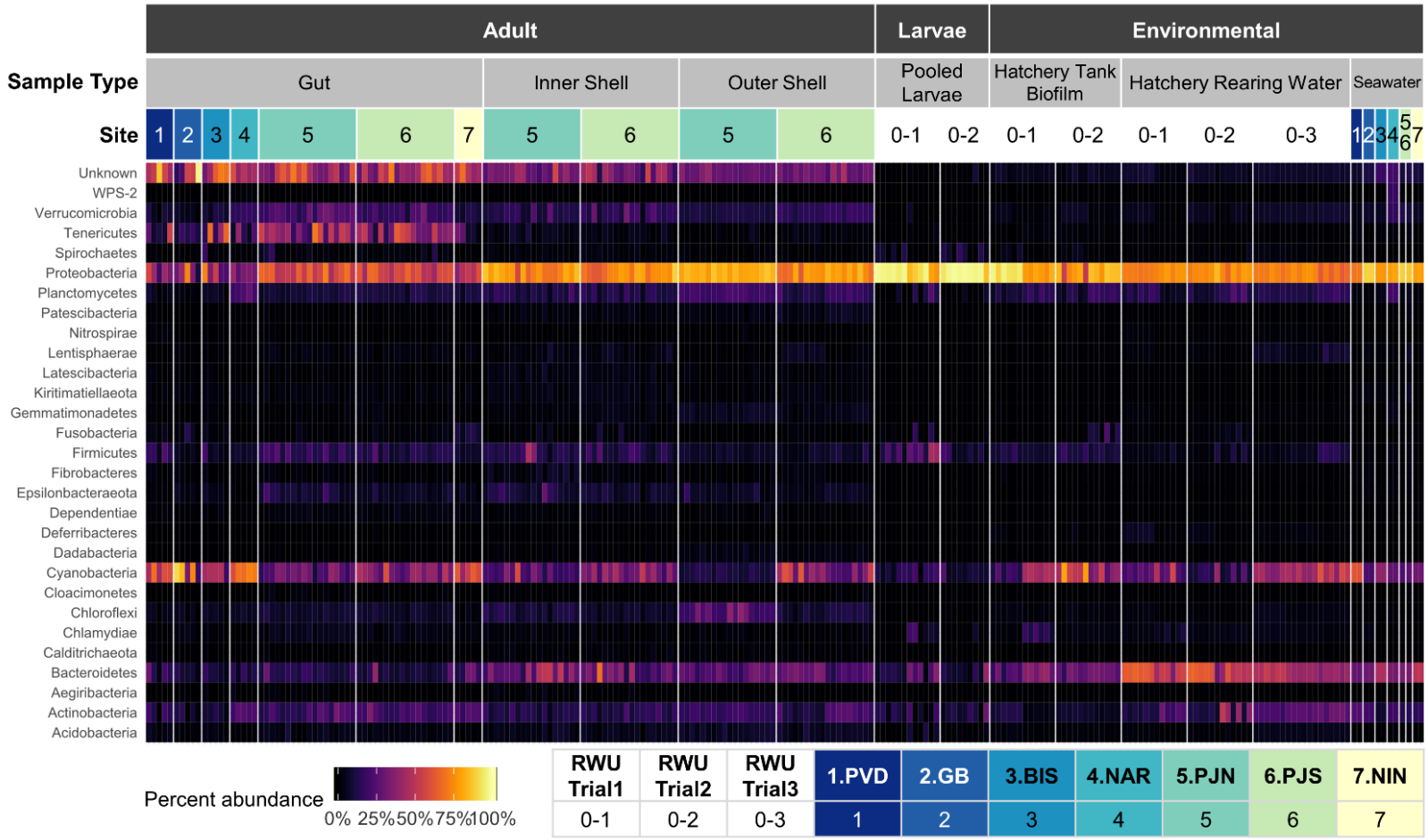

Figure V-6. Relative percent abundance of the 30 most abundant phyla per oyster, larvae, biofilm, and seawater sample at each site (or Trial) using 16S rRNA amplicon data.

The site and/or hatchery trial number is indicated with a color and number, as denoted in the legend.

