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Polycyclic Aromatic Hydrocarbons and the Microbiomes of Two Benthic Species

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POLYCYCLIC AROMATIC HYDROCARBONS AND THE MICROBIOMES OF TWO BENTHIC SPECIES

by

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ABSTRACT

The presence of oil has been shown to affect the microbiomes of the water column, sediments, and organisms, both by altering the diversity and the composition of those microbial communities. If the microbiome is altered it may no longer provide benefits to its host organism, impacting its ability to survive. Thus, it is important to understand the effects of large-scale contamination events including the *Deepwater Horizon* oil spill. This study set out to examine the effects of oil exposure on the microbiome of two benthic species, southern flounder (*Paralichthys lethostigma*) and eastern oysters (*Crassostrea virginica*) to further understand the effects of both short- and long-term oil contamination. The bacterial component of the microbiomes of the two species were analyzed using Next-Generation 16S Sequencing, focusing on diversity and taxonomic analysis of the bacteria present within the samples. Flounder were exposed to oiled sediments as well as a pathogenic bacteria (*Vibrio anguillarum*) challenge in a 17-day fully factorial laboratory experiment. Oysters were collected from reefs across the Mississippi Sound and the concentration of polycyclic aromatic hydrocarbons (PAHs) within the oyster tissue, shell and nearby sediment were measured. Analysis of the flounder microbiome showed significant responses to both the oil and pathogen exposure, but those responses differ between gill and intestine microbiomes. The oyster digestive gland microbiome showed no effect of the measured concentrations of PAHs in either shell, tissue, or nearby sediment. Reef location also showed no effect on the diversity or composition of the oyster microbiome.

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LIST OF ABBREVIATIONS

AHR	Aryl Hydrocarbon Receptor
AIP	Aryl Hydrocarbon Receptor Interacting Protein
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASVs	Amplicon Sequence Variants
ASW	Artificial Seawater
BTE	Basic Transcription Element
CFU	Colony Forming Unit
CYP	Cytochrome P450
GC/MS-SIM	Selective Ion Monitoring Gas Chromatography-Mass Spectrometry
GCRL	Gulf Coast Research Laboratory
GOM	Gulf of Mexico
HPLC	High Performance Liquid Chromatography
OTUs	Operational Taxonomic Units
PAHs	Polycyclic Aromatic Hydrocarbons
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutation Analysis of Variance
POPs	Persistent Organic Pollutants
SFAs	Short-chain Fatty Acids

SSU	Small Sub-Unit
USM	The University of Southern Mississippi
WAF	Water Accommodated Fraction
XRE	Xenobiotic Responsive Element

CHAPTER I - INTRODUCTION

Oil Contamination

The northern Gulf of Mexico has a long history of oil production and processing and is a major producer of oil and gas in the United States (Overton et al. 2004). Today the Gulf of Mexico accounts for 17% of the total U.S. offshore crude oil production out of 1,862 platforms and is responsible for over 45% of the nation's refining capacity (www.eia.gov 2020). In addition to anthropogenic actions of oil extraction, this area is characterized by abundant gas hydrate deposits and natural oil seeps that release between 2.5 and 9.4×10^4 m3 of contaminants such as crude oil, methane, carbon dioxide, and hopanes into the environment per year (Figure 1.1, MacDonald et al. 2015)) (Overton et al. 2004, Roberts et al. 2010, Wang, Liu, et al. 2014).

Oil spills are also major acute sources for oil contamination. On April 20, 2010, the well head underneath the Deepwater Horizon drilling rig exploded, leading to an estimated 5 million barrels of oil being released into the waters of the Gulf of Mexico and the deaths of 11 people (McNutt et al. 2011, US EPA 2019). More than 65% of the of oil reached the surface of the water column where it was acted upon by physical processes such as evaporation, UV degradation, or physical forcing that increased dissolution of oil into uncontaminated waters (Ryerson et al. 2012, Liu et al. 2016). Dissolution was further enhanced by the application of 1.4 million gallons of the dispersant COREXIT® 9500A to the oil slick during clean-up efforts (Kujawinski et al. 2011). The oil that did not reach the surface or dissolve into the water became subsurface plumes at depths between 1.0 and 1.3 km (Camilli et al. 2010, Kujawinski et al. 2011, Valentine et al. 2014). Despite this weathering action, large volumes of oil were washed ashore by currents,

contaminating 1,773 km of coastline from Texas to Florida. Heavy oiling conditions resulted in oiled vegetations mats (dead marsh grass covered over by oil) and wrack lines, conditions that persisted a year later (Michel et al. 2013). Clean-up of the marshes and beaches were intensive efforts that required manual raking and cutting of the grasses or intrusive turnover of beach sands (Michel et al. 2013).

Crude oil is a composite made up of many constituents including gaseous alkanes (methane, ethane, and propane), long-chain alkanes (hexane, heptane, octane, and nonane), monocyclic aromatic hydrocarbons (benzene, toluene, ethylbenzene, and xylene, or BTEX), and polycyclic aromatic hydrocarbons (PAHs) (naphthalene, fluorene, anthracene, phenanthrene, pyrene, chrysene, benzo(a)pyrene) which are considered to be the most toxic constituents (Camilli et al. 2010, Reddy et al. 2012, Sammarco et al. 2013, Beyer et al. 2016, Liu et al. 2016). Certain properties of PAHs can be predicted by the number of rings the molecule has, including physical properties such as evaporation rates (and thus risk to organisms in the immediate environment), or the potential toxicity of the molecule (Wang, Liu, et al. 2014, Liu et al. 2016). PAHs can also have different properties depending on their source of origin. Petrogenic PAHs, those originating as coal or crude oil, are more bioavailable than pyrogenic PAHs, or PAHs that are the products of combustion as evidenced by higher biota-sediment-accumulation factors (BSAF), 1.57 ± 0.53 and 0.25 ± 0.23 respectively (Thorsen et al. 2004). Wang et al. (2014) characterized PAHs within Gulf of Mexico sediments from marshes and along the continental shelf according to distribution and source, using composition analysis and ratios of select PAH isomers (Harrison et al. 1996, Yunker et al. 2002, Barakat et al. 2011). Marsh sediments' PAH composition differed significantly from sediments from

the shelf, with marsh sediments characterized by high-molecular weight PAHs while shelf sediments were enriched with low-molecular weight PAHs. Using ratios of PAHs to determine source, marsh contamination primarily is a result of gasoline and diesel combustion while crude oil is a major contributor for shelf sediments (May and Wise 1984, Li and Kamens 1993, Yunker et al. 2002, Wang, Liu, et al. 2014). Thus, contamination sources for the Gulf are not uniform throughout all habitats therefore the bioavailability potential of PAH contaminants is also non-uniform. The overall concentrations of PAHs also differ across the Gulf contributing to the non-uniformity of contamination bioavailability, with marsh sediments containing higher concentrations (229-379 ng/g) than sediments from the shelf (175-244 ng/g) (Wang, Liu, et al. 2014).

Physical conditions of the environment, such as hypoxia, can affect PAH contamination concentrations. Hypoxia reduces bioturbation of sediments by invertebrates and other benthic infauna, as they move away to avoid the low-oxygen conditions or die (Middelburg et al. 2009). The loss of sediment mixing significantly reduces the rate at which organic matter is degraded by bacteria in two ways, less organic matter is available to the microorganisms as fresh particles are not replaced and the products of degradation can build up and alter the reactivity of the remaining organic matter leading to a 50% increase in the preservation of labile particles in sediments (Jessen et al. 2017). Additionally, when oxygen is no longer available as the electron receptor sulfur takes its place, resulting in increased rates of sulfurization and carbon sequestration into sediments (Burdige 2007, Jessen et al. 2017). Studies have shown that PAH concentration within sediments is controlled by the organic matter content and the grain size of the sediment because the molecules strongly adsorb to these particles (Wang

et al. 2001, Wang, Liu, et al. 2014). When hypoxia results in decreased organic matter degradation rates, it also results in decreased PAH degradation both in this biological manner as well as chemically (Landmeyer and Bradley 2003, Li et al. 2017).

Another factor that impacts the concentration of PAHs in sediments is microbial degradation. Sediments and sand from the northern Gulf contain bacteria that degrade hydrocarbons such as *Pseudomonas*, *Cycloclasticus*, and *Colwellia*, especially in the presence of multiple chemical species including pyrene, fluoranthene, phenanthrene, and toluene (Mueller et al. 1990, Geiselbrecht et al. 1998, Kanaly and Harayama 2000, Mason et al. 2014). High sorption properties of PAH increase the length of exposure to benthic organisms, fortunately this also increases the length of time microbial degradation of PAHs in oxygenated sediments can occur thus reducing availability to disrupt organisms and the functionality of these coastal ecosystems (Hinga 2003, Wang, Liu, et al. 2014).

Measurements of PAHs within sediments of the northern Gulf of Mexico show concentrations below biological effects ranges, or concentrations expected to cause a biological effect 10% of the time, suggesting that current background PAH pollution rates have little risk to organisms (Wade et al. 2008, Wang, Liu, et al. 2014). Santschi et al. (2001) measured persistent organic pollutants (POPs) including PAHs in sediments from Galveston, the Mississippi River Delta, and Tampa Bay to be 0.3 µg/g, 0.6 µg/g, and 6.26 µg/g, respectively, concentrations that were consistent with typical uncontaminated Gulf coast sediments. PAHs measured in offshore sediments from Texas to Florida averaged 0.14 mg/kg (140 ng/g) with a maximum measurement of 1 mg/kg

(1033 ng/g), concentrations well below that which is expected to adversely affect the organisms (Wade et al. 2008).

