Biogeographic Analysis of Eastern Oyster Microbial Associates in the Choptank River

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Science in Ecology with Distinction

Spring 2014

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ACKNOWLEDGMENTS

I would first like to acknowledge my funding sources, the National Science Foundation Biological Oceanography EAGER grant (#1148118) and National Science Foundation EPSCoR grant (IIA-1301765) and the State of Delaware, without which the completion of my project would have been impossible. Additionally, I'd like to thank the DNA Sequencing and Genotyping Center and the Center for Bioinformatics and Computational Biology at Delaware Biotechnology Center for the laboratory preparation and analytical support they have provided throughout my project. I would also like to thank all the members of Wommack and Polson Lab at Delaware Biotechnology Institute for the encouragement, teaching, and guidance they have provided throughout my research experience at the University of Delaware. Furthermore, I would like to thank the other members of my thesis committee, Dr. Deborah Delaney and Dr. Cristina Bacuta for the support they've provided throughout the writing process and the feedback provided about oral presentation strategies. Additionally, I would like to thank the Parks Family for allowing us to utilize fishing equipment during sampling, and the University of Maryland Horn Point Laboratory staff for lending us laboratory space during sample processing. Lastly, I would like to thank the Undergraduate Research Program and the Senior Thesis Program for providing such an opportunity to myself and other ambitious, dedicated, and curious students like me.

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ABSTRACT

The eastern oyster (*Crassostrea virginica*) is a keystone member of the Chesapeake Bay ecosystem, performing critical ecosystem functions. Preservation of this species requires more comprehensive understanding of factors affecting health of populations. Diverse bacterial symbiotic associations with host organisms can provide benefits such as alternate nutrient acquisition and competitive exclusion of pathogens. This study sought to further characterize the eastern oyster extrapallial fluid microflora in a biogeographical context, and to determine if the structure of this community is subject to location specific influences or environmental variation. In June 2011 samples were collected at four oyster bars along the Choptank River near Cambridge, MD and 16S rRNA gene amplicon libraries were constructed with barcoded primers to facilitate multiplex sequencing on the Pacific Biosciences RS2 platform. A number of OTUs were uniquely enriched (Student T-Test; $p < 0.05$) in both oyster extrapallial fluid and the surrounding water column and at specific sample locations (Kruskall-Wallis; $p < 0.05$). Community structure in oyster extrapallial fluid was location-specific (ANOSIM; $p = 0.019$), yet location was not a significant influence in determining community differences in Choptank samples (Mantel Test; $p > 0.05$). A distance-decay relationship exists for community structure between populations separated by approximately 50 km in oyster extrapallial fluid to a far less extent than in water, indicating a degree of conservation of core community structure, subject to transient changes due to environmental variation.

Chapter 1

INTRODUCTION

The Eastern Oyster, *Crassostrea virginica*

Biology and Ecology

The eastern oyster (*Crassostrea virginica*) is a historic economic resource to east coast fishing markets as well as an important functioning member of estuarine ecosystems. The eastern oyster is a bivalve (an invertebrate possessing two calcareous shells) that typically inhabits benthic regions of estuarine and marine waters. These organisms are sessile, and thus obtain most of their food from filtering the water column with ciliated gills, passing flagellates and unicellular algae of certain size to the digestive organs. In optimal conditions, oysters can filter 10 gallons of water in one hour for short periods of time, and may filter 100 gallons of water daily (Visel), reducing turbidity of the surrounding water column and better allowing for subaquatic vegetation (SAV) growth. Large populations of oysters also impact large scale processes like nutrient cycling. For example, Dame and colleagues established the significance of oyster beds in nutrient fluxes correlated with tides by observing direct changes in the ammonia content of water, likely caused by export of oyster waste materials like ammonia and particulate nitrogen in pseudofeces (Dame et. al. 1985). Additional work conducted by Smyth et. al. . indicates that oyster reefs also contribute significantly to estuarine denitrification - more so than any other habitats examined, including salt marshes, SAV beds, intertidal flats and subtidal flats (Smyth et. al.

2013). Furthermore, these same authors have also quantified the roles of individual oysters in contributing to nitrogen source-sink dynamics in benthic sediment; when oysters are present, net nitrogen flux tends to move towards denitrification (nitrogen production), whereas in sediment alone nitrogen flux is composed mostly of nitrogen fixation (nitrogen demand) (Smyth et. al. 2013), showing that oysters may play important roles in recycling nitrogen back to the water column. Additionally, oyster reefs play important roles in benthic ecology by creating complex three-dimensional structures and providing habitats to large numbers of organisms. Harwell et. al. have observed that in comparison to oyster assemblages created by a different introduced oyster species, the Suminoe oyster (*Crassostrea ariakensis*), eastern oyster assemblages supported significantly more biomass than their invasive counterparts in subtidal habitats with mid-range salinities. Additionally, in higher salinity areas, eastern oyster bars supported richer benthic communities per unit of oyster biomass than did Suminoe oyster bars, providing some evidence for species-specificity of reef-associated communities (Harwell et. al. 2010).

Conservation

Despite the importance of this member of estuarine communities to Chesapeake Bay ecosystem function, eastern oyster populations have suffered significant losses relative to historic abundances in pre-colonial America. Based on measurements gathered from available historic data stretching as far back as 1878 and as recent as 2011, Ermgassen et. al. have found that a significant proportion of estuarine areas containing native oyster populations has decreased. While the degree of this decline in geographic area varies by location, the density of market-sized oysters has not changed significantly, owing in part to early historic reports of

extremely large individuals and consequently little economic need to extract oysters of current market size. It is worth noting that this study only analyzed data as early as 1878 (after overfishing of these habitats had already begun), which could mean losses could be even more severe than observed in this study (Ermgassen et. al. 2012). Based on these findings, it would be reasonable to assume that oyster-associated ecosystem services have also declined in this same time frame. In 12 of the 13 estuaries examined in that study, the mean filtration rate of oysters at present density compared to historic density has dropped more than 80%, where previously 6 of those 13 once contained densities that could filter entire estuary volumes in the summer months (Ermgassen et. al. 2013). The implications of this sort of ecosystem service loss are striking, since nutrient loading and climate change present ecological challenges to estuarine systems that depend on the filtering action of oysters to reduce turbidity caused by sediment and phytoplankton blooms.

Another factor which has been investigated in connection with oyster population declines in recent history is protozoan infection by *Perkinsus marinus* ("Dermo"). Dermo has caused a significant degree of mortality, infecting the tissues of host oysters. Infection is spread throughout oyster populations between individuals whether it is directly from necrotic oyster tissue or indirectly from the water column surrounding infected oysters. The most common methods of diagnosis rely on histological examinations or culturing on thioglycollate media, both of which are based on microscopic visual symptoms in processed tissue. Molecular methods, relying on amplification of protozoan genetic material have also been developed (Smolowitz 2013). The protocol described by Penna et. al. . is one example of these types of methods with an added caveat; this procedure also claims to detect other less

common pathogens, "MSX" (*Haplosporidium nelsoni*) and "SSO" (*H. costale*), using a multiplex PCR approach with primers selected to amplify portions of each pathogen's genome specifically (Penna et. al. 2001). Our study attempted to optimize the described procedure for detection of pathogens in our samples, using digested oyster tissue as the source of template DNA for the multiplex reaction. However, the source protocol requires a pathogen culture step before obtaining template DNA (instead of crude oyster tissue DNA extract), the absence of which likely contributed to the relative inconsistency of our developed multiplex assay. In response to these conservation threats, creative efforts must continue to be made in order to preserve this species and, ultimately, to promote the health of the largest estuary in the United States.

Microbiology

Marine Microbial Ecology

In the last 50 years, the scientific perception of the roles of bacteria in natural systems has dramatically changed, especially in marine systems. The understanding established by early microbial ecologists was founded by studies that relied on culture-based techniques, which were not able to give accurate depictions of the abundance of bacterial groups and richness of communities in aquatic systems. For instance, despite high observed abundance of bacteria in direct counts of marine environments, most of these bacteria were assumed to be either dead or inactive due to low concentrations of nutrients in oceanic systems (Kirchman (ed.) 2000). Additionally, since the majority of marine bacteria are essentially unculturable (Kirchman (ed.) 2000), obtaining a true

picture of the relative contributions of different bacterial groups to microbial communities is a difficult and ongoing process. Fluxes in different nutrients like carbon, nitrogen, phosphorus, sulfur, and iron were thought to only be significantly influenced by the "grazing food chain", which included phytoplankton, zooplankton, metazoan consumers, and the dissolved organic matter that they all eventually contribute to during decomposition. This dissolved organic matter was thought to be fairly low in volume, and most primary production was thought to be carried out by phytoplankton, and therefore microorganisms were implied to be negligible contributors to oceanic nutrient cycles. However, more recent and extensive field studies show that a major proportion of biological nutrient cycling is processed in what is called the "microbial loop"; bacterial primary production (now understood to be about 50% of all oceanic primary production) is passed to protozoan predation or released in dissolved organic matter by viral infection, while organic matter and detritus are returned to nutrient cycles by bacterial metabolism (Azam 1998).

We now know that marine environments host diverse microbial communities that account for most of the oceanic biomass and metabolism (Azam 1998), and culture-independent methods of studying these microbial processes are providing new insights into microbial communities in many different environments. The most common method of conducting phylogenetic studies (which utilize the genetic material of organisms to construct evolutionary relationships between groups) in bacteria is by analyzing 16S ribosomal RNA gene sequences from community members. Portions of this DNA sequence are well-conserved across all organisms, making changes that occur in more variable regions useful for elucidating evolutionary relationships and distinguishing certain prokaryotic groups. This technique was

notably used to generate the three domain paradigm of life organization (Woese & Fox 1977) and is used today in many studies to assess the diversity of microbial communities from a variety of environments, including this study of microbial communities of oyster extrapallial fluid. While this method of phylogenetic analysis is effective and cheap, it has important limitations. To obtain numerous copies of this gene, scientists rely on the polymerase chain reaction (PCR) to amplify the desired gene using designed primers that selectively amplify the 16S rRNA gene template from genomic DNA. In downstream analysis, scientists assume that sequences from all bacterial groups in the sample had the same chance of being amplified; this is not necessarily true. If universal primer sequences are suboptimal, it is possible that sequences from certain types of bacteria will be amplified preferentially relative to others, potentially skewing measures of community composition (Klindworth et. al. 2013). Additionally, different bacterial taxa possess a number of different copy numbers of this gene, potentially inflating the prevalence of certain groups over others (Klappenbach et. al. 2001). In short, culture-independent methods have become new avenues for detecting proportions of rare or unknown members of microbial communities, changing scientific thought in microbial ecology research.