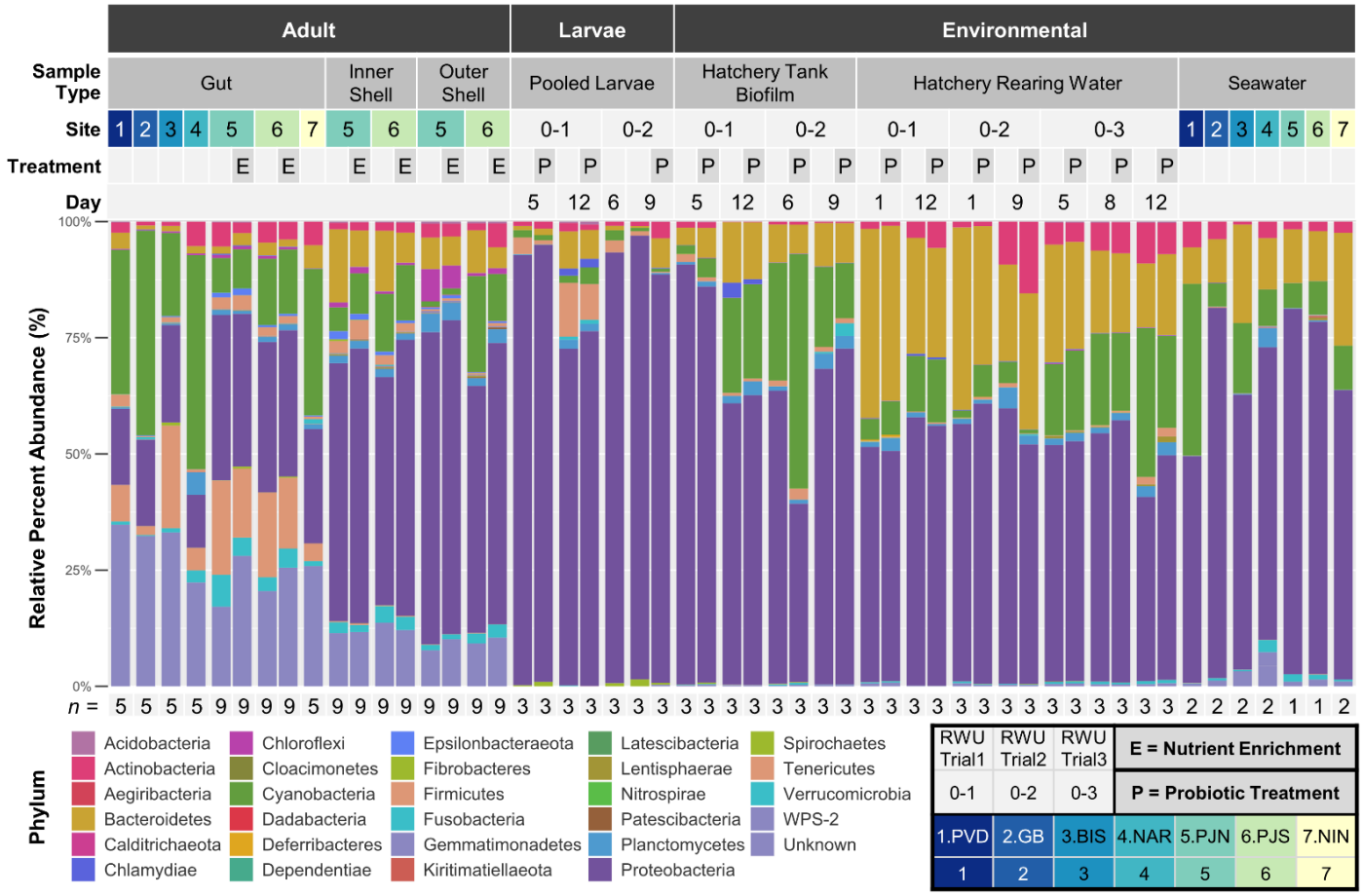

Figure V-7. Averaged relative percent abundance of the 30 most abundant phyla per sampling timepoint, treatment, site/trial, and sample type using 16S rRNA amplicon data.

The number of samples averaged per bar is indicated at the bottom of the bar plot (n=X). The site and/or hatchery trial number is indicated with a color and number, as denoted in the legend.