Despite a natural background level of PAH contamination in the northern Gulf system, acute events such as DHOS or more localized spills result in periods of time during which large quantities of contaminants are present, overwhelming what homeostasis mechanisms local organisms may have. One such mechanism involves the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor for target genes, including genes that code for the cytochrome P450 (CYP) family of proteins, that is activated when a xenobiotic, or ligand such as PCBs or PAHs, enters the cell. The ligand binds to chaperone proteins hsp90 and AHR-interacting protein (AIP) and is transported into the nucleus where the chaperone proteins disassociate and the ligand binds to the AHR nuclear translocator (ARNT) protein (Hankinson 1995, Mimura and Fujii-Kuriyama 2003, Billiard et al. 2006, Whitehead et al. 2017). The ligand-ARNT complex then attaches to promoter regions, the xenobiotic response element (XRE) and basic transcription element (BTE), within the DNA to induce transcription of genes involved in developmental signaling and environmental sensing, and subsequently the translation of xenobiotic-metabolizing enzymes, such as CYP1A1 proteins (Fujisawa-Sehara et al. 1987, Fujii-Kuriyama and Mimura 2005, Thompson et al. 2017). However, if the AHR pathway is continuously activated it can result in sustained misregulation of genes involved in cellular homeostasis, which is a mechanism of PAH toxicity (Billiard et al. 2006, Clark et al. 2010, Whitehead et al. 2017).

Many studies have examined the impacts of oil exposure on different organisms, benthic invertebrates to large marine mammals (Fry and Anderson 2014, Landers et al.

2014, Murawski et al. 2014, Schwacke et al. 2014). Exposure of fish to oil results in a multitude of harmful effects, both lethal and sublethal. These effects range from egg production (Whitehead et al. 2012, Hedgpeth and Griffitt 2016), impaired development and embryogenesis (de Soysa et al. 2012, Le Bihanic et al. 2014, Lo et al. 2014), altered cardiac development and function (Brette et al. 2014, Incardona et al. 2014, Vehniäinen et al. 2019), decreased gill function (Whitehead et al. 2012, Brown-Peterson et al. 2015), and impaired immune responses (Bayha et al. 2017, Jones et al. 2017, Rodgers et al. 2018).

Microbes can also be affected by oil exposure. Deep-sea surveys from the area impacted by the Deepwater Horizon oil spill show that following hydrocarbons entering the system the presence of Proteobacteria closely related to known petroleum degraders increased (Hazen et al. 2010). Similarly, within beach sands the presence of oil degraders, such as *Alcanivorax*, *Marinobacter*, and Rhodobacteraceae, increased significantly in oiled sand (Kostka et al. 2011). Other studies examined the effect of organism associated microbiomes. González-Penagos et al. (2020) exposed zebrafish (*Danio rerio*) to crude oil in the form of water accommodated fraction (WAF) and showed that exposure increased both alpha and beta diversity and relative abundance of *Vibrio*, *Flavobacterium*, and *Novosphingobium*, while abundances of *Aeromonas* and *Cetobacterium* were decreased. The microbiome of a larval sponge (*Rhopaleoeides odorabile*) was also affected by oil exposure and exhibited significantly different microbial communities at concentrations as low as 1.7 ug/L, including increased relative abundances of bacterial classes Gammaproteobacteria, Alphaproteobacteria, and

Chloroflexi, and decreases in the abundances of Deltaproteobacteria, Acidobacteria, and Gemmatimonadetes (Luter et al. 2019).

Microbiomes serve as intermediates with contaminants that the host comes into contact with and often provide immediate bioremediation benefits for the host, however these benefits can be impaired when those same contaminants affect the microbes themselves (Brown-Peterson et al. 2015, Adamovsky et al. 2018). The interaction of host and microbiome to oil contamination is complex, the aim of this thesis is to add to the growing understanding of how microbiomes are impacted by oil contamination and what effect that may have on the host organism. I expect to see alterations in the community structure, or beta diversity of microbiomes of the organisms exposed to oil, such as an increase of oil degrading bacteria taking advantage of a new carbon source or pathogenic bacteria as the symbiotic balance of the microbiome is disrupted allowing opportunistic pathogens to increase in abundance. The response of alpha diversity, or richness, of a microbiome seems to depend on the contaminant and the concentrations therein, therefore the microbiomes of the organisms within my experiments may be impaired by the contaminant, thus oil will decrease the richness, or the additional source of carbon will result in an overall increase in richness as the oil-degrading bacteria are able to utilize the added resource and grow.

Flounder

The southern flounder (*Paralichthys lethostigma*) is an estuarine species of flatfish that inhabits the nearshore waters of North Carolina to northern Mexico (Craig et al. 2015). In the U.S., there are two geographically and genetically distinct populations,

one in the Atlantic and the other in the Gulf of Mexico (Corey et al. 2017). The two populations show some physical differences as well, with the Gulf flounder growing faster and to a larger size. Southern flounder juveniles grow to maturity in estuaries then migrate as adults offshore to batch spawn from November to January, multiple spawning events occur during this season (Murua and Saborido-Rey 2003, Corey et al. 2017). The southern flounder represents a major fishery in the both the Atlantic and Gulf of Mexico, with recreational landings weighing in at 700 metric tons per year (Anderson et al. 2012). However, populations of flounder have been declining in U.S. waters potentially due to bycatch pressure, habitat loss, or overfishing (Wenner and Archambault 2005, Anderson et al. 2012). Another contributing factor to flounder population decline may be related to their benthic habits, as this makes them especially sensitive to contaminants in the sediment (Brown-Peterson et al. 2015).

Exposure to oiled sediments produces a number of deleterious effects in flounder, including decreased growth (Moles and Norcross 1998, Brown-Peterson et al. 2015), reduced testosterone (Truscott et al. 1992), metabolic fatigue (Pulster et al. 2017), and altered gene expression tied to DNA damage and other oxidative stress (Dupuy et al. 2014, Sherwood et al. 2019). Observations following the Deepwater Horizon oil spill suggested an increase in lesions on the skin of flounder (Murawski et al. 2014). As a result, studies were performed examining the effect of oil exposure on the flounder immune response. Chronic exposure of juvenile flounder to environmentally relevant levels of contaminated sediments resulted in increased mortality and decreased growth rates, in addition to impaired condition of liver and gill tissues including hepatic intravascular congestion, macrovascular hepatic vacuolation, telangiectasia of the

secondary lamellae, and lamellar epithelial proliferation (Brown-Peterson et al. 2015). In a sister study, flounder were again chronically exposed to contaminated sediments along with a *Vibrio anguillarum* bacterial challenge in order to examine how oil affects susceptibility to pathogens (Bayha et al. 2017). Flounder exhibited significantly increased mortality in the group exposed to both oil and pathogen, reduced antibody presence in blood plasma as well as overall downregulation of genes related to immune response and organism homeostasis. Additionally, oil further affected the microbiome of both gill and intestine tissues, resulting in increased *V. anguillarum* abundance in the microbiome of flounder exposed to oil and pathogen when compared to those with only pathogen exposure (Bayha et al. 2017). Additionally, flounder have been shown to experience immune effects caused by oil exposure, such as suppressed cholesterol biosynthesis and increased activation of T cells and T cell-related processes, even after a 30-day period of recovery (Rodgers et al. 2021). Taken together, the results of these studies show oil-induced immune effects and system-wide impairment of biological processes and energy budgets.

Oysters

The prominent oyster species in the Gulf of Mexico is the eastern oyster (*Crassostrea virginica*). These oysters range geographically from the St. Lawrence River in Canada to the Atlantic coast of Argentina and can be found in areas with low salinity and in intertidal zones that offer protection from predators (Coen and Luckenbach 2000). Oysters build reefs that provide habitat for a diverse community of epibenthic invertebrates and fishes, as well as other services such as sediment and marsh

stabilization that reduces erosion by water due to vertical relief from the reef itself (Breitburg, Coen, Luckenbach, Mann, et al. 2000, Coen et al. 2007, Grabowski et al. 2012). Oyster reefs also improve water quality by removing phytoplankton and other organic material from the water column, sequestering carbon and reducing the potential scope of eutrophication processes and the hypoxic conditions that may result (Breitburg, Coen, Luckenbach, Mann, et al. 2000, zu Ermgassen et al. 2013). Oyster reefs are economically important in addition to their ecological value and have been valued at between US$5,500 to US$99,000 per hectare per year (Grabowski et al. 2012). The Gulf of Mexico produces more than eighty percent of the oysters sold in the United States (Vanderkooy 2012), thus these populations are of particular interest. However, oyster populations in U.S. waters have been declining since the early 1900s – a decline attributed in most part to overfishing – and with recent disasters in the Gulf of Mexico such as Hurricane Katrina and the Deepwater Horizon Oil Spill, the oyster populations found here are having a particularly difficult time recovering (Jackson et al. 2001, Coen et al. 2007, La Peyre et al. 2014, Posadas et al. 2017, Mississippi Department of Marine Resources 2019).

The Deepwater Horizon oil spill occurred in April and continued into July, corresponding with the spawning season of oysters and potentially exposing larvae to contaminants. Exposure to oil negatively affects the fertilization and embryogenesis of oyster larvae (Laramore et al. 2014, Vignier et al. 2017). It has also been shown that oil has negative effects on all larval stages, affecting survivorship, development, and growth (Laramore et al. 2014, Vignier et al. 2015, Finch et al. 2016). Oyster densities and the overall area of oyster habitat were reduced post-spill (Powers, Grabowski, et al. 2017,

Powers, Rouhani, et al. 2017). Work done before the Deepwater Horizon spill showed both lethal and sub-lethal effects of exposure such as reduced growth, increased susceptibility to disease, and, as discussed earlier, impaired reproduction (La Peyre et al. 2014). In contradiction, Dietl and Durham (2016) showed that when compared to oyster shells in the geohistorical record, the Deepwater Horizon oil spill had no effect on the average body size of oysters. Similarly, there was no evidence of assimilation of oil products into the shells of oysters, a trend that can be seen in mussels and barnacles, as well (Carmichael et al. 2012, Fry and Anderson 2014). There seems to be debate over the long-term effects of the oil spill, yet oyster populations in the Mississippi Sound are still in decline, with especially high mortality rates in the last five years (Bendick et al. 2018).