Microbial Biogeography

One of the central goals of ecological research is to examine biodiversity and how it is regulated spatially. The spatial patterns of biodiversity provide clues as to the mechanisms regulating this diversity (Green & Bohannan 2006). Until recently scientists have assumed microbial diversity is relatively similar on a global scale; that is, most bacterial groups are distributed in a cosmopolitan manner due to high dispersal rates, short generation times, and small physical size, and therefore are

almost always (albeit rarely) present in any environment (Fenchel & Finlay 2004). However, newer scientific findings are reporting trends of spatial regulations to microbial diversity. For example, Cho and Tiedje observed increased genetic variability of *Pseudomonas* strains with increased geographic distance between sample sites on regional scales (5m to 80km) but not on global scales (between continents), indicating that microbial diversity can follow a distance-decay relationship on small scales (Cho & Tiedje 2000). Other studies, such as that by Reche et. al. ., have demonstrated further that the composition of microbial assemblages was significantly influenced by the spatial distribution of lakes, but not significantly influenced by other abiotic factors such as physical constraints, resources, and grazers (Reche et. al. 2005). By sampling water and oysters from multiple sites on a spatial scale, it may help to determine the mode of microbial assembly in oyster extrapallial fluid. For example, if oyster microbiomes are less variable geographically than water communities, it may indicate a degree of selection of observed enriched bacterial groups in oysters, rather than a more passive, opportunistic colonization event of bacterial groups that are dispersed throughout geographic space in the water column.

Oyster Microbiome

One of the most important relatively recent discoveries in microbiology is the existence of symbiotic relationships between microbes and larger host organisms ranging in complexity from protists, like algae, to vertebrates, like humans. At the origin of this field of study, relationships were discovered with motivation to discover disease agents, with the invention of Koch's Laws for isolating disease causing agents (Kirchman (ed.) 2000). We now know that utilizing culture-based methods doesn't

provide scientists with complete snapshots of diverse bacterial communities, let alone indicate major biological relationships with hosts. Yet progress has still been made in determining the roles of specific types of microbes in mutualistic, commensal, and parasitic/pathogenic relationships, and has accelerated with the use of culture-independent methods. In nitrogen-fixing plants, biological nitrogen fixation is carried out by *Rhizobium* and *Bradyrhizobium* populations in root nodules of leguminous plants, and certain strains of which have been shown to have characteristics of plant growth promoting rhizobacteria such as siderophore production, indole 3-acetic acid (IAA) production, and phosphorus solubilization (Antoun et. al. 1998). While this is a very dramatic example of how mutualistic bacteria can directly influence the health and growth of the host organism, similar relationships exist in well-studied systems, like humans.

Bacterial communities likely colonize most surfaces of every organism, and the recently coined term by the scientific community "microbiome" attempts to address the interconnectedness of the microbial communities with the host environment and physiology. Important analysis has started the groundwork for microbiological studies of different human microbiomes and the ecology of these communities. For example, Kurokawa et. al. . compared gut microbiota from adults, children, and unweaned infants and found that a distinct community compositional change occurs after weaning, and that communities were most complex and consistent between adults and weaned child individuals compared to infants, in which usually only a few microbial species/strains dominated the community, which varied widely between individual infants (Kurokawa et. al. 2007). Aagaard and colleagues have also identified unique microbiome signatures in vaginal samples from pregnant women,

dominated by *Lactobacillus* species with generally reduced diversity and richness of all groups, relative to non-pregnant women (Aagaard et. al. 2012). Additionally, five different community "groups" distinguished by the dominant *Lactobacillus* species present in vaginal samples of sexually active, reproductive age women have been shown to correlate to bacterial vaginosis risk among women, with one group defined by higher pH levels and higher diversity of non-*Lactobacillus* taxa. It is thought that this dominance by *Lactobacillus* species aids in protection of the vagina by competitive exclusion, as well as production of lactic acid and bacteriocidal compounds (Ravel et. al. 2010). In well-studied model systems like plants and humans, the relationships between hosts and their microbial communities is dynamic and mutualistic.

Much of the work in bivalve microbiology has focused on disease with potential impact on aquaculture. The most well known bacterial diseases of bivalves are nocardiosis and *Vibrio splendidus* summer mortality in the pacific oyster (*Crassostrea gigas*), Brown Ring Disease in clams of the groups *Ruditapes phillippinarum* and *R. decussatus*, and Juvenile Oyster Disease in the eastern oyster (Paillard et. al. 2004). Numerous approaches have been examined to attempt to detect, treat, and prevent bacterial diseases, such as treatment of water with disinfectants, vitamins, antibiotics, and even probiotics to promote immune health in growing organisms. Additionally, some have attempted to characterize antimicrobial peptides present in the innate immune systems of invertebrates and understand the mode of action of these compounds with regards to the health of invertebrates (Bachere 2003). Some groups have also attempted to characterize native microbial communities of other invertebrates. In pacific oysters, Colwell showed that bacteria are more

concentrated in oysters than in surrounding water, and that almost 50% of this population is able to ferment glucose anaerobically despite low reductive capacity (Colwell & Liston 1960). Antunes et. al. found that in the freshwater mussel (*Anodonta cygnea*), hemocytes in the organism were able to filter and eliminate *Escherichia coli* from hemolymph. Additionally, it was shown that *Vibrio metschnikovii* were able to persist in the mussel, suggesting a potentially commensal relationship (Antunes et. al. 2010). Additionally, Beleneva and colleagues showed that, in the mussel (*Mytilus trossulus*), microbial communities were dominated by organisms that favored anaerobic conditions and that were metabolically active, potentially able to utilize a variety of nutrient substrates for growth (Beleneva et. al. 2003). However, these studies relied on culture-based analytical techniques and almost certainly didn't recognize a large proportion of the community present. LaValley showed that isolation-based methods produce different, even if complementary, results compared to denaturing gradient gel electrophoresis (DGGE) (LaValley et. al. 2009), a method which separates small DNA fragments by small sequence differences in variable regions of the 16S rRNA gene.

Studies that analyzed microbial communities using molecular techniques have further elucidated correlations between microbial communities and organism physiology and ecology. Bourne et. al. have shown, using 16S rRNA gene pyrosequencing, that in a variety of coral reef-dwelling invertebrate taxa, including bivalves, the composition of microbial communities was affected by the presence of photosymbionts. Additionally, the most dominant and frequently found taxa observed and highly associated with photosymbiont presence are all associated with metabolizing complex organic compounds like dimethylsulfoniopropionate (DMSP),

which is involved in climate regulation via cloud formation (Bourne et. al. 2013). Karlinska-Batres & Worheide found that in coralline sponges (*Vaceletia crypta*), microbial taxa (Gammaproteobacteria, Gemmatimonadetes, Actinobacteria, Nitrospira, Deltaproteobacteria, Deferribacteres, and Acidobacteria), many of which contribute to important nutrient cycles, were found to comprise 58% of the sponge-associated microbiome (Karlinska-Batres & Worheide 2013). Additionally, in studies of microbial diversity in oyster gill and digestive tissues using fluorescently labeled group-specific probes, it has been shown that this diversity within tissues is high, including Gammaproteobacteria as principle representatives (Hernandez-Zarate & Olmos-Soto 2006).

This study of the eastern oyster extrapallial fluid microbiome in a local estuary will help to further elucidate the core members of these microbial communities and their potential roles in contributing to important ecological processes. Additionally, analyzing the geographic impact on microbial community structure has the potential to inform decisions regarding optimal conditions for fostering healthy native oyster habitat conditions based on correlations between microbial community profiles and susceptibility to disease or environmentally induced mortality. This study is also important in optimizing a suitable workflow for 16S rRNA gene community profiling on the Pacific Biosciences RS2 platform, which this lab group has only recently begun utilizing. Furthermore, this work would contribute to the growing knowledge of critical associations formed with symbiotic bacteria and the roles these bacteria may play in the maintenance of oyster fitness in its native range.

Chapter 2

METHODOLOGY

Sample Collection

Oysters

Samples were collected on June 28, 2011from 4 oyster bars along the Choptank River in Cambridge, MD. The coordinates for the sample sites are as follows: "Kirby Bar" (GPS Decimal Coordinates: -76.0701, 38.5956), "Dickinson Bar" (GPS Decimal Coordinates: - 76.0927, 38.6022), "Sandy Hill Bar" (GPS Decimal Coordinates: -76.1177, 38.602) and "Howell's Point Bar" (GPS Decimal Coordinates: -76.1254, 38.6099) (Figure 1). Additional environmental measurements are provided in Appendix A. This river was chosen based on observed disease prevalence from previously collected data (Tarnowski (ed.) 2010) in hopes that some collected specimens would be infected and comparisons could be made between healthy speciments. A total of 74 oysters were collected by dredging these locations. 40 live oysters (10 from each site) were randomly selected and cleaned by brush scrubbing and sanitization with 70% denatured ethanol solution. Two holes were then drilled into the extrapallial space of each oyster; one in the anterior portion of the dorsal valve, one in the posterior portion of the dorsal valve near the umbo. Up to 5 mL of extrapallial fluid was then extracted with an 18.5 gauge needle and sterile 5 mL syringe from each oyster sampled. An additional 200 uL of extrapallial fluid was also collected, fixed with formalin, and snap-frozen with liquid nitrogen for use in

epifluorescence microscopy. After extrapallial fluid collection, oysters bodies were shucked and stored in 2 Whirl-Pak bags (Nasco, Wisconsin, USA), and subsequently snap-frozen with liquid nitrogen.

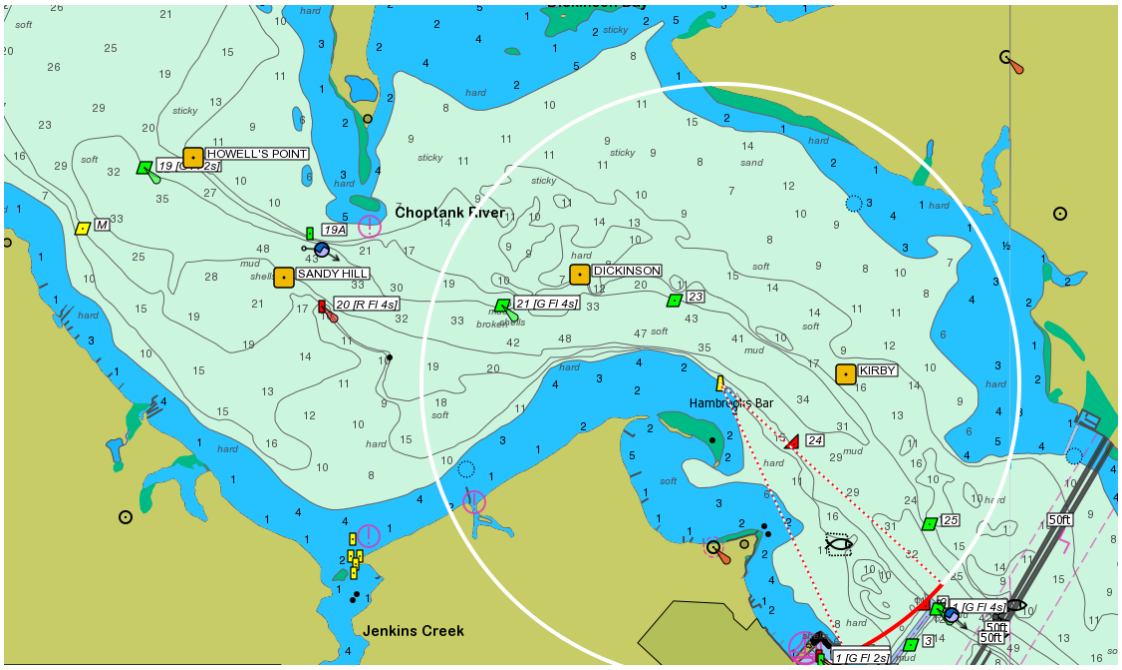

Figure 1 Map of oyster and water sample collection sites in the Choptank River (Cambridge, MD).