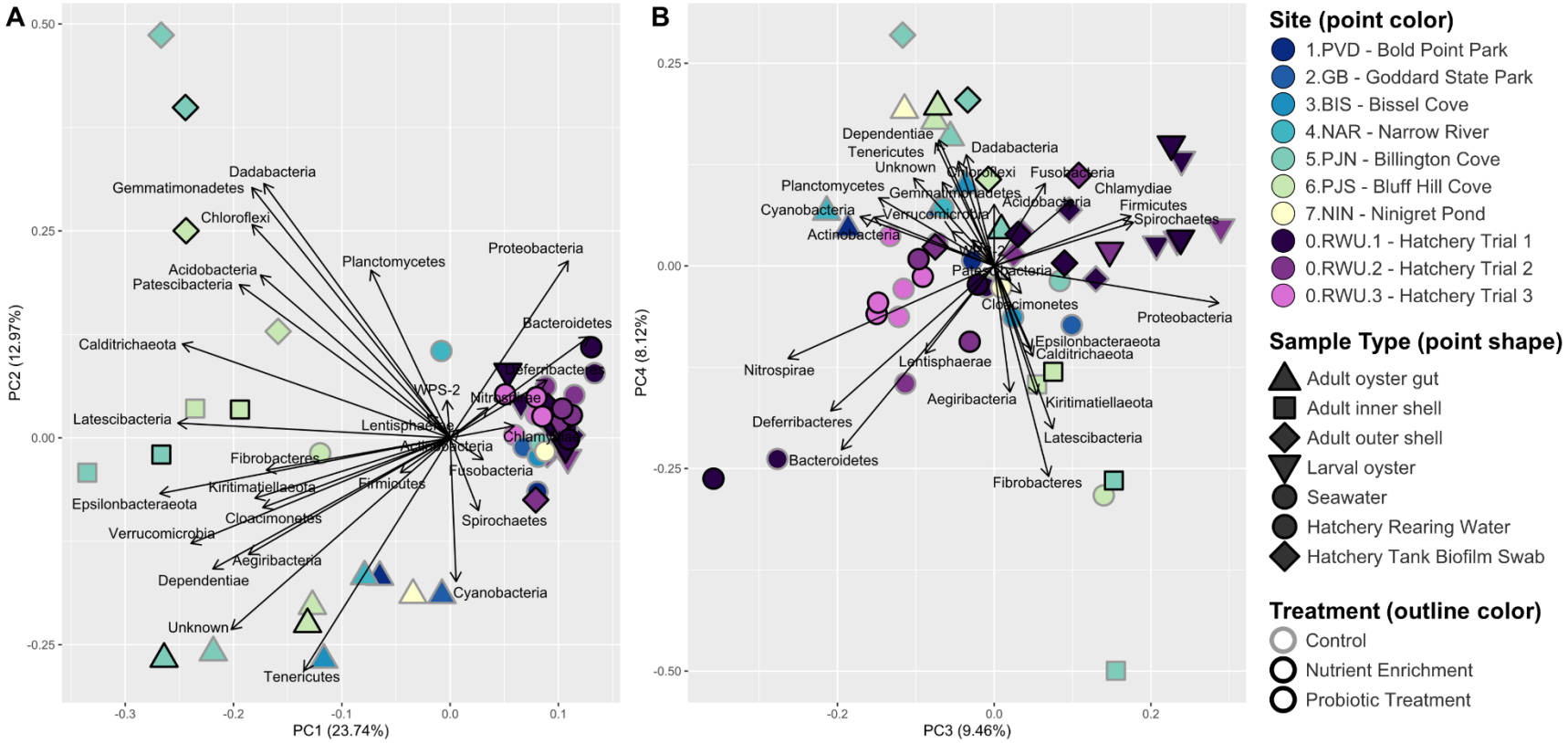

Figure V-8. PCA plot showing correlations for 16S rRNA amplicon data by Phylum across averaged sites, sample types, and treatment.

(A) PCA axes 1 and 2. (B) PCA axes 3 and 4. Each averaged sample group (n=1-9, see Figure V-7) is represented by a colored symbol. Points outlined in black were nutrient enriched or probiotic treated samples. 16S phylas are shown with black arrows.

Table V-1. Summary of all sites surveyed in this dissertation.
Environmental conditions and nutrient concentrations measured are shown. The ambient measurements are shown for 5.PJN & 6.PJS.

Site	0.RWU	1.PVD	2.GB	3.BIS	4.NAR	5.PJN	6.PJS	7.NIN
Full Name	Roger Williams University	Providence River: Bold Point Park	Greenwich Bay	Bissel Cove	Narrow River	Point Judith N: Billington Cove	Point Judith S: Bluff Hill Cove	Ninigret Pond
Location (GPS coordinates)	41.65, -71.26	41.82, -71.39	41.65, -71.45	41.55, -71.43	41.51, -71.45	41.42, -71.50	41.39, -71.51	41.36, -71.69
Dissertation Ch.	2	3	3	3	3	4	4	3
Type of Oysters	Hatchery larval	Wild adult	Wild adult	Wild adult	Wild adult	Farmed adult	Farmed adult	Farmed adult
Sampling date(s)	July 2012 Jan 2013 June 2016	Aug 2017	Aug 2017	Aug 2017	Aug 2017	Jun-Aug 2017	Jun-Aug 2017	Aug 2017
Salinity (psu)	28	24.8±2.1	28.5±0.2	30.5±0.1	18.0±0.4	32.4±0.9	31.7±1.1	28.9±0.9
Temperature (°C)	21-23	23.0±0.8	24.3±1.3	22.7±1.5	25.4±0.3	24.6±1.0	22.3±1.4	23.3±1.6
Dissolved oxygen (mg/L)		4.9±1.5	5.7±3.1	8.2±1.0	7.0±1.9	5.5±1.9	7.1±1.7	9.5±3.5
pH		7.4±0.0	7.4±0.2	7.9±0.0	7.6±0.2	6.9±0.0		8.2±0.0
Chlorophyll-*a* (µg/L)		8.1±4.0	18.8±7.5	4.9±2.8	4.6±1.3	24.0±20	7.6±42	3.8±0.4
Ammonium (µM)		7.6±0.1	5.6±0.9	45.8±0.8	1.6±1.1	37.6±6.0	22.5±5.9	13.9±0.1
Nitrite (µM)		0.7±0.0	0.0±0.0	0.1±0.2	0.0±0.0	0.09±0.03	0.09±0.02	0.0±0.0
Nitrate (µM)		9.7±0.1	1.9±0.2	2.1±0.3	2.3±0.1	0.7±0.2	0.3±0.3	0.9±0.1
Phosphate (µM)		3.7±0.1	1.6±0.1	0.7±0.1	0.1±0.0			0.2±0.0

Appendix A: Health Status of Oysters in Narragansett Bay

In addition to the microbial communities, we also assessed the disease levels and overall oyster health using histology and qPCR at each site. We hypothesized that the environmental gradient in Narragansett Bay will cause differential responses in oyster health and disease levels.

Methods

Sample Collection and Processing

A total of 150 oysters were collected from 5 sites (30 per site) and processed on the day of collection (Figure 1). Ten oysters per site were randomly selected for histological analysis of tissue health, inspection for pathogen presence and prevalence, and assessment of their reproductive status. Cross-sectional tissue samples were collected from these 10 oysters and preserved in 10% formalin for 24 hours, after which they were transferred to 70% ethanol until processing. Gill, mantle, and rectum tissues from all 30 oysters per site were preserved in 100% ethanol for pathogen detection through qPCR, and stored at -80 °C until DNA extractions.

Histological Analysis

Ten tissue samples for each site (50 total) were embedded in paraffin on microscope slides and subsequently stained with hematoxylin and eosin (H&E) according to standard procedures by Mass Histology Service, Worcester, MA (Luna, 1992). The resulting 50 slides were analyzed on qualitative scales for tissue atrophy and necrosis levels, pathogen detection, sex, and maturity using the standard NOAA rankings (Kim et al., 2006).