Microbiome

Bacteria and other microorganisms are found throughout the environment as well as on and within organisms. Communities of these microbes, including archaea, fungi, and viruses, that live in a particular environment are called microbiomes (Colston and Jackson 2016). The study of the microbiome is an emerging field in the evaluations of organismal health and is being increasingly used to evaluate the organismal effects of contaminants (Jin et al. 2017, Adamovsky et al. 2018). Most of this previous work has been done with microbiomes of humans and other vertebrates, but invertebrates play host to microbial populations as well (Funkhouser and Bordenstein 2013, Llewellyn et al. 2016, Adamovsky et al. 2018). The microbiome provides a variety of benefits to their host that includes providing necessary nutrients, aiding in digestion, and fighting

pathogens (Prieur et al. 1990, Pujalte et al. 1999, Newton et al. 2007, Cruz-López and Maske 2016).

Bacteria produce enzymes that the host cannot; for example, members of the phyla Bacilli, Actinobacteria, γ-Proteobacteria, and β-Proteobacteria breakdown plant polysaccharides into short-chain fatty acids (SFAs), a source of energy for the host (Lee et al. 2015). The microbiome is also able to synthesize essential vitamins for the host, such as those involved in host enzyme metabolism of amino acids or convert dietary vitamins into forms that can be taken up by the host, such as Vitamin B12 or iron (Schaible and Kaufmann 2004, Kau et al. 2011). The microbiome is also able to provide the host with nutrients such as the SFAs directly, which improve the intestinal epithelial barrier thus preventing infections from pathogens such as *Escherichia coli* (Peng et al. 2007, Kau et al. 2011, Takiishi et al. 2017). Some products of microbial metabolism are agonists or antagonists of immune cell receptors, (Kau et al. 2011, Schubiger et al. 2015). For example, metabolites produced by bacteria in the skin microbiome of rainbow trout induces antibody production within the host (Sepahi et al. 2016). T cells exhibit increased metabolic needs when they are stimulated, requiring more glucose and amino acids; when these needs are not met the T cells have decreased functionality and activation success. Microbial SFAs both stimulate the T cells and provide metabolic energy source (Kau et al. 2011). SFAs can also interfere with pathogenic gene expression, inhibiting certain pathogens' ability to invade the intestinal epithelial cells (Lawhon et al. 2002, Gantois et al. 2006).

In addition to aiding in the hosts' immune response, the microbiome can play a role in pathogen defense itself (Gomez et al. 2013, Greenspan et al. 2019). Two species

of pathogenic fungus are growth-inhibited by commensal bacteria on the skin of rainbow trout (Lowrey et al. 2015). In another example, *Staphylococcus epidermis* is able to utilize glycerol naturally produced by the host to produce SFAs, specifically succinic acid in this case, and inhibit the growth of pathogenic *Propionibacterium acnes* when its population became too large (Wang, Kuo, et al. 2014). In other instances, the bacteria synthesize small molecules that interfere with pathogenic gene expression. *Ruminococcus obeum* interferes with cellular communication mechanisms of *Vibrio cholerae*, preventing it from expressing certain genes it needs to colonize human intestines (Hsiao et al. 2014). Microbiomes can also provide resistance to pathogens through competition for nutrients (Ubeda et al. 2017). In the mouse intestine, two strains of commensal *E. coli* utilize the sugars needed by a pathogenic *E. coli* strain. When both commensal strains are present, the pathogenic strain is outcompeted, however, if one of the commensal strains is missing the pathogen has access to some of the nutrients it needs and is able to colonize the mouse intestine (Maltby et al. 2013).

Another benefit the microbiome provides to its host is that of the biotransformation of toxicants. Bacteria can perform many chemical transformations including reduction, hydrolysis, dehydroxylation, acetylation, demethylation, etc. on compounds such as pharmaceuticals (Claus et al. 2016) and metals (Mishra et al. 2017, Coryell et al. 2018). The microbiome acts as a mediator between exposure to toxicants and the response of the host and can influence properties of the toxicant such as dose and availability (Adamovsky et al. 2018).

Most of the focus in host-associated microbiome studies has been on the gastrointestinal organs (stomach, intestine), but microbiomes are also present in other

tissues including skin, respiratory organs like lungs or gills, and reproductive tissues (Lokmer, Kuenzel, et al. 2016, Adamovsky et al. 2018). Additionally, the microbiome within an individual can change as a result of development, for example in zebrafish the microbiome of larvae was similar to the microbiome of the surrounding environment but as the individuals aged the microbiomes diverged (Trabal Fernández et al. 2014, Stephens et al. 2016). This change suggests that the environment can be a factor in shaping the microbial community, but also that the strength of environmental influence can change through time (Adamovsky et al. 2018).

The community composition of microbes within a host can also be affected by other external factors. Temperature stress has been shown to significantly disrupt bacterial communities in oyster hemolymph (Lokmer and Wegner 2015). Diet can also affect the microbiome, salmon fed with a plant-based feed had reduced species richness and diversity than those fed a fish meal diet, changes that could contribute to the negative health outcomes observed when the plant-based diet is used (Krogdahl et al. 2010, Desai et al. 2012). Location plays a major role in shaping both the internal and external microbiomes of organisms, oyster adductor muscle and gastrointestinal tract communities most strongly varied by site (Trabal Fernández et al. 2014, King et al. 2019), similarly the skin microbiome of aquatic species showed geographical variation is the factor with the strongest influence (Bierlich et al. 2018, Chiarello et al. 2019).

Prokaryotes have been the subject of study for centuries but with the more recent advent of genetic sequencing that study has been able to take leaps and bounds from its humble culture-based origins (Givens et al. 2014, Adamovsky et al. 2018). The primary tools of study now are Next Generation Sequencing platforms that generally make use of

sequencing-by-synthesis, a process that utilizes fluorescently tagged nucleotides to identify the genetic sequence as DNA is replicated (Siqueira et al. 2012, Ghanbari et al. 2015). One common method of microbiome study has been of marker gene analysis, more specifically sequencing of the 16S rRNA gene, which allows for the identification of bacterial taxonomic groups and the phylogenetic relationships of those organisms (Woese and Fox 1977, Pace 2009, Dickson et al. 2016). Universal primers are used to amplify the gene during polymerase chain reaction (PCR) steps prior to sequencing, then the sequences of hypervariable regions within the 16S gene allow for identification (Adamovsky et al. 2018).

The 16S region is a part of the small subunit (SSU) of the prokaryotic ribosome and contains areas of conserved sequences and variable sequences, which are used for phylogenetic mapping (Pace 1997, Srinivasan et al. 2015). Once the sample DNA is sequenced, it can then be aligned and then clustered into Amplicon Sequence Variants (ASVs), groups of sequences that share sequence similarity and are akin to genus or species level phylogeny (Dickson et al. 2016). ASVs are then taxonomically classified using databases that contain near full length 16S gene sequences of known organisms (Srinivasan et al. 2015). Using bioinformatics analysis, it is then possible to identify the bacteria present in the sample, how many of each type there are, the relative abundances of all the bacteria, and the diversity of the sample (Dickson et al. 2016).

Goals

The first goal of my thesis was to examine the effect of chronic sediment-associated oil exposure on pathogen challenge effects on the flounder microbiome. A

previous study examined the effect of chronic exposure to environmentally relevant oil concentrations of juvenile southern flounder and found system-wide impairment of the biological processes involved in growth, liver and gill function, microbiome regulation, and even increased mortality (Brown-Peterson et al. 2015). A follow-up study that aimed to further elucidate the immune system impact of short-term oil exposure in combination with a pathogen challenge on juvenile southern flounder found similar, adverse effects including near complete mortality in the treatment with both oil and pathogen exposure as well as impairment of multiple biological systems including the regulation of immune related genes and disruption of the microbiome (Bayha et al. 2017). Using results from these two previous studies, the parameters for a third study which constitutes the second chapter of this thesis, were determined to fine tune the sublethal effects of both oil and pathogen exposure on southern flounder.

The second goal of my thesis was to investigate the presence of lasting effects of oil contamination on the oyster microbiome, a potential factor contributing to the decline of local oyster populations. The northern Gulf of Mexico has a long history of oil production and in combination with natural seeps results in persistent contamination of this region (Overton et al. 2004, Kennicutt 2017). When that contamination becomes buried in the sediment it has the potential to cause harm to organisms, especially benthic organisms like oysters, for much longer periods of time (Carmichael et al. 2012, Liu et al. 2012, Brown-Peterson et al. 2015). Because the microbiome plays an important role in nutritional and immune processes for the host understanding how it is affected is an important part of understanding the overall impact of oil exposure.