Water

Replicate 5 liter ambient bottom water samples from each oyster bar were obtained using a Niskin bottle. Approximately 5 mLs of ambient water was collected and fixed with formalin, and snap-frozen with liquid nitrogen. The collected water was sequentially passed through 5 um, 1.2 um, and 0.22 um Sterivex filters (Millipore, MA, USA). 1.5 mL of SET Buffer (50 mM Tris HCl (pH 8), 50 mM NaEDTA, 20%

sucrose) was added and filters were frozen at -80C as soon as facilities were available. The 0.22 um filtrate was also collected and inoculated with iron chloride, and incubated for 1 hour. Post-incubation, the solution was passed through a 0.8 um filter to capture flocculated viral particles. These filters were stored in the dark at 4C.

Sample Processing

Epifluorescence Microscopy

1 mL of formalin-fixed water samples were thawed and vacuum filtered through 0.02 um filters. In the analogous preparation of oyster extrapallial fluid, this fluid is diluted to a 1:10 concentration with autoclaved and 0.22 um filtered 1X phosphate-buffered saline (PBS) solution. 1 mL of this extrapallial fluid dilution is then passed through 0.02 um filters. In both workflows, the filter is then stained for 15 minutes with 400 uL of 2.5X SYBR Gold DNA Stain (Life Technologies, New York, USA). Filtration resumes and, after approximately 10 minutes of drying the stained filter, slides are made to be analyzed for direct counts of bacterial and viral abundance.

DNA Extraction and Isolation

One mL of DNA extraction buffer (DEB) (100 mM Tris buffer (pH 8), 100 mM NaEDTA (pH 8), 100 mM phosphate buffer (pH 8), 1.5 M NaCl, 1% CTAB) with 10 uL of proteinase-K (10 ug/mL) and 20 uL of lysozyme (100 ug/mL) was prepared. Once Sterivex filters were thawed, the SET buffer was pushed through and stored until after DNA extraction was completed. One or two mL of DEB was added and the filter was frozen at -80C for 15 minutes and thawed at 37C for 5 minutes in a cyclical manner 3 times. After these 3 freeze thaw cycles, the DEB was collected and incubated in a 37C water bath for 30 minutes. 100 uL of 10% sodium dodecyl sulfate

(SDS) solution was added, mixed via inversion into the collected DEB and incubated in a 65C water bath for 2 hours. After this incubation, remaining tube space was filled with a 50:49:1 phenol/chloroform:isoamyl alcohol solution, vortexed and centrifuged for 5 minutes at 3000 rpm. The aqueous layer was decanted, and treated again with the phenol/chloroform solution. This process was repeated, and then treated similarly a third time with the treatment solution of exclusively chloroform. 60% of the resulting decanted layer volume from the third treatment was estimated; this estimated volumetric proportion of 100% isopropanol was then added, inverted, and incubated at room temperature for at least 2 hours. This solution was then centrifuged for 30 minutes at 13000 rpm. After this step, a pellet of precipitated DNA could sometimes be observed. The isopropanol and buffer was then decanted without perturbation of the pellet. The pellet is washed with a 70% ethanol solution and centrifuged at 13000 rpm for 10 minutes. This solution is decanted, and the ethanol wash process is repeated. The pellet is then dried for approximately 15-30 minutes and resuspended in 250 uL of EB Buffer (10 mM tris, pH 8.5), vortexed for optimal elution, and stored at 4C until elution is complete.

16S rRNA PCR and Sequencing

16S rRNA gene amplicon libraries were created for all replicate water samples and between 7-8 oyster samples per sample site via polymerase chain reaction. Primers for amplification were chosen to amplify the V1-V3 hypervariable region of the gene, a commonly targeted region for pyrosequencing on Roche 454 platforms, which was the traditional platform for samples processed in this area by our lab group. The 27F (5'-AGAGTTTGATCMTGGCTCAG-3') forward primer and 534R (5'-ATTACCGCGGCTGCTGG-3') reverse primer were utilized, with 534R outfitted with

a variety of unique 6-8mer barcode sequences on the 5' end to facilitate multiplex sequencing on the Pacific Biosciences SMRTcell platform post library preparation. The specific sequences for utilized barcodes are outlined in (Table 1).

PCR reactions were prepared as follows: 18.25 uL sterile water, 2.5 uL 10X PCR Buffer, 2.5 uL dNTP solution, 0.25 uL 27F 10 uM primer stock (final concentration: 0.1 uM), 0.25 uL 534R 10 uM primer stock (final concentration: 0.1 uM), 0.25 uL ExTaq DNA Polymerase (Takara, Japan), and 1-2 uL template microbial DNA. If, based on multiple attempts, a degree of PCR inhibition was apparent, 1 uL of bovine serum albumin would also have been added. Cycling conditions were as follows: 95C for 5:00, 30 cycles of denaturation (95C for 45s), annealing (55C for 1:00), and elongation (72C for 45s), 72C for 6:00, and a continuous storage setting at 4C. After PCR amplification, samples were purified via gel electrophoresis. PCR products were loaded into 1.8% agarose gels electrophoresed at 100V. Gels were stained with SYBR Gold DNA stain and bands at the expected product size were cut. DNA within gel slices was extracted using either the Qiagen QIAquick Gel Extraction Kit (QIAGEN, Germany) or the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, California) and purified with the Agencourt AMPure XP kit (Beckman Coulter, California, USA). After DNA Quantification via the Qubit dsDNA High Sensitivity Assay (Life Technologies, New York, USA), approximately 100ng of each DNA sample was pooled and submitted to colleagues at the DNA Sequencing and Genotyping Center (University of Delaware) for DNA sequencing on the Pacific Biosciences RS platform (California, USA), utilizing one SMRTcell.

Table 1 534R reverse primer sequences and 5’ adapted barcodes utilized in amplicon library preparation, facilitating multiplex sequencing on Pacific Biosciences SMRTcell platform.

Plate Position	Barcode Sequence	PacBio Primer Sequence (5'--3')
A3	TGAAGC	TGAAGCATTACCGCGGCTGCTGG
A5	TCACAC	TCACACATTACCGCGGCTGCTGG
A8	CCTCTC	CCTCTCATTACCGCGGCTGCTGG
A12	AGCTTC	AGCTTCATTACCGCGGCTGCTGG
B2	CAAGAAC	CAAGAACATTACCGCGGCTGCTGG
B8	ACAAGGC	ACAAGGCATTACCGCGGCTGCTGG
B10	ATACCAC	ATACCACATTACCGCGGCTGCTGG
C3	AAGGTGC	AAGGTGCATTACCGCGGCTGCTGG
C5	TAATCTC	TAATCTCATTACCGCGGCTGCTGG
C11	TGCGTTC	TGCGTTCATTACCGCGGCTGCTGG
D1	CCAGGAC	CCAGGACATTACCGCGGCTGCTGG
D3	TTCCTGC	TTCCTGCATTACCGCGGCTGCTGG
D5	CGTCGTC	CGTCGTCATTACCGCGGCTGCTGG
D7	AACAACTC	AACAACTCATTACCGCGGCTGCTGG
E4	ACGAAGTC	ACGAAGTCATTACCGCGGCTGCTGG
F2	TCCAGAAC	TCCAGAACATTACCGCGGCTGCTGG
F5	ACTAATTC	ACTAATTCATTACCGCGGCTGCTGG
F12	TTGTGTTC	TTGTGTTCATTACCGCGGCTGCTGG
G6	TTCTCAAC	TTCTCAACATTACCGCGGCTGCTGG
H9	TTAAGATTC	TTAAGATTCATTACCGCGGCTGCTGG

Statistical and Bioinformatic Analysis

Pairwise comparisons of bacterial and viral abundance in samples were determined via Student's T-Test with an alpha of 0.05. Circular consensus DNA sequences were filtered and checked for chimeras to ensure the use optimal quality reads. The bioinformatic software tool QIIME (Quantitative Insights into Microbial Ecology, Version 1.7.0) (Caporaso et. al. 2010) was utilized to perform taxonomy assignments and diversity index calculations. Sequences were clustered into

operational taxonomic units (OTUs) at 97% similarity and a representative sequence from each cluster was selected to be assigned to a discrete taxonomic unit using the uclust taxonomy classifier. Sequences were then aligned to a reputed reference sequence using the PyNAST alignment tool, and lanemask filtered to remove unnecessary gaps in alignment. An OTU table was then created in matrix form to visualize the relative abundance of sequences belonging to determined taxonomic identifiers.

Comparisons of relative abundances between treatments (source environment) and sample sites were performed using Student's T-Test and Analysis of Similarity (ANOSIM), respectively, both with an alpha of 0.05. Comparisons of relative abundances of OTUs between treatments (water and oyster) were performed via Student's T-Test using an alpha of 0.05. Relationships between metadata categories and weighted UniFrac distance (metric of community dissimilarity) were analyzed using the Mantel test and an alpha value of 0.05. Alpha diversity calculations were conducted using the EstimateS web tool (Chao et. al. 2005) by estimating species richness, Shannon Index, and Chao1 Index over 100 randomizations of rarefied OTU table data. Treatment- and class-specific enrichment statistical analysis was performed using the LEfSe (LDA Effect Size) module for Galaxy web-based software (Goecks et. al. 2010, Blankenberg et. al., Giardine et. al. 2005). The Kruskall-Wallis test was utilized to determine significant differences in the relative abundances of certain taxa based on sample site, using an alpha value of 0.05. Significant results from this step are then ranked based on the relative effect size of each taxa on how different the classes (sample site or treatment) are from each other using Linear Discrimination Analysis.

Chapter 3

RESULTS

Direct Counts

According to counts acquired by epifluorescence microscopy, bacterial concentrations are generally higher in oyster extrapallial fluid (N = 30) than the surrounding water column (N = 16). At Kirby, Sandy Hill, and Howell's Point bars, bacterial concentrations of oyster extrapallial fluid exceeded that of the water column, while at Dickinson bar, bacterial abundance in water was slightly higher than that within oysters. The highest mean bacterial abundance for a sample site was observed at Kirby bar, with bacterial concentrations averaging 2.04×10^7 bacteria/mL ($\pm 1.05 \times 10^7$ bacteria/mL). Additionally, at this site, bacterial concentrations were significantly different between oyster extrapallial fluid and water samples ($p = 0.05$). No other sites showed significant differences between bacterial concentrations of oyster extrapallial fluid and water samples ($p > 0.05$). The lowest mean bacterial abundance observed at any site was observed in oyster extrapallial fluid from oysters at Dickinson bar, averaging 3.73×10^6 bacteria/mL ($\pm 3.73 \times 10^6$ bacteria/mL). When comparing the mean bacterial concentrations of water samples from different sites, no significant differences were observed ($p > 0.05$). Mean bacterial concentrations of oyster extrapallial fluid from Dickinson oysters differed significantly from those of Kirby oysters ($p = 0.042$) and Sandy Hill oysters ($p = 0.015$). No other significant differences were observed between pairwise comparisons of oyster extrapallial fluid bacterial concentrations from different sites (Figure 1).