Tissue DNA Extraction and Disease qPCR

Total DNA was extracted from 30 mg of preserved gill, mantle, and rectum tissues (n=30 per site; 150 total) using the Qiagen DNeasy Blood and Tissue kit with the following modifications. After the addition of Buffer ATL and proteinase K, the tissues were lysed overnight at 56 °C, then vortexed for 15 seconds. An RNA digestion was performed according to protocol recommendations, and two final elutions were done using 100 µL of Buffer AE each. Following extraction, DNA concentrations were quantified with a Nanodrop 2000 instrument and quality was confirmed using gel electrophoresis. Each sample was then normalized to 10 ng/µL for downstream analysis.

A standardized qPCR analysis of infection levels of *Perkinsus marinus*, the causative agent of Dermo disease, was performed in duplicate with 100 ng of purified oyster DNA according to the method described in De Faveri et al. (2009). Levels of amplification were compared to a standard curve of DNA extracted from known amounts (10^1-10^7) of *P. marinus* cells from laboratory cultures (the limit of detection is 10 *P. marinus* cells). Concentrations were then converted to disease intensity per oyster following the Mackin Index (0 for no infection – 5 for heavy infections), and also disease weighted prevalence per site based on both intensity and prevalence. Histology and *P. marinus* infection results were analyzed using R and RStudio to determine within- and between-site variability in oyster tissue health, and correlations with environmental parameters.

Results and Discussion

A summary of histological and disease measurements collected and their correlation with the trophic gradient is in Table 1. Histological analysis showed evidence of widespread digestive tissue atrophy, necrosis, and perivascular inflammation at all sites (Figure 2). In general, the gonadal status, and therefore maturity, of the oysters decreased down the Bay (Figure 2). Standardized qPCR analysis of *P. marinus* revealed that more than 83% of the oysters from populations in 1.PVD, 3.BIS, and 4.NAR scored positive for *P. marinus* infection (Figure 3). 3.BIS oysters showed the highest prevalence of Dermo (97%), including 3 highly infected oysters by *P. marinus*. The 2.GB population had no infected oysters, and the 5.NIN oysters had only 23% disease prevalence. Examined histology slides showed increased atrophy of digestive diverticula and various degraded connective tissue at the more Northern sites (Figure 2). Analysis also showed residual phagolysosomes, indicative of ongoing intracellular pathogen destruction.

A PCA analysis of *P. marinus* infection levels as determined by qPCR and histological indices, combined with the environmental parameters measured at each site, revealed correlations between the environment parameters and health status (Figure 4). Factors positively correlated with *P. marinus* infection included salinity and ammonium, while temperature was negatively correlated with *P. marinus* infection. The phenotype and histology samples from 1.PVD and 2.GB were associated with the highest levels of nitrate, nitrite, and phosphate, confirming these northernmost sites as the most eutrophic. The southernmost site (5.NIN) had the highest dissolved oxygen and pH values.

Our results showed that the environment influenced oyster health along the eutrophication gradient. 4.NAR and 5.NIN had the Southern "healthier" environmental ecotype: higher pH and increased DO. 1.PVD, 2.GB, 3.BIS had the Northern "unhealthy" environmental ecotype: high nutrient levels, tissue atrophy and necrosis, and high disease levels. Our hypothesis that Dermo would be more prevalent at more eutrophic sites (1.PVD and 2.GB) was proven incorrect. Instead, there are likely other environmental factors, as well as a genetic component of the oyster host, that have more of an impact on *P. marinus* infections and their associated microbial communities (Pierce et al., 2016; Trabal Fernández et al., 2014). The influence of the environment on organism health is consistent with previous studies (Widdows et al., 1981), where a negative influence of eutrophication on tissue health, inflammation was observed.