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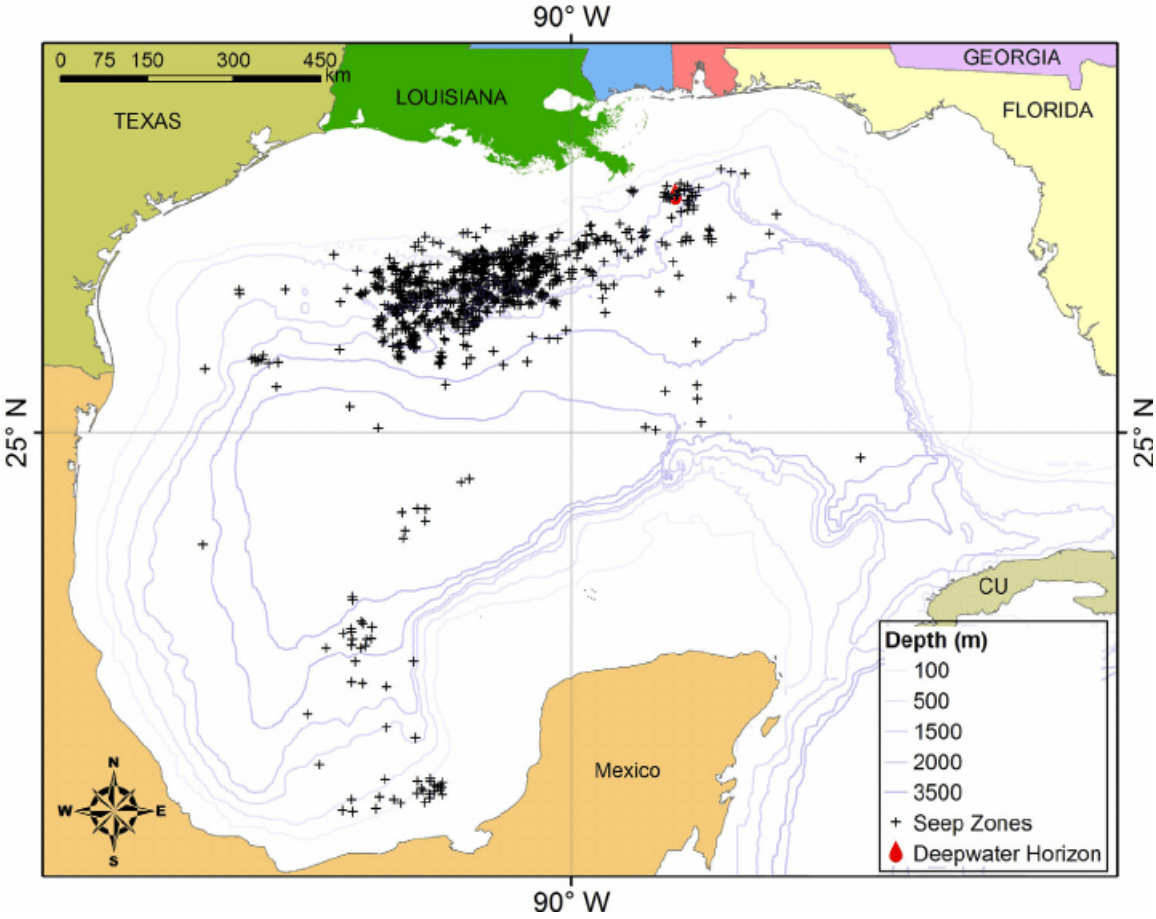

Figure 1.1 *Gulf of Mexico Oil Seep Zones*

A map of the known natural oil seep zones in the Gulf of Mexico (After MacDonald et al. 2015).

CHAPTER II – EFFECTS OF CRUDE OIL AND PATHOGENIC BACTERIA ON THE MICROBIOME OF SOUTHERN FLOUNDER

Introduction

The southern flounder (*Paralichthys lethostigma*) is a benthic flatfish that can be found in the nearshore waters of the east coast of North America from North Carolina to Mexico (Corey et al. 2017). Juveniles grow in the estuaries until they move offshore as adults to spawn between November and January (Murua and Saborido-Rey 2003). Southern flounder exhibit batch spawning, or multiple spawning events within one season (Corey et al. 2017). There is evidence of two distinct populations of southern flounder, one in the Atlantic and the other in the Gulf of Mexico (Anderson et al. 2012). Flounder from the Gulf of Mexico exhibit higher growth rates than those in the Atlantic, as well as younger age-at-maturity (Corey et al. 2017).

Southern flounder represent a major recreational fishery in the Atlantic and Gulf of Mexico, in addition to a commercial fishery that lands 12,000 metric tons per year (Anderson et al. 2012). Unfortunately, populations of southern flounder have been in decline from Texas to North Carolina due to overfishing, by-catch pressure, or other environmental factors like habitat loss (Wenner and Archambault 2005, Anderson et al. 2012). Flounder and other benthic organisms are also particularly susceptible to contamination from sediment that can be reintroduced into the water column during storms or dredging activities, further contributing to population declines (Roberts 2012). There are many methods available to study the effects contamination has on benthic organisms like flounder, one relatively new field is the study of the microbiome.

Microbiomes are the collection of bacteria, viruses, and other microbes in a given location. Microbiomes provide a number of benefits to their host including aiding in digestion, providing nutrients, and fighting pathogens. The bacteria within the digestive system can aid in breaking down food by producing enzymes the host is unable to produce (Lee et al. 2015) and can produce essential vitamins/molecules the host needs metabolically (Kau et al. 2011). Commensal bacteria also provide protection from pathogens (Adamovsky et al. 2018). This occurs in two major ways, directly and indirectly. Direct pathogen fighting involves the microbes themselves acting upon the pathogen, and can happen through active measures against the pathogen itself (e.g. phagocytosis) or inactively through competition or by passively producing byproducts that impair the pathogen (Hsiao et al. 2014, Lowrey et al. 2015). Indirect pathogen fighting involves affecting the immune system of the host. This can happen a few ways, the microbes can produce molecules that activate the hosts' immune response or improve the intestinal barrier through induced cell differentiation and mucus production, thus making it harder for pathogens to enter the host and cause harm (Wrzosek et al. 2013, Sepahi et al. 2016, Takiishi et al. 2017).

In the past, most host-associated microbiome studies focused on the intestine and other digestive organs, but microbiome communities can be found among many tissues of host organisms such as the skin or gills (Hoffmann et al. 2013, Dickson et al. 2016, Reverter et al. 2017, Bierlich et al. 2018, Evariste et al. 2019). The microbial make-up or community composition differs between these locations due to environmental or competitive selection pressure within the microbial community (Larsen et al. 2015, Pratte et al. 2018, Maher et al. 2019). Additionally, microbial communities within an organism

can change through time, for example the intestinal microbiome of zebrafish is similar to that of the water column at the larval stages but as the organism grows through development its microbiome becomes more distinct (Lokmer, Goedknegt, et al. 2016, Stephens et al. 2016).

While the majority of microbiome studies have been focused on human or other mammalian model organisms, the importance of understanding microbiomes has led to its use in studying other organisms as well, including fish and other marine organisms (Larsen et al. 2013, Bahrndorff et al. 2016, Coryell et al. 2018). The microbiome can be used as tools to study the health of the host organism including the role of the microbiome in disease, or in environmental studies as biomarkers to contamination (Kau et al. 2011, Adamovsky et al. 2018). The microbiome has been shown to change due to contamination, for example when exposed to oil a higher abundance of oil-degrading bacteria is present (Thomas et al. 2014). Therefore, contaminant-induced alterations in the microbiome of an organism can be used to inform researchers on both the health of the organism and the environment in which the organism is found. However, this field is still relatively new and needs continuing investigation.

The *Deepwater Horizon* oil spill resulted in 62,159 km2 of oiled surface waters and 2,100 km of oiled coastline along Texas, Louisiana, Mississippi, Alabama, and Florida (Michel et al. 2013, Beyer et al. 2016). Up to 47% of oil not recovered from the spill ended up in the sediments of the northern Gulf (Romero et al. 2017). However, PAH degradation is reduced in these sediments due to hypoxic conditions (Landmeyer and Bradley 2003). Additionally, PAH concentration within sediments is controlled by the organic matter content and grain size as PAHs strongly adsorb to sediment particles

(Wang, Liu, et al. 2014). This reduces the microbial bioavailability and thus biodegradation rates, contributing to persistent contamination (Wang, Liu, et al. 2014). Chronic contamination of sediments has lasting impacts on the benthic organisms that live there (Landmeyer and Bradley 2003).

Exposure to oil is harmful for many species including fish and can result in both lethal and sublethal effects. Impaired egg production, embryogenesis, and development can result, as well as altered development and function of the gills and heart, impaired immune responses, and increased mortality (de Soysa et al. 2012, Whitehead et al. 2012, Brette et al. 2014, Lo et al. 2014, Brown-Peterson et al. 2015, Jones et al. 2017). When juvenile southern flounder were chronically exposed to contaminated sediments, they exhibited a suite of impacts including increased mortality, reduced growth, impaired liver and gill tissue, upregulated gene expression of the *Cyp1a* gene, and significantly altered microbiome communities in both gill and intestine tissues (Brown-Peterson et al. 2015). To further examine the effect of contaminated sediments on southern flounder, Bayha et al. (2017) conducted a 7-day exposure followed by a challenge with pathogenic *Vibrio anguillarum*. Immune function was significantly impaired resulting in down-regulation of genes relating to immune function including antibodies, increased presence of *V. anguillarum* in the microbiome, vibriosis, and death (Bayha et al. 2017).

Southern flounder are exposed xenobiotics such as PAHs via contaminated sediments due to their benthic nature. This chapter aims to examine the effect of chronic oil exposure in the presence of a pathogenic bacteria on the gill and intestine microbiomes of flounder through contaminated sediment and a pathogen challenge to determine if there are implications of exposure on the flounder immune system. Southern

flounder were exposed to two environmentally relevant concentrations of oil contaminated sediment for 7 days, followed by a 1-hour *V. anguillarum* pathogen challenge. At four time points post-bacterial inoculation flounder were sacrificed and samples for microbiome analysis were collected.