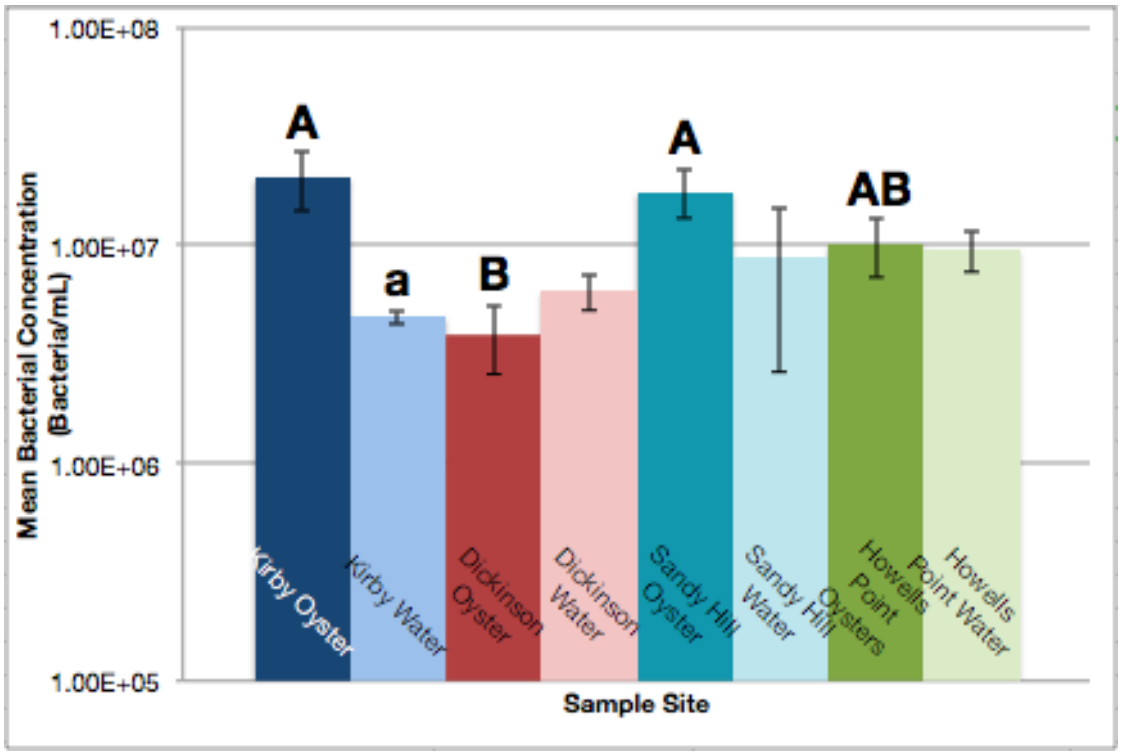

Figure 2 Mean bacterial concentrations of oyster (*Crassostrea virginica*) extrapallial fluid samples and water samples from 4 oyster bars along the Choptank River (Cambridge, MD) determined by epifluorescence microscopy. Columns without matching letters indicate significantly different bacterial concentrations (Student's T-Test; $p > 0.05$). Only one instance was observed where extrapallial fluid and water samples differed significantly ($p = 0.05$) within a site (Kirby bar); this is denoted by a matching lowercase letter.

16S rRNA Community Profiling

After chimera screening and quality filtering of output sequences, a total of 153,299 sequences from 43 samples (Oyster and Water), including sequences from 5 samples obtained by a colleague from a more distant sampling location (Smithsonian Environmental Research Center, Edgewater, MD) were used to create OTU tables for community profiling. These sequences were assigned to 23,962 OTUs. The number of

sequences retrieved for each sample varied widely, within a range of 113 to 11,258 sequences, averaging 3,565 sequences per sample (Figure 3).

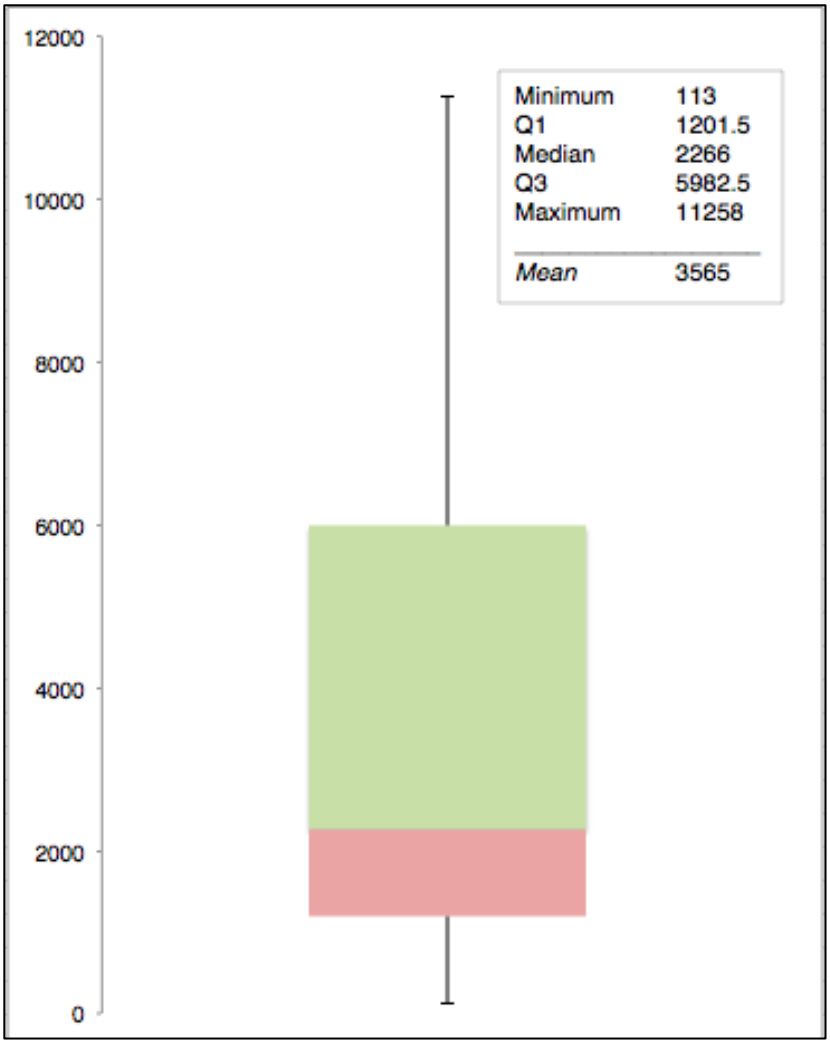

Figure 3 Distribution of sequence density among all samples sequenced (N=43).

Treatment (Water v. Oyster)

In water, the 10 most abundant classes, representing 92% of sequences, were Alphaproteobacteria (39%), Actinobacteria (18%), Flavobacteriia (8%), Synechococcophycideae (6%), Betaproteobacteria (5%), Sphingobacteria (3%), Acidimicrobiia (3%), Chloroplast (2%), Gammaproteobacteria (2%), and other (5%). The top 10 most abundant classes in oyster extrapallial fluid, composing 81% of sequences, were as follows: Alphaproteobacteria (25%), Synechococcophycideae (14%), Actinobacteria (8%), Acidimicrobiia (8%), Betaproteobacteria (6%), Gammaproteobacteria (5%), Flavobacteriia (4%), Deltaproteobacteria (3%), Spirochaetes (2%), and other (7%) (Figure 4).

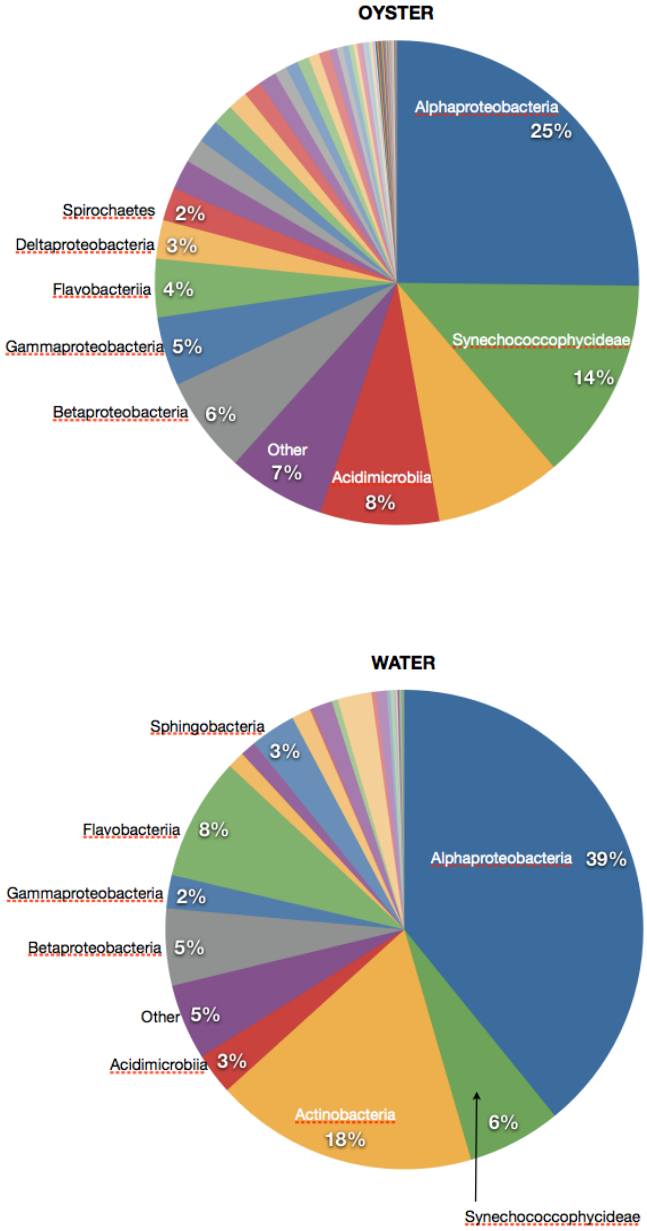

Figure 4 Mean relative abundance of bacterial classes in oyster extrapallial fluid and water from oyster bars in the Choptank River.

Of all classes present in samples of both treatments (N=142), 32 were significantly different in mean relative abundance between the two sample types ($p < 0.05$). Among these 32 classes include major groups, such as members of the phylum Proteobacteria [Alphaproteobacteria ($p = 0.026$), Gammaproteobacteria ($p = 0.004$), Betaproteobacteria ($p = 0.037$)], Actinobacteria ($p = 0.009$), and Acidimicrobiia ($p = 0.000002$), Additionally, many classes belonging to a particular phylum were significantly different, such as classes of Cyanobacteria [S15B-MN24 ($p = 0.002$), Gloeobacterophycideae ($p = 0.022$), 4C0d-2 ($p = 0.042$), other ($p = 0.042$)], Verrucomicrobia [Opitutae ($p = 0.035$), Methylacidiphilae ($p = 0.040$), Pedosphaerae ($p = 0.044$), Verruco-5 ($p = 0.045$)] and OD1 [ABY1 ($p = 0.000001$), ZB2 ($p = 0.000002$), Unclassified ($p = 0.003$)] (Table 2).