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Figures and Tables

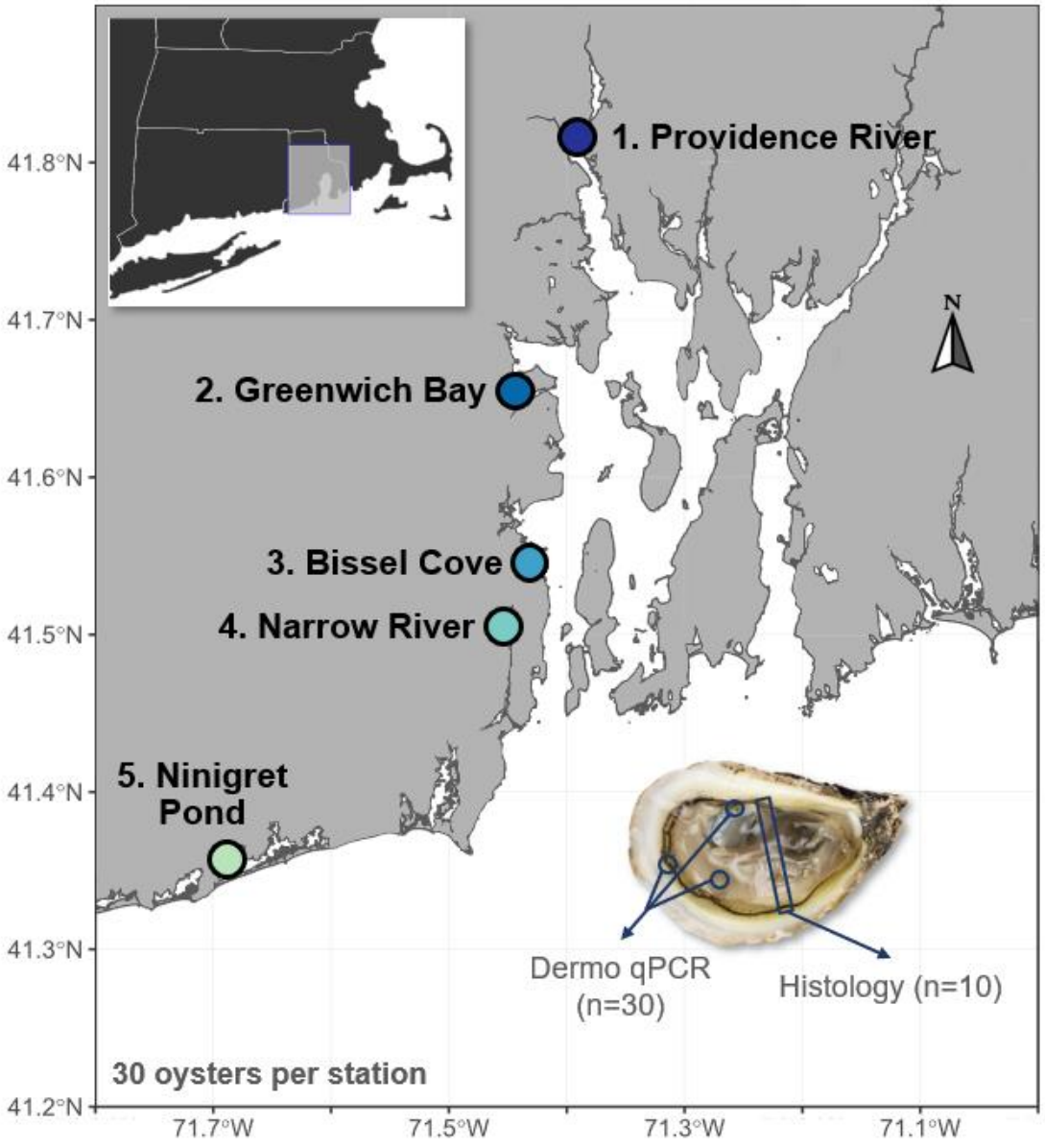

Figure 1. Map of study area with 5 sampling locations. A schematic of the tissue samples collected from each oyster is shown in the bottom right.