Materials and Methods

This study utilized a fully factorial, six treatment design to implement an oil-contaminated sediment exposure (Fig 2.1). Field collected weathered Louisiana MC252 crude oil (BP code 00003277), was mixed with non-oiled, field collected, sediment from Alabama marshes (30 22’ 45.72” N; 88 18’ 24.40” W) following the procedure laid out in Brown-Peterson et al (2015). Target nominal concentrations were 10 mg/kg and 25 mg/kg, and the respective treatments were labeled Low Oil and High Oil; for 1.8 kg of sediment in each tank 5.38 g of oil per kg of sediment and 13.5 g oil per kg of sediment were used respectively. Mixed sediments were allowed to settle overnight following the addition of artificial seawater. The treatments are as follows: (1) Control, (2) Low Oil, No Bacteria, (3) High Oil, No Bacteria, (4) No Oil, Bacteria (bacterial control), (5) Low Oil, Bacteria, (6) High Oil, Bacteria. The exposures took place in four 10-gal tanks with three fish per tank, fresh artificial seawater was consistently replenished at a rate of 9% of the total tank volume to maintain optimal water quality throughout the experiment.

Following a 7-day exposure, fish were removed from the exposure tank and placed into the bacterial challenge tank (or control tank with artificial seawater (ASW) for those treatments without the bacterial challenge). The bacterial challenge consisted of a 1-hour exposure to 20 L of ASW with 2.65 x 105 colony-forming unit (CFU)/L *V.*

anguillarum (ATCC strain 19264). The fish were then rinsed and returned to the exposure tanks until sampling time points at 1-, 4-, 7-, and 10-days post-challenge (Table 2.1). One fish per tank was sacrificed and dissected at each time point and samples were stored at -20 °C until further processing. Samples of oiled sediment and water were collected the day before fish were placed into tanks and on the last day of the exposure, samples were stored at -20 °C until shipped to ALS Environmental (Kelso, WA) for PAH analysis via gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM). DNA was extracted with PowerSoil DNA Isolation Kit (Thermo Scientific) and the DNA concentration of each sample was measured using NanoDrop (Thermo Scientific). Extracted DNA was sequenced at Texas Tech University Center for Biotechnology & Genomics at the V3-V4 variable region of the gene encoding 16S rRNA using Illumina MiSeq sequencing platform.

Raw sequences were run through the QIIME2 platform as follows. First sequences were demultiplexed using q2-demux and trimmed (Forward trimmed at 0, truncated at 295; reverse trimmed at 0, truncated at 213) and denoised for quality control using DADA2 (Callahan et al. 2016), and output of a feature table or Amplicon Sequence Variant (ASV) table was produced and summarized for visualization (Supplementary ASV Table). Sequences were then run through phylogenetic analysis via q2-phylogeny to identify relationships and generate a phylogenetic tree, then ASVs were assigned taxonomic labels based on the SILVA 99% 138 reference database using the q2-feature-classifier (Bokulich et al 2018) classify-consensus-vsearch (Quast et al. 2013, Rognes et al. 2016, Bolyen et al. 2019). Shannon diversity index was used to determine the alpha diversity of the microbial communities in conjunction with the Kruskal-Wallis statistical

test. Analyzing differences between the microbial assemblages of each treatment was done with PERMANOVA based on a Bray-Curtis dissimilarity matrix (using q2-diversity) in conjunction with Principal Coordinates Analysis (PCoA) generated in R (R Core Team 2020).

Results

Sediment PAH Results

A total of 75 PAHs were measured from the sediment (Table 2.2). The measured ΣPAH75 was 0.085 mg/kg for the Control treatment, 11.61 mg/kg for Low Oil treatment, and 32.96 mg/kg for High Oil treatment at the beginning of the experiment. The High Oil treatment had the highest concentrations of PAHs with the most abundant being C3-Phenanthrenes/Anthracenes (2830 µg/kg; Figure 2.2). PAH concentrations also decreased from the beginning of the experiment to the end by 4.15 mg/kg in the Low Oil treatment and 6.46 mg/kg in the High Oil treatment.

Vibrio anguillarum Results

The challenge tanks for treatments Control No Bacteria, Low Oil No Bacteria, and High Oil No Bacteria contained a bacterial density of 40 ± 3.27 CFU/L (Table 2.3). *V. anguillarum* concentrations in the Control Bacteria, Low Oil Bacteria, and High Oil Bacteria tanks were 5.30 x 105 ± 1.81 x 105 CFU/L. There was no flounder mortality associated with the bacterial treatment. Fish were observed to jump out of the treatment tanks during the 3 days preceding the bacterial challenge, but not after the challenge for the remainder of the experiment.

Sequencing Summary

71 samples from the gill microbiome returned 1,980,333 raw sequences, from which 3,633 unique ASVs were assigned; 64 samples from the intestine microbiome returned 1,760,219 raw sequences, from which 1,870 unique ASVs were assigned (Table 2.4). 9 samples from the sediment microbiome returned 586,874 sequences and 9 samples from the water microbiome returned 605,634 sequences. There were 13 intestine samples removed during sequence quality control due to low read yields or poor sequence quality, leaving a total of 51 intestine samples remaining. The highest number of ASVs in one gill sample was 85,369 while the average for all gill samples was 14,843 ASVs, and 159 ASVs were unable to be assigned (Table 2.4). The maximum ASVs identified in a single intestine sample was 66,459 ASVs, overall intestine samples had an average 11,674 ASVs and 3,183 ASVs were unassigned.

Water and Sediment Microbiome Results

Sediment samples were primarily characterized by having a higher Shannon diversity than the other three matrices with no single dominant taxa, the most abundant taxa across sediment samples were *Gramella*, *Psychrobacter*, and *Hoppeia*. Water samples were similarly structured, though overall less diverse with communities made up of primarily *Marivivens*, *Silicimonas*, and a Rhodobacteraceae taxa (Figure 2.3).

Sediment sample microbiomes showed the highest alpha diversity, while the water samples microbiome had an alpha diversity very similar to gill (Figure 2.4A). There were significant differences between the alpha diversity of the sediment

communities and the communities of all other matrix type, additionally water and intestine samples differed significantly as well (Figure 2.4A). All four matrix types showed significant differences in beta diversity, showing differences in the community composition of sediment, water, gill, and intestine microbiomes (p-value = 0.001, Figure 2.4B).

Changes in Flounder Microbiome Over Time

Alpha diversity using the Shannon index for both gill and intestine samples show no significant changes through the 4 time points, despite a general decrease through time shown in the box plots for both tissues (Figure 2.5A). The low number of samples may be responsible for this pattern. Beta diversity based on Bray-Curtis dissimilarity also shows no differences through time for both tissues. Neither gill nor intestine microbiome samples showed any significant treatment effects on clustering using PCoA (Figure 2.6). Small sample sizes in both gill and intestine samples prevented the calculation of confidence ellipses for these analyses, potentially impairing determination of finer details of changes of the microbiome through time after a disturbance. Because there was no statistical effect of time for neither gill nor intestine, moving forward all timepoints for each tissue in each treatment were be pooled for analysis.

Gill Microbiome Results

Gill samples contained a large number of bacteria identified in each sample, however four genera comprised 31% of the microbiome of the gill samples, *Photobacterium*, *Pseudoalteromonas*, *Alcanivorax*, and *Vibrio* (Figure 2.7A). Figure 2.8

shows the most abundant 10 taxa over all samples by treatment for gill (A) and intestine (B) respectively, here the two tissues differ from each other. Gill samples are characterized by a majority of *Vibrio*, *Pseudoalteromonas*, *Photobacterium*, and *Alcanivorax*; each treatment has three taxa that make up the majority of each community along with a much lower relative abundance of the remaining bacteria (Figure 2.8A). The control group contains *Photobacterium*, *Vibrio*, and *Pseudoalteromonas* as most abundant genera, which is very similar to the bacterial control group (No Oil, Bacteria) which had the same three primary members but a larger abundance of other, less abundant genera such as *Alcanivorax* (0.05% in Control, 0.19% in bacterial control; Figure 2.8A), *Marivivens* (0.00% in Control, 0.11% in bacterial control), and *Mesoflavibacter* (0.02% in Control, 0.12% in bacterial control). The remaining treatments showed very similar patterns to each other, all four had *Vibrio* (Low Oil No Bacteria 1.77%, High Oil No Bacteria 2.08%, Low Oil Bacteria 1.77%, High Oil Bacteria 2.06%; Figure 2.8A), *Alcanivorax* (Low Oil No Bacteria 1.23%, High Oil No Bacteria 1.46%, Low Oil Bacteria 1.35%, High Oil Bacteria 1.33%; Figure 2.8A), and *Pseudoalteromonas* (Low Oil No Bacteria 1.48%, High Oil No Bacteria 1.08%, Low Oil Bacteria 1.70%, High Oil Bacteria 1.01%; Figure 2.8A) as the majority constituents with similar, lower abundances of the remaining genera (less than 0.5% relative abundance for *Acinetobacter*, *Shewanella*, *Catenococcus*, and *Mesoflavibacter*; Figure 2.8A).

Intestine Microbiome Results

Intestine microbiomes showed a different pattern from those in the gill, *Vibrio* made up 25% of all intestine samples and the next most common genus was

Photobacterium making up 2.4% of the intestine microbiome (Figure 2.8B). Intestine samples are much less diverse than the gill samples (Figure 2.7). The single primary genus across all treatments was *Vibrio* (Control 3.89%, Low Oil No Bacteria 4.74%, High Oil No Bacteria 7.67%, No Oil Bacteria 2.81%, Low Oil Bacteria 3.26%, High Oil Bacteria 3.80%; Figure 2.8B), while the control groups both had a small secondary contributor of *Photobacterium* (0.83% and 0.93%, respectively; Figure 2.8B). Interestingly, the Low Oil, Bacteria group had a relatively large number of *Shewanella* (0.70%; Figure 2.8B) that is not seen in other treatments.