Table 2 Bacterial classes with significantly different relative abundance between oyster extrapallial fluid and water samples (Student's T-Test; $p < 0.05$).

	Relative Abundance		p-value		Relative Abundance		p-value
OTU	Oyster	Water		OTU	Oyster	Water	
(OD1) ABY1	0.25%	0.00%	0.000001	**Nitrospira**	0.21%	0.00%	0.009
Acidimicrobiia	7.94%	2.80%	0.000002	**Thermoleophilia**	0.03%	0.00%	0.009
(OD1) ZB2	0.11%	0.00%	0.000002	**Actinobacteria**	8.41%	17.81%	0.009
koll11	0.04%	0.00%	0.000050	**(GN02) Other**	0.05%	0.01%	0.014
Bacteroidia	0.84%	0.01%	0.00040	**Ignavibacteria**	0.06%	0.01%	0.015
Phycisphaerae	0.20%	0.05%	0.00050	**Gloeobactero-phycideae**	0.03%	0.01%	0.022
[Brachyspirae]	1.62%	0.01%	0.00050	**Flavobacteriia**	3.90%	8.29%	0.023
(GN02) BD1-5	0.05%	0.00%	0.0010	**(Firmicutes) Bacilli**	0.19%	0.02%	0.023
(Unclassified) Other	0.41%	0.02%	0.00200	**Alpha-proteobacteria**	25.18%	39.12%	0.026
(Cyanobacteria) S15B-MN24	0.14%	0.04%	0.0020	**Opitutae**	0.38%	0.21%	0.035
(OD1) Unclassified	0.03%	0.00%	0.0030	**Beta-proteobacteria**	6.45%	5.20%	0.037
Gemm-2	0.16%	0.03%	0.0030	**[Methylacidiphilae]**	0.07%	0.02%	0.040
Gamma-proteobacteria	4.59%	2.23%	0.004	**(Cyanobacteria) Other**	1.99%	1.04%	0.042
Planctomycetia	0.72%	0.31%	0.006	**(Cyanobacteria) 4C0d-2**	0.01%	0.00%	0.042
(GN02) 3BR-5F	0.01%	0.00%	0.007	**[Pedosphaerae]**	0.08%	0.03%	0.044
Fibrobacteria	0.05%	0.00%	0.007	**Verruco-5**	0.05%	0.00%	0.045
Gemm-1	0.06%	0.00%	0.008	**(Firmicutes) Other**	0.07%	0.02%	0.046

Higher overall OTU richness was observed in water samples compared to oyster samples. With a sampling depth of 34,082 sequences, 8,396 OTUs were observed in water samples, while 7,757 OTUs were observed at a sampling depth of

35,710 sequences in extrapallial fluid. However, based on alpha diversity calculations generated by EstimateS, bacterial communities of oyster extrapallial fluid were more diverse than those of the surrounding water column (Shannon Index). While there were more calculated individuals in oyster extrapallial fluid samples in total, at the greatest sampling depth of water samples (34,802 sequences), extrapallial fluid communities still exhibited a mean Shannon index of 6.15 (33,329 sequences) while that of water was 5.73 (Figure 5).

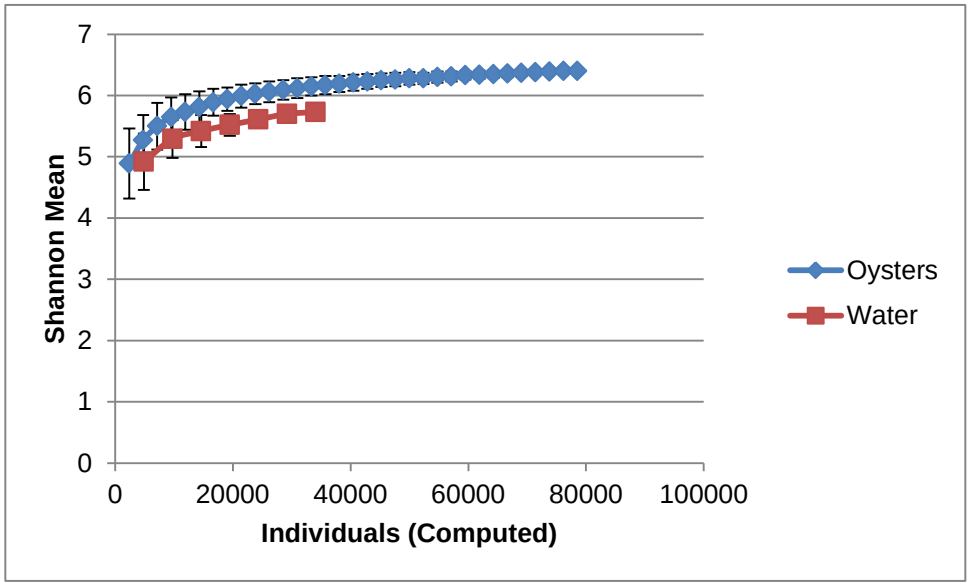

Figure 5 Shannon Index of Diversity of all bacterial OTUs of oyster extrapallial fluid and water from the Choptank River (Cambridge, MD).

Biogeography

The average relative abundance of bacterial classes of oysters at each specific site varied depending on the sample site, yet there were no trends in changes of relative abundance moving downstream showing significant correlation. In the

Choptank River, Alphaproteobacteria decreased by a net 8.21% in relative abundance moving downstream, beginning at 28.34% in Kirby oysters, slowly decreasing to 20.13% in Howell's Point oysters. Additionally, Synechococcophycideae relative abundance increased dramatically from 10.15% in Kirby oysters to 20.21% in Howell's Point oysters, increasing by a net 9.87% (Figure 6). When taking into account oyster samples from the more distant Rhode River, the relative abundance of 27 classes were significantly different than of those in samples from the Choptank River. Notable groups included in these significant classes were Synechococcophycideae ($p = 0.004$), Actinobacteria ($p = 0.007$), Dehalococcoidetes ($p = 0.032$), and Acidimicrobiia ($p = 0.032$).

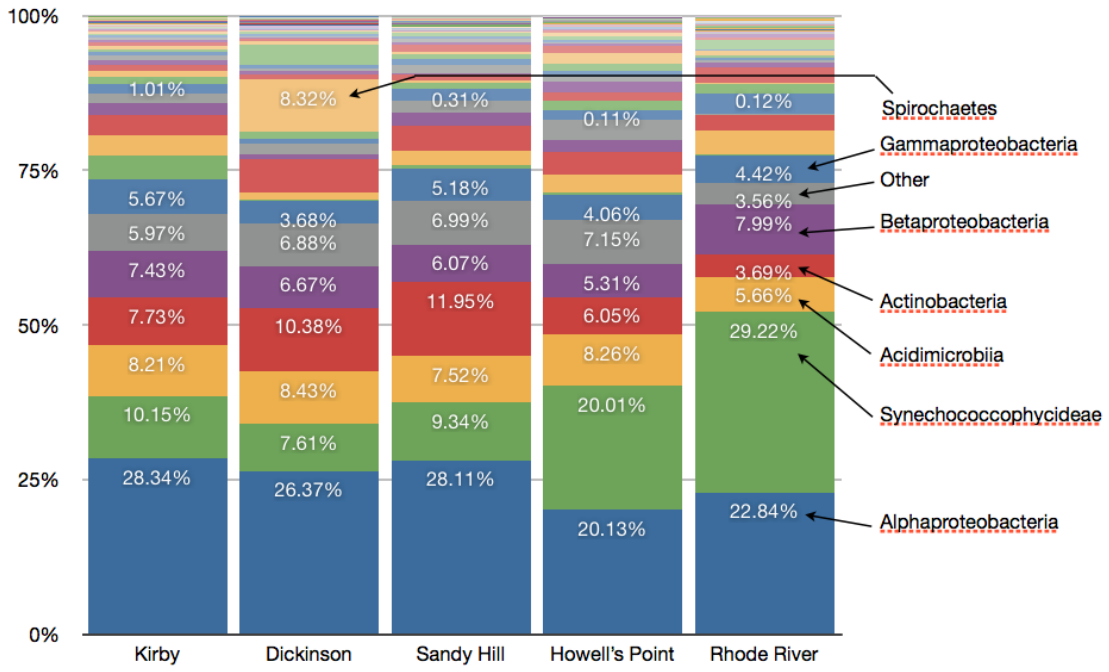

Figure 6 Relative abundance of bacterial classes from oyster extrapallial fluid from oysters at separate oyster bars in the Choptank River (Cambridge, MD), and a sampling site at the Smithsonian Environmental Research Center (Edgewater, MD). 27 classes were observed to be significantly different in relative abundance between Choptank River and Rhode River oysters. Community composition of oysters are location-specific, and statistically significantly different from each other (ANOSIM; $p = 0.019$)

According to Analysis of Similarity (ANOSIM) conducted on weighted UniFrac distance between samples from the Choptank River, the bacterial community composition of oysters from a given bar significantly differs from oysters at the others ($R = 0.101$; $p = 0.019$). Based on rarefied Shannon Index estimations of diversity on a site-by-site basis, it is apparent that bacterial communities are most diverse in oysters at Kirby bar, and least diverse in the water at the same location. Among oyster samples, there is no downstream trend in diversity of bacterial communities, supported by non-adjacent sites having more similar indeces of diversity than adjacent sites in all

cases. However, in water samples, diversity was highest in the most downstream site sampled for water (Sandy Hill) and lowest in the most upstream site (Kirby) (Figure 7).

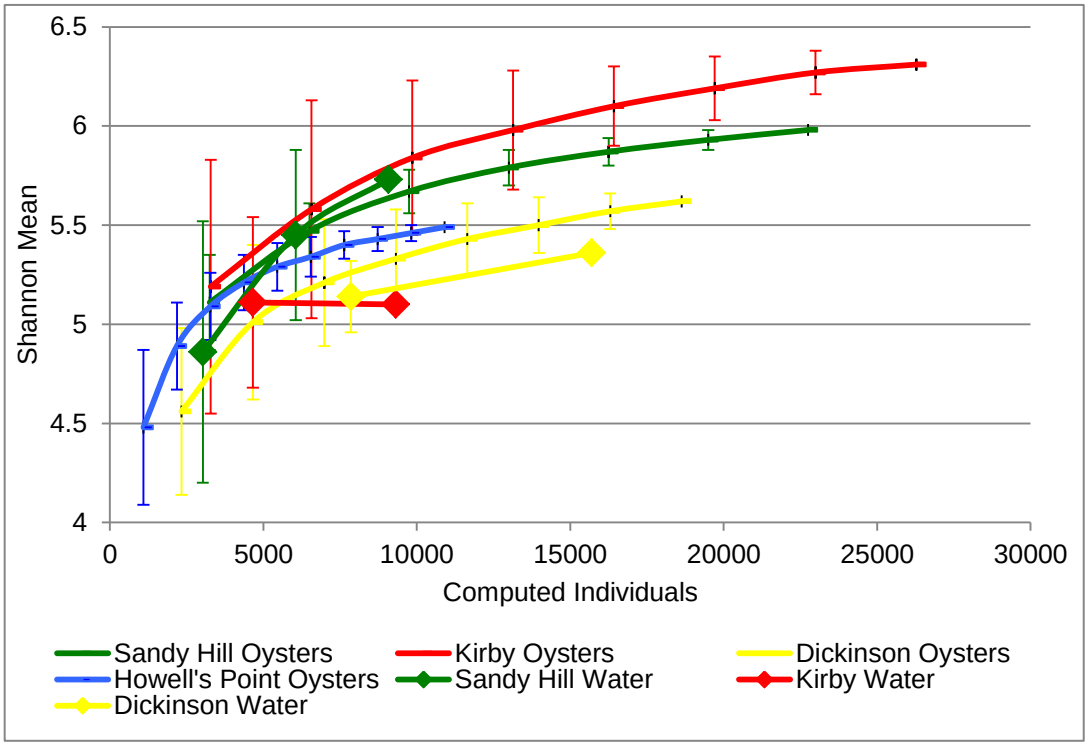

Figure 7 Shannon Index of diversity of bacterial OTUs from oyster extrapallial fluid and water samples from 4 sites along the Choptank River.