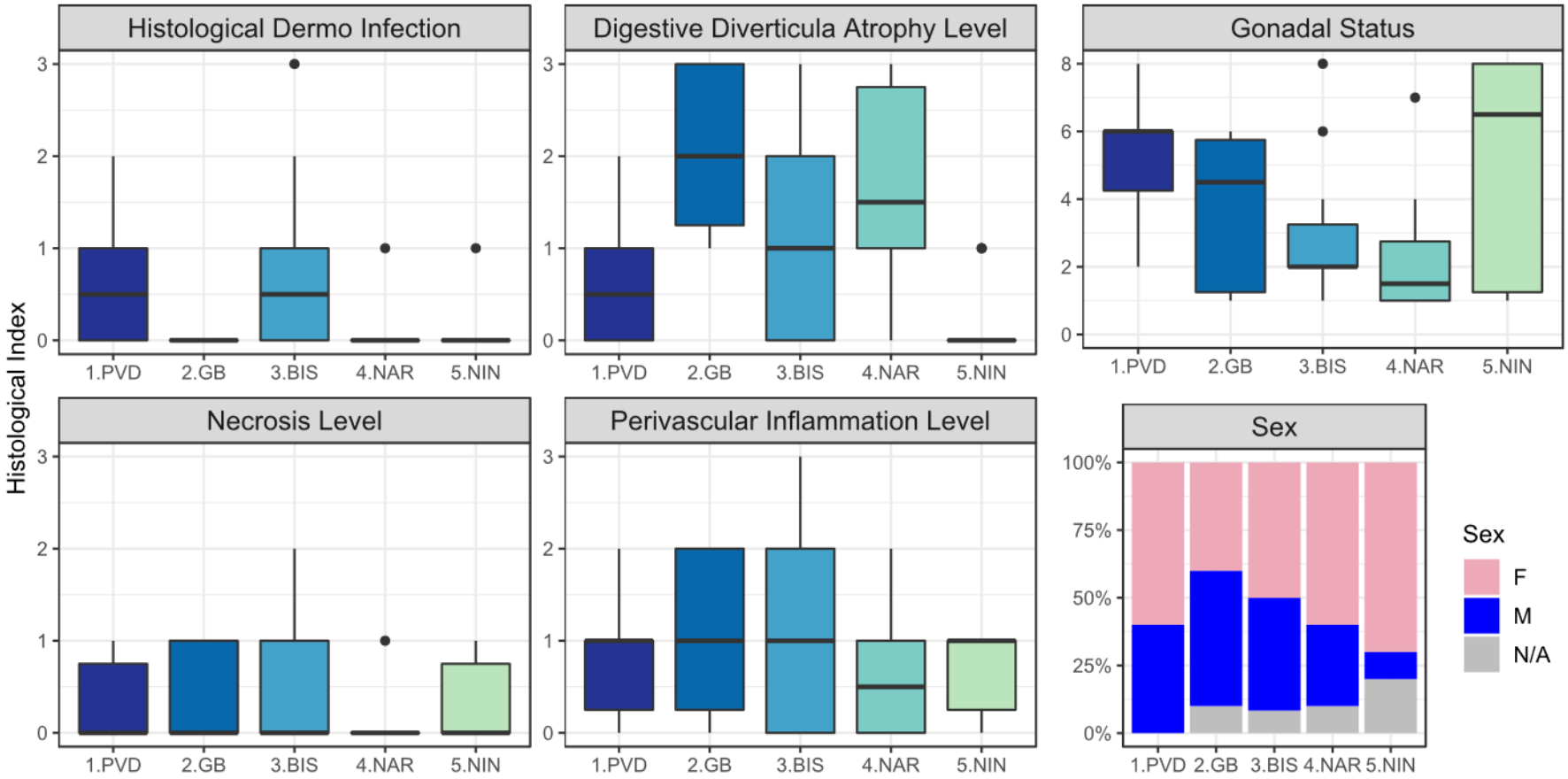

Figure 2. Summary of all histological parameters observed in the oysters from each site (n=10). Qualitative classification using a 0-3 severity scale indicated differences in oyster health status between sites. Gonadal Status was determined using rankings from 1-8, observing stages of maturity. Sex was also recorded for each oyster with N/A being sexually nondifferentiable.

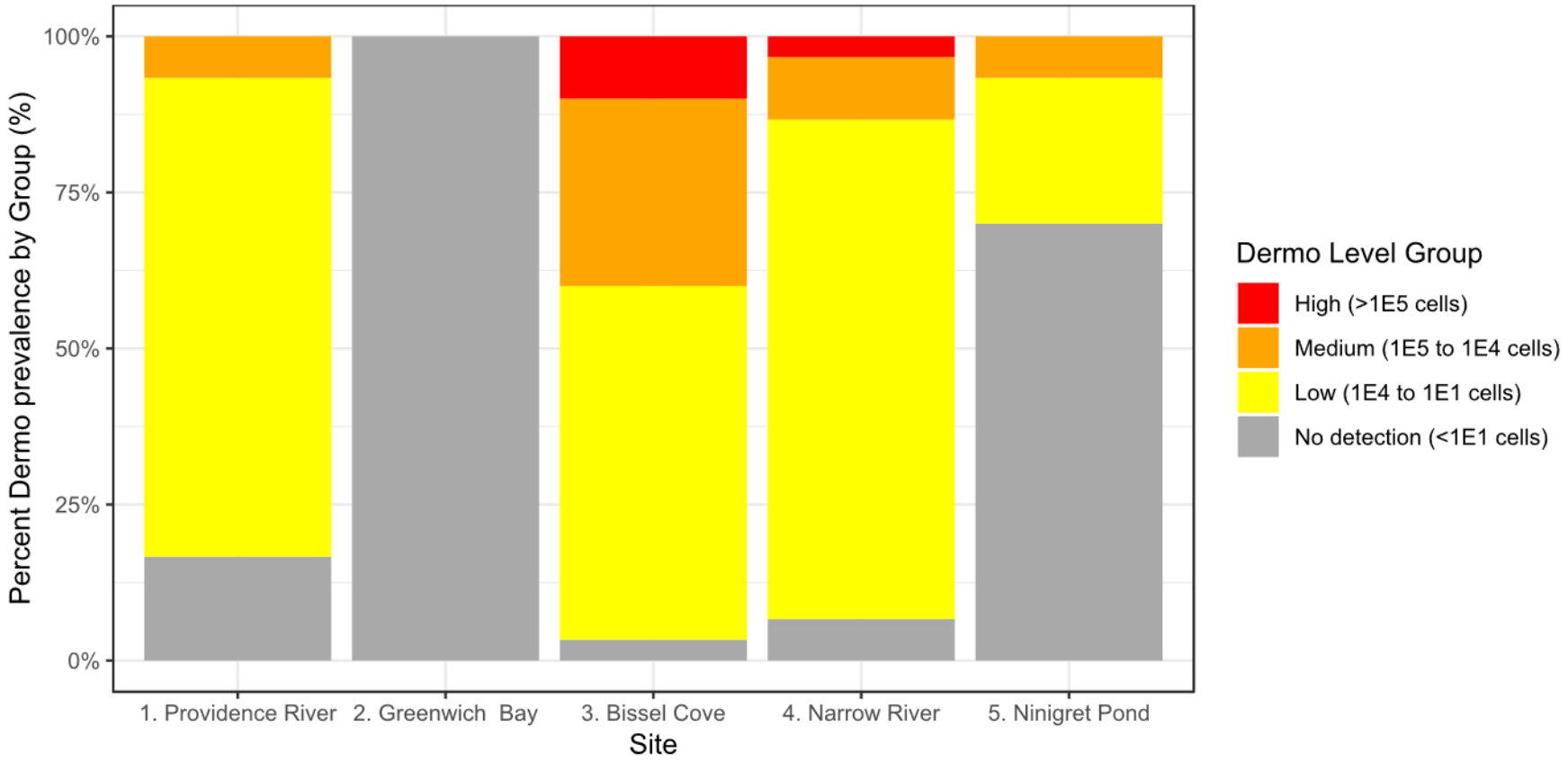

Figure 3. Percent prevalence of different stages of severity of *Perkinsus marinus* infection (causative agent of Dermo disease) at the 5 sites based on qPCR data (n=30). High levels of infection correspond to >1E5 *P. marinus* cells, medium levels of infection range from 1E5 to 1E4 cells, low 1E4 to 1E1 cells, and no detection <10 cells.