Comparison of Gill and Intestine Microbiomes Under Control Conditions

In the Control treatment, the alpha diversity of the gill, intestine, and water microbiomes are similar, but all three are significantly different from the sediment microbiome (Gill-Intestine p-value = 0.298, Gill-Sediment p-value = 0.004, Intestine-Sediment p-value = 0.008, Water-Sediment p-value = 0.021; Figure 2.4A). The PCoA shows some grouping of the intestine, water, and sediment samples, while gill samples have a much wider spread (Figure 2.4B). However, the PERMANOVA shows significant differences between all four matrices, including gill and intestine microbiomes (p-value = 0.006, Figure 2.4B). Despite similar alpha diversities, gill and intestine samples showed significantly different composition, for example gill samples had much higher relative abundances of *Alcanivorax*, *Pseudoalteromonas*, and *Photobacterium* than intestine samples, suggesting the two tissues are capable of hosting similar numbers of different microbes but characteristics of the tissues influence the specific members that are present.

Effects of Oil Exposure on the Gill Microbiome

Within the gill samples, oil exposure groups are characterized by a slightly different set of primary members from that of the Control group – *Alcanivorax, Vibrio,* and *Pseudoalteromonas* – in addition to containing many other genera in lower abundances such as *Shewanella* (Control 0.20%, Low Oil 0.29%, High Oil 0.50%; Figure 2.10A), *Acinetobacter* (Control 0.02%, Low Oil 0.16%, High Oil 0.50%), and *Mesoflavibacter* (Control 0.02%, Low Oil 0.17%, High Oil 0.25%; Figure 5A, Figure 2.10A).

Gill samples show an increase in Shannon diversity as a result of exposure to oil, with both oil groups significantly more diverse than the control (10 mg/kg p-value = 0.0012, 25 mg/kg p-value = 0.0001; Figure 2.10A), and the higher oil level higher not significantly more diverse (p-value = 0.67) than the lower oil level (p-value = 0/67; Figure 2.10A). Significant q-values (p-values adjusted for multiple comparisons) confirm significant difference (Figure 2.10A). The PCoA for Oil Concentration shows clustering for gill samples and PERMANOVA confirms differences between the treatment groups (p-value = 0.001; Figure 11A). Oil exposure does affect the composition of flounder gill microbiome in addition to an increase in Shannon diversity, oil-exposed treatments showed decreased *Photobacterium* abundance, while *Alcanivorax, Shewanella,* and *Acinetobacter* increased in abundance.

Effects of Oil Exposure on the Intestine Microbiome

Intestine samples show very similar communities between the oiled groups, however with increasing oil concentration the relative abundance of *Vibrio* increased

(Control 3.90%, Low Oil 4.74%, High Oil 7.67%; Figure 2.8B), while *Photobacterium* was inhibited (Control 0.83% Low Oil 0.37%, High Oil 0.07%; Figure 2.8B).

There were no significant differences between the three oil concentrations for alpha diversity within intestine samples (Control-Low Oil p-value = 0.38, Control-High Oil p-value = 0.24, Low Oil-High Oil p-value = 0.94; Figure 2.10B). Intestine samples were less clustered than gill samples in the PCoA (Figure 2.11B), and only the highest level of oil was significantly different from the control (p-value = 0.018; Figure 2.11B). These results suggest the bacterial community make-up in the intestine if less sensitive to oil than that of the gill, the higher pseudo-F values in the gill samples suggest larger differences between groups and contribute to that suggestion (Figure 2.11B).

Effects of Pathogen Challenge on the Gill Microbiome

Gill samples show very similar bacterial assemblages between treatments with and without the bacterial challenge (Figure 2.8A). The bacterial control group shows lower relative abundances of *Pseudoalteromonas* (Control 1.41%, bacterial control 1.09%; Figure 2.8A) and *Shewanella* (Control 0.20%, bacterial control 0.12%; Figure 2.8A), while other taxa such as *Vibrio* (Control 1.75%, bacterial control 2.49%; Figure 2.8A), *Marivivens* (Control 0.00%, bacterial control 0.11%; Figure 2.8A), and *Mesoflavibacter* (Control 0.02%, bacterial control 0.13%; Figure 2.8A) have higher relative abundance. There is no statistical significance in alpha diversity between the two treatments in gill samples (p-value = 0.614; Figure 2.12A). Gill samples showed slight clustering in the PCoA, however the PERMANOVA results show no statistical significance between groups (p-value = 0.591, Figure 2.13A).

Effects of Pathogen Challenge on the Intestine Microbiome

Intestine samples differ slightly between the No Bacteria and Bacteria challenge groups. Overall, the pathogen challenge treatments have less *Vibrio* than their No Bacteria counterparts (Control 3.90%, bacterial control 2.81%; Figure 2.8B), and the oiled treatments had slightly increased abundances of the other taxa, such as *Shewanella* (Low Oil No Bacteria 0.13%, Low Oil Bacteria 0.70%; Figure 2.8B) and *Marivivens* (High Oil No Bacteria 0.20%, High Oil Bacteria 0.42%; Figure 2.8B).

Intestine samples show similar responses to the bacterial challenge in terms of community alpha diversity as the gill samples, despite the box plots appearing to increase in alpha diversity after bacterial challenge in gill samples and decreasing in intestine samples (Figure 2.12B). There is no statistical significance in alpha diversity between the two treatments in intestine samples (p-value = 0.428; Figure 2.12B). Beta diversity in both tissues show opposite patterns in response to the pathogen challenge than the oil exposure, the intestine samples clustered in the PCoA, confirmed by significant p-values (p-value = 0.003, Figure 2.13B). A higher pseudo-F value in the intestine samples suggest this community is more sensitive to the pathogen challenge than to oil exposure (Gill pseudo-F = 0.836, intestine pseudo-F = 4.439; Figure 2.13B). Overall, the pathogen challenge showed little effect on the gill samples, while intestine samples were impacted, showing lower abundances of *Acinetobacter*, *Catenococcus*, and *Shewanella* in the pathogen challenge treatment.

Effects of Oil Exposure on the Microbiomes' response to Pathogen Challenge

To examine if there was any impact of the microbiome response to a pathogenic bacteria due to oil exposure, a PERMANOVA test was performed on all treatments and their interactions (Table 2.5). Sample matrix and oil concentration account for over 22% of the variation within the samples (15.95% and 6.36%, respectively). Four interactions showed statistical significance and together they account for 13% of the variation between samples: Sampling Point-Matrix (4.90%, p-value = 1.00E-04), Oil Concentration-Matrix (4.26%, p-value = 0.023), Oil Concentration-Bacterial Challenge (2.06%, p-value = 0.004), and Oil Concentration-Sampling Point (1.82%, p-value = 0.012).

Recall that in the gill samples, exposure to the pathogen did not affect the alpha diversity of the microbiome, and that exposure to oil increases the alpha diversity of the gill microbiome. This same pattern of increasing alpha diversity as a result of oil exposure is also exhibited in the pathogen challenge treatments, which suggests that oil has little effect on the response of the gill microbiome to the pathogen challenge (Fig 2.14A, Table 2.6). Similarly, in the beta diversity analysis there were no differences between the Control and the No Oil, Bacteria challenge treatments as well as complimentary significant differences between the respective oiled treatments meaning oil also had little effect on the beta diversity response of the gill microbiome to the pathogen challenge (Fig 2.15A, Table 2.7).

There were no significant differences in alpha diversity between all treatments in intestine samples, suggesting neither oil exposure, pathogen, nor the combination of the two stressors had an effect on the Shannon index of the intestine microbiome (Fig 2.14B,

Table 2.8). The beta diversity of the intestine samples was only significantly impacted by oil exposure at the highest concentration, and there were significant differences in composition following the pathogenic bacteria challenge. The PCoA shows lots of overlap between treatments, however the PERMANOVA (Fig 2.15B, Table 2.9) results showed significant differences between the No Oil, Bacteria and the two Oil, Bacteria treatments, suggesting that the combination of pathogen and oil can cause changes in beta diversity of the intestine microbiome.

Discussion

The microbiome communities of all four sample types, sediment, water, gill and intestine, within this study differed from each other. Sediment samples exhibited the highest diversity with *Gramella* being the most abundant taxa within this sample type. *Gramella* are found in nutrient rich marine environments where they specialize in the degradation of high molecular weight compounds in organic matter (Bauer et al. 2006). Gill and water samples had similar alpha diversities, however the community compositions differed significantly from each other. While the two environments might be able to support similar numbers of different organisms, characteristics of the environments favor a different community composition. A recent study from Pratte et al. (2018) involving reef fish found that gill samples had significantly fewer ASVs than the surrounding seawater and suggested that the gill microbiome is distinct from the external environment. Other comparisons also found that animal associated microbiomes were less diverse than the free-living microbiome of the water column (Thompson et al. 2017). The gills are in constant contact with the environment and collect nutrients more densely,

thus providing an environmental gradient that may select for a different assemblage of bacteria (Reverter et al. 2017).

The microbiome of the intestine showed the lowest diversity of the four sample types and differed significantly from the gill samples, despite having similar alpha diversities. Higher diversity observed in the gills compared to the digestive tract is consistent with other studies of the teleost microbiome, including rainbow trout (*Oncorhynchus mykiss*) and seabream (Lowrey et al. 2015, Thompson et al. 2017, Brown et al. 2019, Ruiz-Rodríguez et al. 2020). Individual differences within fish may be responsible for this pattern but characteristics of the tissues themselves are more likely to have a stronger influence (Legrand et al. 2020). The gills experience a few unique circumstances that can result in increased microbiome diversity including experiencing higher levels of oxygen and nutrients concentrated from the external water column than the intestine (Pratte et al. 2018). Similarly, the gills are the site of gas exchange, waste excretion, and site-specific immune activity, this complex environment allows for complex assemblages to form (Maina 2002, Llewellyn et al. 2014, Salinas 2015, van Kessel et al. 2016, Pratte et al. 2018).