After incorporation of oyster samples from the more distant Rhode River, these location-specific differences remain significantly different ($R = 0.184$; $p = 0.005$). Based on enrichment analysis using LDA Effect Size, there are a number of bacterial groups that are significantly enriched (Kruskall-Wallis Test; $p < 0.05$) on a location-specific basis. At Kirby bar, the families Bacteriovoracaceae and Francisellaceae were

both significantly enriched. The ACK-M1 group of Actinobacteria were the only enriched group from oysters at Dickinson Bar. At Sandy Hill, a number of groups from the phyla Actinobacteria, OD1, and Planctomycetes were significantly enriched, as well as specialized groups such as Sulfurimonas and Cryobacteria. Members of the phylum Clostridia, as well as the genus Nitrospira were significantly enriched in Howell's Point oysters. Rhode River oysters, being the most geographically isolated, had the broadest degree of taxon enrichment, including many groups of Cyanobacteria, Chlorobi, Sphingobacteria, TM7 and the class Deltaproteobacteria (Figure 8).

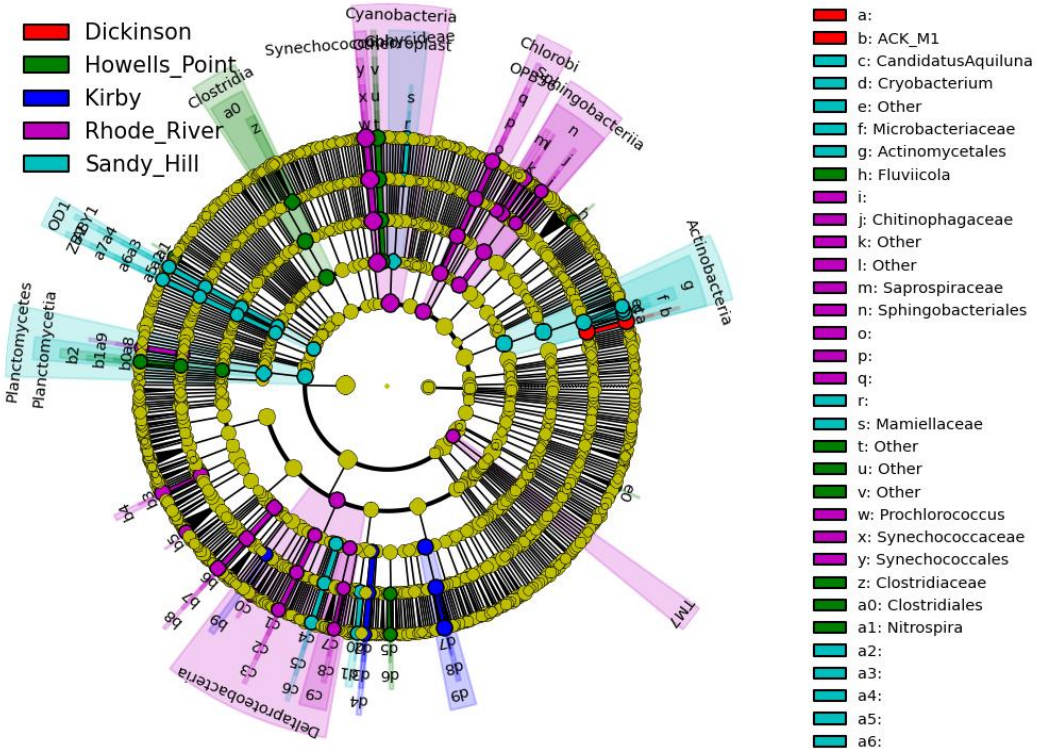

Figure 8 LDA enrichment cladogram highlighting bacterial groups significantly enriched (Kruskall-Wallis Test; $p < 0.05$), hierarchically on a sample site-specific basis.

The geographic distance between two communities was compared to how similar the communities were (1 – weighted UniFrac distance), and the results were plotted for all pairs of communities of each treatment (water and extrapallial fluid). According to the Mantel test, geographic distance did not significantly relate to community dissimilarity in Choptank oysters ($R = -0.003$; $p = 0.98$) or water samples ($R = 0.368$; $p = 0.10$). However, geography did significantly relate to community dissimilarity between Choptank River and Rhode River samples in both oysters ($R = 0.63$; $p = 0.01$) and water ($R = 0.795$; $p = 0.01$).

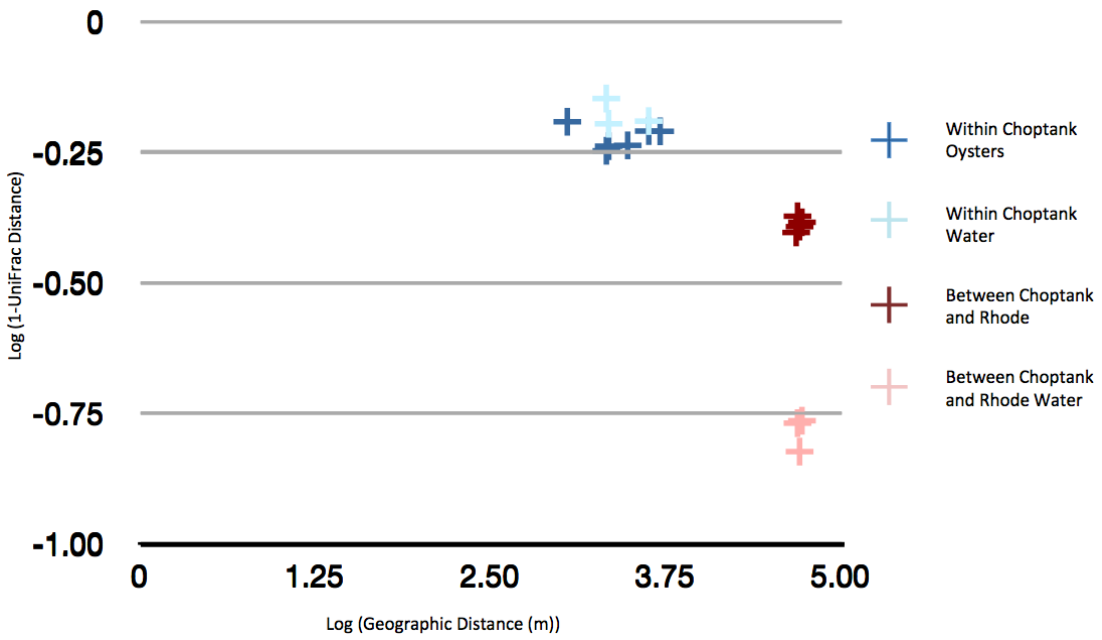

Figure 9 Distance-decay relationship of geographic distance (x) with community similarity (y). All pairwise comparisons of Choptank River sample sites, (both water and oyster samples) were made, as well as between Choptank sites and Rhode sites (water and oyster).

Chapter 4

DISCUSSION

Direct Counts

Epifluorescence microscopy direct count data supports a slightly higher bacterial density in extrapallial fluid than in water, despite only one significant difference between these environments at Kirby bar ($p = 0.05$). The amount by which communities were more dense in extrapallial fluid than by the surrounding water varied widely based on site, with Dickinson bar even showing higher bacterial abundance in water compared to the extrapallial fluid. Both Kirby and Sandy Hill bars had significantly higher bacterial abundance than Dickinson bar, while Howell's Point bar was not significantly different from any. Based on these observations, geography plays little role in determining microbial density of oyster extrapallial fluid, probably due to the relative uniformity of microbial density in the water (the assumed source of colonizing bacteria) observed in the Choptank River at the time of sampling.

16S rRNA Community Profiling

Higher OTU richness was observed in water samples compared to all oyster extrapallial fluid, while the greatest diversity was observed in oysters as a whole compared to the surrounding water column. These findings support the presence of a relationship between rare bacterial OTUs in the water column and a commensal role in oysters, since bacterial density is apparently generally lower in water than in oysters. While these bacteria are maintained at low numbers in the water, it is possible that

post-colonization of the oyster extrapallial fluid, they are allowed to grow in number. A number of OTUs significantly more abundant in oyster extrapallial fluid than in the water have been shown to be associated with organismal microflora. For example, OTUs within the class Bacteroidia, composing 0.84% of extrapallial fluid communities are tightly associated with mucus linings. Due to these characteristics, they are frequently cited as sources of opportunistic infections in the upper respiratory tract and mouth of humans (Falagas & Siakavellas 2000). Such a group would not flourish in an aquatic environment; the anaerobic, nutrient rich fluid of oysters could facilitate a colonization event of such bacteria. Another group of bacteria significantly enriched in oyster extrapallial fluid compared to the water was Cyanobacteria. While Synechococcophycideae were not statistically significantly different between extrapallial fluid and water, *Prochlorococcus* composed 13.62% of the extrapallial fluid community, compared to 1.38% in the surrounding water column. This is an unexpected result, as Cyanobacteria are a well-characterized aquatic, photosynthetic group, and should normally compose a major portion of an estuarine water community. However, based on annual data collected by a colleague, a large accumulation of Cyanobacteria in oysters is normal during this sampling time period (Sakowski [unpublished]). Three classes witin the phylum OD1 were significantly enriched in oyster extrapallial fluid. Recent findings have indicated that this group may play a role in sulfur reduction in relatively anoxic, sulfur-rich environments, based on whole-genome analysis (Wrighton et. al. 2012). Enrichment of this phylum in oyster extrapallial fluid would align with these findings, as oysters often form bars in dense, benthic, sulfur-rich sediment (Visel) and may indicate that oysters house active sulfur-reducing bacteria in small concentrations.