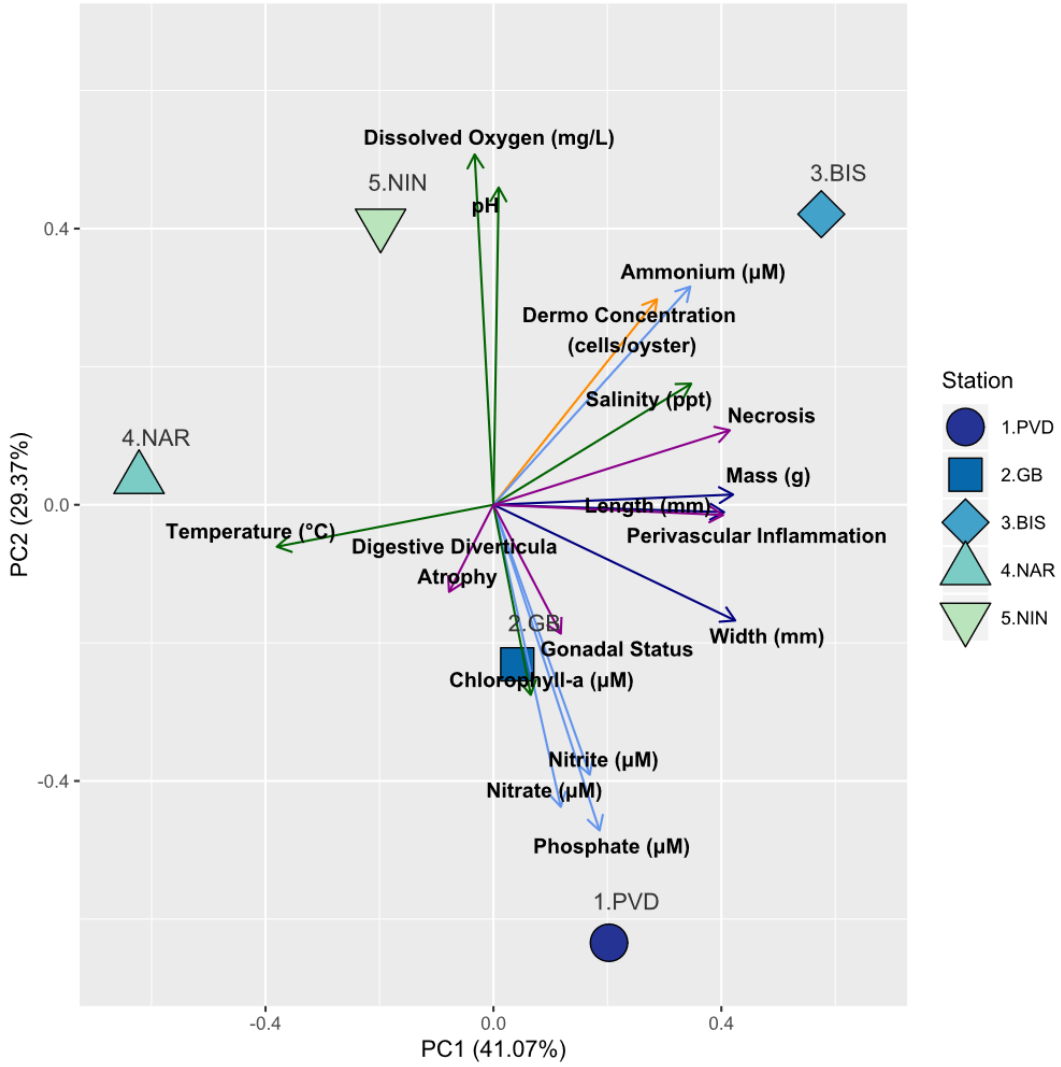

Figure 4. PCA of environmental and oyster health factors, averaged per site. Each site is represented by a colored symbol and each environmental condition is represented with an arrow. Green arrows indicate average environmental values measured *in situ* during the sampling week (n=2); light blue arrows are nutrient concentrations measured from water samples (n=3); dark blue arrows are oyster measurements collected after sampling (n=30); the orange arrow is qPCR-measured Dermo levels (n=30); purple arrows are histological indices observed from oyster tissues (n=10).

Table 1. Summary of all health-related parameters collected per site. Histological indices are rated on a scale from 0-3, with the exception of gonadal status, which is rated from 0-8. Spearman's correlation coefficient (-1 to 1) was calculated for the association between each parameter and Latitude. A value closer to 1 indicates that the parameter decreases from North-South (1.PVD to 5.NIN) and a value closer to -1 indicates that the parameter increases from North-South. A correlation coefficient of 0 means there is no linear association and that the value does not consistently change along the estuarine gradient.

	1.PVD	**2.GB**	**3.BIS**	**4.NAR**	**5.NIN**	**Spearman's Coefficient**
Location (GPS coordinates)	41.816, -71.391	41.654, -71.445	41.545, -71.431	41.505, -71.453	41.358, -71.689	1
Dermo infections (n=30)						
qPCR-detected *P. marinus* cells/oyster	1976.0 ±5736.8	0.0 ±0.0	66619.7 ±230326.9	9416.0 ±26784.8	2924.4 ±10895.7	-0.5
Histological Analysis (n=10)						
Digestive Diverticula Atrophy	0.7±0.8	2.1±0.9	1.1±1.1	1.7±1.1	0.2±0.4	0.4
Gonadal Status	5.3±2.1	3.7±2.2	3.0±2.1	2.3±1.9	5.0±3.2	0.3
Necrosis	0.3±0.5	0.4±0.5	0.4±0.7	0.1±0.3	0.3±0.5	0.3
Perivascular Inflammation	0.9±0.7	1.1±0.9	1.2±1.0	0.6±0.7	0.7±0.5	0.5
Percent Females	60%	40%	50%	60%	70%	-0.6

Appendix B: Metatranscriptomic Analysis of Oyster Tissues

The analysis presented in Chapter 4 focused on the effect of nutrient enrichment on oyster microbiomes and nitrogen metabolism. Here, we also explored the effect of sample type on overall microbial function in oyster microbiomes to better understand how each microenvironment shapes the microbial response.