The flounder intestine samples showed a high incidence of *Vibrio* compared to all other sample types. *Vibrio* was also identified as the most abundant bacteria in the microbiome of the red snapper (*Lutjanus campechanus*) (Arias et al. 2013, Tarnecki et al. 2016). *Vibrio* is a genus that is highly prevalent in marine environments and are known to be opportunistic pathogens to many organisms including fish, coral, and humans (Campbell and Wright 2003, Austin and Zhang 2006). *Vibrio* causes vibriosis, an intestinal disease that can result in diarrhea, vomiting, and fever in humans as well as

deadly hemorrhagic septicemia in fish and aquatic invertebrates (Frans et al. 2011, Washington State Department of Health 2021). It is difficult to identify *Vibrio* species using 16S rRNA, thus determining the pathogenicity of the *Vibrio* present within these flounder intestine is difficult (Reverter et al. 2017). However, taking into account the increased abundance beyond that of the control with increasing oil exposure coupled with a depressed abundance of *Vibrio* following exposure to a known pathogenic species (*V. anguillarum*), suggests that the flounder intestine microbiome is dominated by symbiotic, not pathogenic, *Vibrio* species.

All oil exposure treatments showed large abundances of *Alcanivorax* within the gill microbiome and was not seen in either of the control groups, this pattern is consistent with previous studies of flounder microbiomes (Bayha et al. 2017). *Alcanivorax* is an oil degrading bacterium whose populations increase in sediments, sand, and the water column following oil contamination (Kostka et al. 2011, Gutierrez et al. 2013, Liu and Liu 2013). *Alcanivorax* preferentially degrade branched and straight-chained hydrocarbons, like those found in the crude oil from the DHOS and do so rapidly in timescales of weeks to months after the spill (Kasai et al. 2002, Kostka et al. 2011, Gutierrez et al. 2013). It is very likely the large abundances of *Alcanivorax* seen in these flounder gill microbiomes are a result of transport from the oil-contaminated sediment to the gill and during the length of the experiment this genus was able to utilize the hydrocarbons and propagate, while *Alcanivorax* was not observed in either the control or bacterial control because those treatments did not contain any of the crude oil.

Pseudoalteromonas was also very abundant in the gill microbiome, composing one of the three most abundant genera in all treatments but seen in much lower

abundances in all other sample types. *Pseudoalteromonas* are true marine bacteria and have been found as a majority of the microbiomes of other marine species such as copepods, seabass (*Dicentrarchus labrax*), and thresher sharks (*Alopias vulpinus*), and are typically associated with healthy individuals (Moisander et al. 2015, Offret et al. 2016, Doane et al. 2017, Rosado et al. 2019). The presence of *Pseudoalteromonas* can inhibit the growth of *Vibrio* and *Photobacterium* species via antimicrobial metabolites that are associated with host homeostasis, this may be the reason for such high abundances observed in the gill microbiome where the flounder are most directly exposed to the oil contamination (Offret et al. 2016, Rosado et al. 2019).

Photobacterium was the most abundant genus in the gill microbiome of both control groups and second most abundant genus in the intestine microbiome for both control groups but appeared in much lower abundances in all other treatments for both tissues. *Photobacterium* are luminescent marine bacteria that are commensal members of fish microbiomes, however some species can be pathogenic and cause septicemia (Fouz et al. 2000, Romalde 2002, Rosado et al. 2019, Hagedorn 2020). *Photobacterium* appear in the skin microbiome of farmed seabass and have also been found as a majority in the gut of Atlantic cod (*Gadus morhua*), making up over 50% of the microbiome (Rosado et al. 2019). It is interesting that in the flounder microbiome *Photobacterium* is only present in the non-oiled treatments and *Alcanivorax* appears to replace it in the presence of hydrocarbons, this may be due to environmental pressure, *Photobacterium* are potentially sensitive to the toxic effects of the xenobiotics, or to competition pressure of the *Alcanivorax* upon the *Photobacterium* in which the increased carbon source allows *Alcanivorax* to outgrow and impede the growth *Photobacterium*, or some other bacterial

interaction. The ubiquity of both these genera, along with *Vibrio*, lends to the importance of looking into this interaction as a direction for future study.

Other prominent genera in the flounder microbiome were *Shewanella* and *Marivivens*. *Shewanella* has been found in the microbiomes of fish and plants in marine environments and is a large contributor to mineral recycling and bioremediation of metals (Lovley and Phillips 1988, Dikow 2011). *Shewanella* appears in the microbiomes of other fish including damselfish, pinfish, hogchokers, and carp (Givens et al. 2015, Liu et al. 2016, Parris et al. 2016, Pratte et al. 2018). *Marivivens*, the primary constituent of the water samples, is a marine genus in family Rhodobacteraceae and is globally distributed in seawater from the Gulf of Mexico, Bay of Bengal, and the Khazar Sea (Slightom and Buchan 2009, Park et al. 2016, Ebrahimi et al. 2020).

Gill samples were more sensitive to the oil exposure than the pathogen challenge in this study. It is known that these stressors can affect the microbiome by altering composition, such as increasing abundance of hydrocarbon degrading bacteria with exposure to oil (Thomas et al. 2014) and decreased phylogenetic diversity with oil (Greenspan et al. 2019) and pathogenic infection (Green and Barnes 2010, Lokmer and Wegner 2015). The taxonomic bar plots show a reduction of 28% in *Vibrio* and an increase of 12% in *Photobacterium* following the bacterial challenge in the intestine. For the same community, alpha diversity was also depressed (though not significantly), a significant shift in microbiome structure following pathogen exposure was also observed in recent studies of oysters, fish, and frogs (Green and Barnes 2010, Jani and Briggs 2014, Lokmer and Wegner 2015). Such shifts could result in detrimental effects to the

host if the nutritional and immune benefits provided by the microbiome are impaired when the community changes (Sekirov and Finlay 2009).

The microbiome of the gills was more sensitive to oil exposure than that of the intestine, consistent with previous studies (Brown-Peterson et al. 2015). These results not surprising because the gills are exposed to the exterior of the fish and are usually in continuous contact with the contaminated sediment. Fish behaviors such as burrowing into the sediment and other bioturbation actions can mix sediment and contaminants into the water column where it can then enter the body via absorption through the gills. Whereas the fish must ingest contaminated sediments before the xenobiotic can interfere with the intestine and associated microbiome, actions that generally occur with less frequency especially during times of stress (Fletcher et al. 1981, Pérez-Casanova et al. 2008, Yuan et al. 2017). Establishing the relative importance of each stressor is useful for putting the results of laboratory experiments, like these, into context within the wider complexity that is environmental science.

Conclusions

Vibrio, *Pseudoalteromonas*, *Photobacteria*, and *Alcanivorax* make up primary taxa within the gill microbiome of juvenile southern flounder, while *Vibrio* dominated the intestine microbiome. Gill and intestine microbiomes also exhibited different responses to the oil and pathogen exposures, the gill samples showed a higher alpha diversity than intestine samples and were more sensitive to oil exposure showing increased Shannon diversity and altered composition. However, intestine samples did exhibit changes in composition following the pathogen challenge, despite having no change in alpha

diversity. When the effect of oil exposure on the response to a pathogen was examined, both gill and intestine samples showed no effect of the pathogenic *V. anguillarum* challenge on the microbiome.

Lower concentrations of oil (10 mg/kg and 25 mg/kg) and *V. anguillarum* (5.30 X 105 ± 1.81 X 105 CFU/mL) allowed for the capture of effects of oil on the flounder microbiome at concentrations sublethal to flounder. A previous study saw near complete mortality after 48 hours following an exposure to oil and pathogenic *Vibrio,* however they used oil and pathogen concentrations of 57.4 mg/kg of oil and 9.03 X 105 ± 5.09 X 104 CFU/mL *V. anguillarum*, which were much higher than the concentrations used in this study (Bayha et al 2017). Taken together, these results narrow down the limits of oil and pathogen exposure effects on the southern flounder microbiome and contribute to the overall understanding of what occurred in the Gulf of Mexico following the *Deepwater Horizon* oil spill, filling in the gaps between increased marine snow deposition to the elevated mortality of charismatic macrofauna such as bottlenose dolphins and sea turtles (Antonio et al. 2011, Carmichael et al. 2012, Daly et al. 2016).

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Table 2.1 *Experiment schedule for flounder exposure.*

Day	
-1	Sediment, Water samples
0	Fish into tanks
7	Bacterial Challenge
8	Gill & Intestine samples
11	Gill & Intestine samples
14	Gill & Intestine samples
17	Gill, Intestine, Sediment, Water

Table 2.2 *List of PAHs measured in sediment and water samples*.

cis/trans-Decalin	C1 - Fluoranthenes/Pyrenes
C1-Decalins	C2 - Fluoranthenes/Pyrenes
C2-Decalins	C3 - Fluoranthenes/Pyrenes
C3-Decalins	C4-Fluoranthenes/Pyrenes
C4-Decalins	Naphthobenzothiophene
Benzo(b)thiophene	C1-Naphthobenzothiophenes
C1-Benzothiophenes	C2-Naphthobenzothiophenes
C2-Benzothiophenes	C3-Naphthobenzothiophenes
C3-Benzothiophenes	C4-Naphthobenzothiophenes
C4-Benzothiophenes	Benz(a)anthracene
Naphthalene	Chrysene
C1-Naphthalenes	C1 - Chrysenes
C2-Naphthalenes	C2 - Chrysenes
C3-Naphthalenes	C3 - Chrysenes
C4-Naphthalenes	C4 - Chrysenes
Biphenyl	Benzo(b)fluoranthene
Dibenzofuran	Benzo(j+k)fluoranthene
Acenaphthylene	Benzo(a)fluoranthene
Acenaphthene	Benzo(e)pyrene
Fluorene	C30-Hopane
C1 - Fluorenes	Benzo(a)pyrene
C2 - Fluorenes	Perylene
C3 - Fluorenes	Indeno(1,2,3-cd)pyrene
Anthracene	Dibenz(a,h)anthracene
Phenanthrene	Benzo(g,h,i)perylene
C1-Phenanthrenes/Anthracenes	4-Methyldibenzothiophene
C2-Phenanthrenes/Anthracenes	2-Methyldibenzothiophene
C3-Phenanthrenes/Anthracenes	1-Methyldibenzothiophene
C4-Phenanthrenes/Anthracenes	3-Methylphenanthrene
Retene	2-Methylphenanthrene
Dibenzothiophene	2-Methylanthracene
C1 - Dibenzothiophenes	9-Methylphenanthrene
C2 - Dibenzothiophenes	1-Methylphenanthrene
C3 - Dibenzothiophenes	2-Methylnaphthalene
C4 - Dibenzothiophenes	1-Methylnaphthalene
Benzo(b)fluorene	2,6-Dimethylnaphthalene
Fluoranthene	2,3,5-Trimethylnaphthalene
Pyrene	Carbazole