The most abundant groups in the water column were composed of Alphaproteobacteria (mainly Pelagibacter and other Rickettsiales), Actinobacteria (ACK-M1 (15.72%)), and Flavobacteria. This is expected as Alphaproteobacteria and Flavobacteria (along with Beta- and Gammaproteobacteria) often compose major proportions of aquatic microbial communities, having been observed in high abundance in rivers (Kenzaka et. al. 1998), as well as particulate detritus (DeLong et. al.1993). Observations from data in Choptank River are reinforced by these findings. Actinobacteria (specifically in the ACK-M1 family (15.72%)) were greatly enriched in water samples as well. ACK-M1 relative abundance is reported in oligosaline (salinity ~ 0.5) bodies of water (Wu et. al. 2006), which aligns with the salinities observed in the Choptank River at sampling time (~0.6-0.7).

The community composition of oyster extrapallial fluid, as well as the surrounding water sampled at different oyster bars along the Choptank river, exhibited varying degrees of fluctuation. Oysters from all Choptank River sampling sites exhibited significantly different community structure from the others ($R = 0.101$; $p = 0.019$), and varying levels of Shannon diversity (Figure 7). While diversity is highest (Kirby and Sandy Hill bars) and lowest (Dickinson and Howell's Point) in oysters from non-adjacent populations, there is a slight trend in communities of the surrounding water to increase in diversity moving downstream. Furthermore, the degree to which communities in oysters are more diverse than the surrounding water column at given site exhibits a slight downstream trend; Kirby oyster bacterial communities are far more diverse than water, Dickinson oyster bacterial communities are much closer in diversity to those of the water, while the diversity in the water at Sandy Hill exceeds that in oyster extrapallial fluid. Based on these observations, it is

possible that diversity may fluctuate more widely in the water surrounding oyster bars, and while certain aspects fluctuate, bacterial diversity remains more constant in extrapallial fluid of oysters. It is important to consider that Shannon diversity was calculated based on sequence sampling depth from each site, of which there is apparent disparity in water samples compared to oyster for most sites. Therefore, sampling depth may be a confounding variable to be considered in comparing diversity estimates between treatments at discrete sampling sites.

Downstream trends in relative abundance of certain OTUs were observed in some cases in the Choptank River, particularly in Synechococcophycideae (mainly *Prochlorococcus*). Cyanobacteria relative abundance tended to increase moving closer to the mouth of the river, beginning at 10.16% at Kirby, increasing to 20.01% at Howell's Point (Figure 6). However, compared to oysters sampled from the Rhode River, a more northern estuary on the western side of the Chesapeake Bay, even the highest observed abundance of Synechococcophycideae in the Choptank River was much lower than those of the Rhode River (29.22%). Interestingly, relatively high abundance of Cyanobacteria (31.70%) was observed in water samples from Rhode River. Based on these results, relative abundance of Synechococcophycideae could negatively correlate with salinity, since salinity was much lower in the Rhode River water (0.41) than in the Choptank (0.62-0.67). Another possibility is that, because water was collected at the surface at the Rhode River site, while bottom water was collected using a Niskin bottle at Choptank sites, Rhode River water samples actually represent a different depth profile of the water column than Choptank River samples. Based on recent data collected by Fortunato and colleagues, the former explanation is most likely, as *Prochlorococcus* has been labeled as an indicator genus in epipelagic

enivronments, and weakly correlated with salinity and dissolved oxygen (Fortunato et. al. 2013).

Since environmental conditions such as salinity and temperature were so similar between Choptank sites, it is likely that any enrichment unique to a particular sample site is either transient or due to environmental fluctuations not quantified in this study, such as dissolved oxygen, available nutrients (nitrogen, phosphorus, potassium, sulfur), turbidity, or dissolved organic carbon. For example, according to LEfSe analysis (Figure 8), certain OTUs of the phylum OD1 and Actinobacteria were significantly enriched at Sandy Hill bar. Knowing that OD1 abundance could indicate active sulfur metabolism (Wrighton et. al. 2012), it is possible that sediment at this site was particularly sulfur-rich, resulting in increased levels of these OTUs relative to other classes. Additionally, numerous members of Deltaproteobacteria were significantly enriched in Rhode River oysters, including members of the family Syntrophaceae. Studies have implicated important roles of Deltaproteobacteria in the degradation of alkanes in partnership with methanogenic bacteria (Cheng et. al. 2013 (44 & 45 in References)), exhibiting syntrophic activity. The presence of this group in high abundance in oysters may implicate remarkable roles of Deltaproteobacteria community members, allowing the oyster to potentially cope with potentially toxic chemicals to a small extent. Under conditions at the Rhode River, this group was able to flourish to greater extent than in Choptank oysters. One potential reason for this may be the proximity of the sampling site on the Rhode River to a parking lot; this potential source of diverse organic compounds could facilitate increased syntrophic demand in extrapallial fluid where compounds may accumulate to a greater density than in the water column.

Alternatively, Actinobacteria such as *Candidatus aquiluna* were also enriched in Sandy Hill oysters. Phylogenetic analysis of this species indicates close relationships with other groups of freshwater Actinobacteria, and 3 out of 4 strains cultured were isolated from freshwater habitats (with the other from seawater) (Hahn 2009). Transient water intrusion into the pallial space of the oysters at this site could explain enrichment of a typically freshwater bacterioplankton species in oysters. The same phenomena could also explain enrichment of ACK-M1 in Dickinson bar oysters, since ACK-M1 is a well-characterized member of oligosaline water communities (Wu et. al. 2006).

A challenge in identifying biogeographical relationships with microbial community structure is the cosmopolitan distribution of many groups of bacteria, and the immense role environmental conditions in niches plays in determining the effective community structure of microhabitats. One way to begin to determine the drivers of differential community structure, such as dispersal history and environmental heterogeneity, between geographic locations is to evaluate the degree of a distance-decay relationship of microbial community similarity (Green & Bohannon, 2006). Such a relationship appears to exist both in oyster extrapallial fluid and water samples (Figure 9), but not within such a small distance as 2-5 km. Due to the essential nonexistence of a community structure distance-decay relationship within the Choptank River, environmental heterogeneity within the Choptank River is not significant enough to cause large fluctuations in bacterial community structure. However, when comparing Rhode River samples to Choptank River samples, the relationship changes dramatically; a distance close to 50 km causes significant changes in community structure of both oyster extrapallial fluid and water. One reason

for this may be a larger dispersal barrier between the two bodies of water (i.e. the Chesapeake Bay), resulting in decreased likelihood of gene flow between the two sites. Another possibility, which has already been discussed as a contributing factor to differences in relative abundance of specific phyla between locations, is environmental variability. Distance matrices generated both salinity and temperature metadata categories were compared to differences in weighted UniFrac distances and were also significantly correlated to community differences in both water (Salinity: $R = 0.791$; $p = 0.01$, Temperature: $R = 0.803$, $p = 0.03$) and oyster (Salinity: $R = 0.539$; $p = 0.01$, Temperature: $R = 0.504$; $p = 0.01$). It is quickly apparent that such large distances create differences in microbial communities, the causes of which are diverse and vary in influence. However, geographic distance, temperature, and salinity all affect the degree to which oyster extrapallial fluid communities differ between locations to a lesser extent than in water communities. What this may indicate is a true conservation of some elements of a distinct community composition in oysters compared to the water column.

Chapter 5

CONCLUSIONS

Oyster extrapallial fluid communities exhibit many important differences from the communities in the surrounding water column. Water communities exhibit a higher degree of OTU richness, the rarer members of which possibly intrude the pallial space and are allowed to flourish due to more diverse and concentrated nutrient sources. The relative abundance of certain OTUs within oyster extrapallial fluid helps to confirm this phenomenon, such as members of the phylum Bacteroidia, various classes of OD1, and ACK-M1 actinomycetes. Additionally, oyster extrapallial fluid bacterial communities appear somewhat unique to the sampling location, but the degree to which they differ is insignificant over distances of less than 5 km. Furthermore, many taxa were enriched in oyster extrapallial fluid on a location-specific basis, but these increases are likely due to transient environmental variations favoring increased abundance of small groups and not due to dispersal limitations on geographic basis. Significant differences in community similarity were correlated well with geographic distance, temperature, and salinity in both oysters and water from the Choptank River and Rhode River. These factors influence community changes in water to a far greater extent than in oyster extrapallial fluid, suggesting conservation of a core community in oyster pallial fluid, and overall community structure subject to environmental fluctuations to a smaller extent.

REFERENCES

1. T.C. Visel. Life History of The American or Eastern Oyster *Crassostrea virginica*. The Sound School Regional Vocational Aquaculture Center.

2. R.F. Dame, T.G. Wolaver, S.M. Libes. 1985. The summer uptake and release of nitrogen by an intertidal oyster reef. *Netherlands Journal of Sea Research*. 19:265-8.

3. A.R. Smyth, S.P. Thompson, K.N. Siporin, W.S. Gardner, M.J. McCarthy, M.F. Piehler. 2013. Assessing nitrogen dynamics throughout the estuarine landscape. *Estuaries and Coasts*. 36:44-55.

4. A.R. Smyth, N.R. Geraldi, M.F. Piehler. 2013. Oyster-mediated benthic-pelagic coupling modifies nitrogen pools and processes. *Marine Ecology Progress Series*. 493:23-30.

5. H.D. Harwell, P.R. Kingsley-Smith, M.L. Kellogg, S.M. Allen, S.K. Allen, D.W. Meritt, K.T. Paynter, Jr., M.W. Luckenbach. 2010. A Comparison of *Crassostrea virginica* and *Crassostrea ariakensis* in Chesapeake Bay: Does oyster species affect habitat function? *Journal of Shellfish Research*. 29:253-269.

6. P.S.E. zu Ermgassen et. al. . 2012. Historical ecology with real numbers: past and present extent and biomass of an imperiled estuarine habitat. *Proc. R. Soc.* B. 279:3393-3400.

7. P.S.E. zu Ermgassen, M.D. Spalding, R.E. Grizzle, R.D. Brumbaugh. 2013. Quantifying the loss of a marine ecosystem service: filtration by the eastern oyster in US estuaries. *Estuaries and Coasts*. 36:36-43.

8. R. Smolowitz. 2013. A review of current state of knowledge concerning *Perkinsus marinus* effects on (Gmelin)(the Eastern Oyster). *Veterinary Pathology*. 50:404-411.

9. M. Penna, M. Khan, R.A. French. 2001. Development of a multiplex PCR for the detection of *Haplosporidium nelsoni*, *Haplosporidium costale* and *Perkinsus marinus* in the eastern oyster (*Crassostrea virginica*, Gmelin, 1971). *Molecular and Cellular Probes*. 15:385-390.

10. D.L. Kirchman (*Editor*). 2000. *Microbial ecology of the oceans*. Wiley-Liss, Inc., New York.

11. F. Azam. 1998. Microbial control of oceanic carbon flux: the plot thickens. *Science*. 280:694-6.

12. C.R. Woese, G.E. Fox. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *PNAS*. 74:5088-5090.

13. A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F.O. Glockner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*. 41:1-11.

14. J.A. Klappenbach, P.R. Saxman, J.R. Cole, T.M. Schmidt. 2001. rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Research*. 29:181-4.

15. J. Green, B.J.M. Bohannan. 2006. Spatial scaling of microbial biodiversity. *TRENDS in Evolution and Ecology*. 21:501-7.