Methods

Samples were collected, processed, and sequenced as described in Chapter 4. All gut, inner shell, and outer shell metatranscriptomic samples were combined per type for this analysis, regardless of field site or nutrient enrichment. Differential expression and relative normalized read counts per sample were calculated using DeSeq2 (Love et al., 2014; Zhu et al., 2019). The expression of each group (n=12) was compared to the average of the other groups to calculate relative log fold changes in expression per sample type.

Results

The most significant differentially expressed pathways were observed in the gut samples (Figure 1). Relative to the other samples, the gut samples significantly upregulated genes involved in respiration, membrane transport, DNA metabolism, dormancy and sporulation, phages and transposable elements, and photosynthesis (Benjamini-Hochberg adjusted $p<0.05$; Figure 5B). Genes involved in carbohydrates and virulence pathways were significantly downregulated in the gut samples (adjusted $p<0.05$). The inner shell samples significantly upregulated fatty acid, lipids, and

isoprenoid pathways, and phages and transposable element pathways (adjusted $p<0.05$; Figure 5B). In the outer shell, motility and chemotaxis was downregulated, while sulfur metabolism, DNA metabolism virulence and iron acquisition were upregulated (adjusted $p<0.05$; Figure 5B). These differences in expression patterns per sample type indicate that the oyster microenvironment may limit which functions are suitable, which may further select for colonization by certain microbes.

Figures

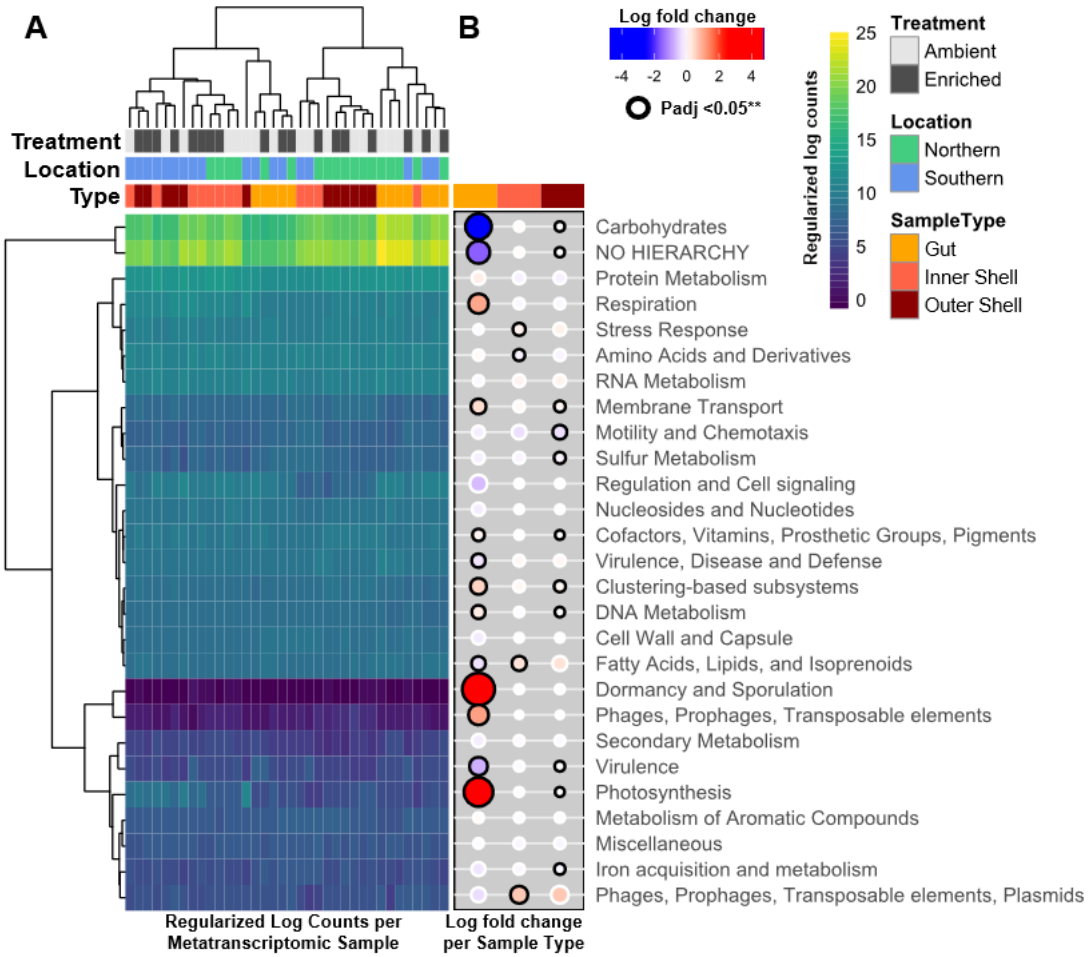

Figure 1. Differential Expression of All Pathways in each Sample, per Type. (A) Heatmap of regularized log counts per pathways in each metatranscriptome sample. The type, site, and treatment of each sample is indicated by colors along the top of the heatmap. **(B)** Log fold change of each pathway per sample type, relative to the mean of the others. Red indicates upregulation and blue indicates downregulation. The size of the point is relative to the magnitude of the fold change and points outlined in black are significantly differentially expressed.