16. T. Fenchel, B.J. Finlay. 2004. The ubiquity of small species: patterns of local and global diversity. *BioScience*. 54:777-784.

17. J.C. Cho, J.M. Tiedje. 2000. Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. *Applied and Environmental Microbiology*. 66:5448-5456.

18. I. Reche, E. Pulido-Villena, R. Morales-Baquero, E.O. Casamayor. 2005. Does ecosystem size determine aquatic bacterial richness? *Ecology*. 86:1715-1722.

19. H. Antoun, C.J. Beauchamp, N. Goussard, R. Chabot, R. Lalande. 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (*Raphanus sativus* L.). *Plant and Soil*. 204:57-67.

20. K. Kurokawa, T. Itoh, T. Kuwahara, K. Oshima, H. Toh, A. Toyoda, H.Takami, H. Morita, V.K. Sharma, T.P. Srivastava, T.D. Taylor, H. Noguchi, H. Mori, Y.Ogura, D.S. Ehrlich, K. Itoh, T.Takagi, Y. Sakaki, T. Hayashi, and M. Hattori. 2007. Comparative Metagenomics Revealed Commonly Enriched Gene Sets in Human Gut Microbiomes. *DNA Res*. 14:169:181.

21. K. Aagaard, K. Riehle, J. Ma, N. Segata, T-A. Mistretta. C. Coarfa. S. Raza, S. Rosenbaum, I. Van den Veyver, A. Milosavljevic, D. Gevers, C. Huttenhower, J. Petrosino, J. Versalovic. 2012. A Metagenomic Approach to Characterization of the Vaginal Microbiome Signature in Pregnancy. *PLoS ONE*. 7: e36466.

22. Ravel, J. et. al. . 2010. Vaginal microbiome of reproductive-age women. *PNAS USA Early Edition*. DOI:10.1073/pnas.1002611107.

23. C. Paillard, F. Le Roux, J.J. Borrego. 2004. Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution. *Aquatic Living Resources.* 17:477-498.

24. E. Bachere. 2003. Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. *Aquaculture*. 227:427-438.

25. R.R. Colwell, J. Liston. 1960. Bacteriological study of the natural flora of pacific oysters (*Crassostrea gigas*). *Microbiology of Shellfish*. 8:104-9.

26. F. Antunes, M. Hinzmann, M. Lopes-Lima, J. Machado, P. Martins da Costa. 2010. Association between environmental microbiota and indigenous bacteria found in hemolymph, extrapallial fluid and mucus of *Anodonta cygnea* (Linnaeus, 1758). *Microbial Ecology*. 60:304-309.

27. I.A. Beleneva, N.V. Zhukova, and E.F. Maslennikova. 2003. Comparative study of microbial communities from cultured and natural populations of the mussel *Mytilus trossulus* in Peter the Great Bay. *Microbiology*. 72:472-7.

28. K.J. La Valley, S. Jones, M. Gomez-Chiarri, J. Dealteris, M. Rice. 2009. Bacterial community profiling of the eastern oyster (*Crassostrea virginica*): comparison of culture-dependent and culture-independent outcomes. *Journal of Shellfish Research*. 28:827-835.

29. D.G. Bourne, P.G. Dennis, S. Uthicke, R.M. Soo, G.W. Tyson, N. Webster. 2013. Coral reef invertebrate microbiomes correlate with the presence of photosymbionts. *The ISME Journal*.7:1452-8.

30. K. Karlinska-Batres, G. Worheide. 2013. Microbial diversity in the coralline sponge *Vaceletia crypta*. *Antonie van Leeuwenhoek.* 103:1041-1056.

31. G. Hernandez-Zarate, J. Olmos-Soto. 2006. Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent *in situ* hybridization and polymerase chain reaction. *Journal of Applied Microbiology*. 100:664-672.

32. M. Tarnowski (ed.). 2010. Maryland Oyster Population Status Report: 2009 Fall Survey. MDNR Publication No. 17-8172010-471.

33. J.G. Caporaso et. al. . 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 7:335-6.

34. A. Chao, R. L. Chazdon, R. K. Colwell, and T.-J. Shen. 2005. A new statistical approach for assessing compositional similarity based on incidence and abundance data. *Ecology Letters.* **8**:148-159.

35. J. Goecks, A. Nekrutenko,J. Taylor and The Galaxy Team. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol*. 11:R86.

36. D. Blankenberg, G. Von Kuster, N. Coraor, G. Ananda, M. Mangan, A. Nekrutenko, J. Taylor. Galaxy: a web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology*. 19(19.10):1-21.

37. B. Giardine et. al. . 2005. Galaxy: a platform for interactive large-scale genome analysis. *Genome Research*. 15:1451.

38. M.E. Falagas & E. Siakavellas. 2000. Review: *Bacteroides, Prevotella,* and *Porphyromonas* species: a review of antibiotic resistance and therapeutic options. *International Journal of Antimicrobial Agents.* 15:1-9.

39. K.C. Wrighton et. al. . 2012. Fermenation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla. *Science.* 337:1661-5.

40. T. Kenzaka, N. Yamaguchi, K. Tani, M. Nasu. 1998. rRNA-targeted in situ hybridization analysis of bacterial community structure in river water. *Microbiology (UK).* 144:2085-2093.

41. E.F. DeLong, D.G. Franks, A.L. Alldredge. 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine microbial assembly. *Limnology and Oceanography.* 38:924-934.

42. Q.L. Wu, G. Zwart, M. Schauer, M.P. Kamst-van Agterveld, M.W. Hahn. 2006. Bacterioplankton community composition along a salinity gradient of sixteen high-mountain lakes located on the Tibetan plateau, China. *Applied and Environmental Microbiology.* 72:5478-5485.

43. C.S. Fortunato, A. Eiler, L. Herfort, J.A. Needoba, T.D. Peterson, B. Crump. 2013. Determining indicator taxa across spatial and seasonal gradients in the Columbia River coastal margin. *ISME Journal*. Pg. 1-13.

44. M.W. Hahn. 2009. Description of seven candidate species affiliated with the phylum Actinobacteria, representing planktonic freshwater bacteria. *International Journal of Systematic and Evolutionary Microbiology.* 59:112-117.

45. L. Cheng, C. Ding, Q. Li, Q. He, L. Dai, H. Zhang. 2013. DNA-SIP reveals that Syntrophaceae play an important role methanogenic hexadecane degradation. *PLOS One*. 8:e66784.

46. L. Cheng, J. Rui, Q. Li, H. Zhang, Y. Lu. 2013. Enrichment and dynamics of novel syntrophs in a methanogenic hexadecane-degrading culture from a Chinese oilfield. *FEMS Microbiology Ecology.* 83:757-766.

Appendix

Environmental Metadata

Sample Name	Sample Type	Bar	Salinity (ppt)	Latitude	Longitude	Depth (ft)	Fluid Volume (mL)	Shell Length (mm)	Bacterial Count (per mL)
Oyster_1	Oyster	Kirby	6.2	38.5956	-76.0701	16	2.6	107	4.01E+07
Oyster_2	Oyster	Kirby	6.2	38.5956	-76.0701	16	2	82	2.61E+07
Oyster_3	Oyster	Kirby	6.2	38.5956	-76.0701	16	4.5	70	
Oyster_4	Oyster	Kirby	6.2	38.5956	-76.0701	16	2	100	3.26E+07
Oyster_5	Oyster	Kirby	6.2	38.5956	-76.0701	16	4.5	95	7.25E+06
Oyster_7	Oyster	Kirby	6.2	38.5956	-76.0701	16	3.3	87	1.47E+07
Oyster_8	Oyster	Kirby	6.2	38.5956	-76.0701	16	5	106	
Oyster_9	Oyster	Kirby	6.2	38.5956	-76.0701	16	4.2	110	1.78E+06
Water_1A	Water	Kirby	6.2	38.5956	-76.0701	16	0	0	5.12E+06
Water_1B	Water	Kirby	6.2	38.5956	-76.0701	16	0	0	4.27E+06
Oyster_11	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	5	105	3.84E+06
Oyster_12	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	5	120	1.59E+05
Oyster_14	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	5	110	6.40E+06
Oyster_15	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	2.75	115	2.43E+06
Oyster_16	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	5	95	4.84E+06
Oyster_17	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	4.5	107	1.19E+06
Oyster_19	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	5	116	1.14E+07
Oyster_20	Oyster	Dickinson	6.3	38.60215	-76.0927	7-12	5	115	7.14E+05
Water_2A	Water	Dickinson	6.3	38.60215	-76.0927	7-12	0	0	5.55E+06
Water_2B	Water	Dickinson	6.3	38.60215	-76.0927	7-12	0	0	6.83E+06
Oyster_21	Oyster	Sandy Hill	6.6	38.602	-76.117733	30	2.4	85	1.54E+07
Oyster_22	Oyster	Sandy Hill	6.6	38.602	-76.117733	30	4.2	117	5.97E+06
Oyster_23	Oyster	Sandy Hill	6.6	38.602	-76.117733	30	3.6	105	1.37E+07
Oyster_24	Oyster	Sandy Hill	6.6	38.602	-76.117733	30	3.3	112	1.43E+07
Oyster_25	Oyster	Sandy Hill	6.6	38.602	-76.117733	30	2.9	80	5.22E+06
Oyster_27	Oyster	Sandy Hill	6.6	38.602	-76.117733	30	5	103	3.31E+07
Oyster_28	Oyster	Sandy Hill	6.6	38.602	-76.117733	30	3.7	105	3.92E+07
Water_3A	Water	Sandy Hill	6.6	38.602	-76.117733	30	0	0	6.40E+06

Sample Name	Sample Type	Bar	Salinity (ppt)	Latitude	Longitude	Depth (ft)	Fluid Volume (mL)	Shell Length (mm)	Bacterial Count (per mL)
Water_3B	Water	Sandy Hill	6.6	38.602	-76.117733	30	0	0	6.40E+06
Water_3B_2	Water	Sandy Hill	6.6	38.602	-76.117733	30	0	0	3.41E+06
Oyster_31	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	4.6	115	1.60E+07
Oyster_33	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	4.2	137	5.22E+06
Oyster_34_1	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	3.5	110	1.59E+06
Oyster_34_2	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	3.5	110	1.59E+06
Oyster_35	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	4	100	1.55E+07
Oyster_37	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	3.8	85	1.39E+07
Oyster_38_1	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	1.5	86	4.45E+06
Oyster_38_2	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	1.5	86	4.45E+06
Oyster_39	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	5	101	1.81E+04
Oyster_40	Oyster	Howell's Point	6.7	38.609917	-76.125417	12	3.4	94	2.44E+07
June_Oyster1	Oyster	Rhode River	4.11	38.887273	-76.539566	5			1.28E+07
June_Oyster2	Oyster	Rhode River	4.11	38.887273	-76.539566	5			2.94E+07
June_Oyster3	Oyster	Rhode River	4.11	38.887273	-76.539566	5			2.30E+07
June_Water1	Water	Rhode River	4.11	38.887273	-76.539566	5			1.04E+07
June_Water2	Water	Rhode River	4.11	38.887273	-76.539566	5			5.42E+06