Table 2.3 *Bacterial density within pathogen challenge tanks.*

Treatment	Target Density (CFU/L)	Actual Density (CFU/L)
Control	0	8.00
Low Oil, No Bacteria	0	4.00
High Oil, No Bacteria	0	0.00
No Oil, Bacteria	2.65E+6	4.25×10^5
Low Oil, Bacteria	2.65E+6	3.80×10^5
High Oil, Bacteria	2.65E+6	7.84×10^5

Table 2.4 *Summary statistics for all flounder microbiome samples.*

Sample	Treatment	Time Point	Replicate	Matrix	Raw Sequence Counts	ASVs	Shannon Diversity	Good's Coverage Index
1A-1.297	1	1	A	Gill	25927	12378	4.084	1.00
1A-2.477	1	2	A	Intestine	30969	11214	4.088	1.00
1A-2.479	1	2	A	Gill	10690	7252	3.455	1.00
1A-3.703	1	3	A	Gill	4008	2359	3.996	1.00
1A-4.895	1	4	A	Gill	22058	11664	3.622	1.00
1B-1.304	1	1	B	Intestine	29608	13279	5.075	1.00
1B-1.306	1	1	B	Gill	61602	49	0.000	1.00
1B-2.486	1	2	B	Intestine	19459	8322	4.000	1.00
1B-2.488	1	2	B	Gill	19324	12259	4.345	1.00
1B-3.710	1	3	B	Intestine	17522	11934	5.001	1.00
1B-3.712	1	3	B	Gill	13782	5444	4.683	1.00
1B-4.904	1	4	B	Gill	17237	8706	4.359	1.00
1C-1.313	1	1	C	Intestine	70756	32751	6.159	1.00
1C-1.315	1	1	C	Gill	35727	23284	5.565	1.00
1C-1.497	1	2	C	Gill	13394	8287	3.370	1.00
1C-2.495	1	2	C	Intestine	31776	1143	0.000	1.00
1C-3.708	1	3	C	Gill	13745	7273	5.013	1.00
1C-3.719	1	3	C	Intestine	42534	19793	5.289	1.00
1C-4.911	1	4	C	Intestine	103306	66459	2.722	1.00
1C-4.913	1	4	C	Gill	22438	12090	3.540	1.00
2A-1.322	2	1	A	Intestine	13926	8272	2.926	1.00
2A-1.324	2	1	A	Gill	27671	16510	6.916	1.00
2A-2.506	2	2	A	Gill	8154	5344	5.540	1.00
2A-3.728	2	3	A	Intestine	11745	5596	5.465	1.00
2A-3.730	2	3	A	Gill	15080	9142	6.367	1.00
2A-4.920	2	4	A	Intestine	14041	8496	5.585	1.00
2A-4.922	2	4	A	Gill	22759	11702	6.564	1.00
2B-1.331	2	1	B	Intestine	54105	99	0.000	1.00
2B-1.333	2	1	B	Gill	31020	17625	7.036	1.00
2B-2.513	2	2	B	Intestine	14703	7386	3.034	1.00
2B-2.515	2	2	B	Gill	21779	13015	6.552	1.00
2B-3.737	2	3	B	Intestine	13111	6761	5.043	1.00
2B-3.739	2	3	B	Gill	15217	6121	3.809	1.00
2B-4.929	2	4	B	Intestine	26044	17559	3.299	1.00
2B-4.931	2	4	B	Gill	10910	7022	6.059	1.00
2C-1.340	2	1	C	Intestine	12393	7203	5.171	1.00
2C-1.342	2	1	C	Gill	108458	54612	7.996	1.00
2C-2.522	2	2	C	Intestine	31231	23276	3.065	1.00
2C-2.524	2	2	C	Gill	4129	1293	2.889	1.00

Table 2.4
(continued)

2C-3.746	2	3	C	Intestine	9281	4982	3.983	1.00
2C-3.748	2	3	C	Gill	15860	9189	4.917	1.00
2C-4.938	2	4	C	Intestine	9175	6552	3.548	1.00
2C-4.940	2	4	C	Gill	6262	3801	5.160	1.00
3A-1.351	3	1	A	Gill	49701	23424	6.993	1.00
3A-2.531	3	2	A	Intestine	38464	20892	3.846	1.00
3A-2.533	3	2	A	Gill	9002	5930	5.856	1.00
3A-3.755	3	3	A	Intestine	23055	11298	6.067	1.00
3A-3.757	3	3	A	Gill	13391	8116	5.735	1.00
3A-4.947	3	4	A	Intestine	39986	28905	2.652	1.00
3A-4.949	3	4	A	Gill	20059	12601	6.395	1.00
3B-1.358	3	1	B	Intestine	11910	8395	3.096	1.00
3B-1.360	3	1	B	Gill	48884	25394	7.157	1.00
3B-2.540	3	2	B	Intestine	19605	9064	4.456	1.00
3B-2.542	3	2	B	Gill	15116	8742	5.835	1.00
3B-3.764	3	3	B	Intestine	55744	16374	4.975	1.00
3B-3.766	3	3	B	Gill	15317	8698	4.865	1.00
3B-4.956	3	4	B	Intestine	36162	23642	3.264	1.00
3B-4.958	3	4	B	Gill	170685	85369	6.501	1.00
3C-1.367	3	1	C	Intestine	18673	11285	4.067	1.00
3C-1.369	3	1	C	Gill	123012	59855	6.892	1.00
3C-2.549	3	2	C	Intestine	13362	9742	2.451	1.00
3C-2.551	3	2	C	Gill	28669	15966	6.588	1.00
3C-3.775	3	3	C	Gill	49930	27869	7.026	1.00
3C-4.965	3	4	C	Intestine	75486	45637	5.620	1.00
3C-4.967	3	4	C	Gill	1112	559	4.298	1.00
4A-1.378	4	1	A	Gill	5409	4127	3.238	1.00
4A-2.558	4	2	A	Intestine	18385	211	0.000	1.00
4A-2.560	4	2	A	Gill	5210	3273	5.088	1.00
4A-3.782	4	3	A	Intestine	12978	4926	3.515	1.00
4A-3.784	4	3	A	Gill	5971	3830	4.358	1.00
4A-4.974	4	4	A	Intestine	113	26	2.067	1.00
4A-4.976	4	4	A	Gill	29527	15824	4.943	1.00
4B-1.385	4	1	B	Intestine	29719	17681	3.364	1.00
4B-1.387	4	1	B	Gill	27188	17084	3.389	1.00
4B-2.569	4	2	B	Gill	7185	4794	3.729	1.00
4B-3.791	4	3	B	Intestine	19046	11211	4.141	1.00
4B-3.793	4	3	B	Gill	7797	5227	5.849	1.00
4B-4.983	4	4	B	Intestine	44298	485	0.000	1.00
4B-4.985	4	4	B	Gill	34049	17825	6.836	1.00
4C-1.396	4	1	C	Gill	10910	7248	5.063	1.00

Table 2.4
(continued)

4C-2.576	4	2	C	Intestine	38413	877	0.000	1.00
4C-2.578	4	2	C	Gill	25625	16108	4.778	1.00
4C-3.802	4	3	C	Gill	7542	5278	4.303	1.00
4C-4.992	4	4	C	Intestine	51630	35483	2.894	1.00
4C-4.994	4	4	C	Gill	12436	7659	5.775	1.00
5A-1.405	5	1	A	Gill	9178	5258	5.524	1.00
5A-2.587	5	2	A	Gill	11597	6881	5.038	1.00
5A-3.809	5	3	A	Intestine	25184	14560	5.160	1.00
5A-3.811	5	3	A	Gill	74026	43078	5.533	1.00
5A-4.1001	5	4	A	Intestine	17154	8387	4.955	1.00
5A-4.1003	5	4	A	Gill	6818	3634	4.403	1.00
5B-1.414	5	1	B	Gill	17578	9957	6.247	1.00
5B-2.594	5	2	B	Intestine	8296	6653	1.876	1.00
5B-2.596	5	2	B	Gill	12042	7155	4.953	1.00
5B-3.818	5	3	B	Intestine	74116	29782	6.868	1.00
5B-3.820	5	3	B	Gill	17609	10234	5.913	1.00
5B-4.1010	5	4	B	Intestine	57857	27818	5.488	1.00
5B-4.1012	5	4	B	Gill	7276	4777	5.742	1.00
5C-1.421	5	1	C	Intestine	41088	28022	3.438	1.00
5C-1.423	5	1	C	Gill	17199	9713	6.201	1.00
5C-2.603	5	2	C	Intestine	29311	17997	5.489	1.00
5C-2.605	5	2	C	Gill	9851	5659	5.903	1.00
5C-3.829	5	3	C	Gill	82799	48492	7.090	1.00
5C-4.1019	5	4	C	Intestine	22591	13442	6.110	1.00
5C-4.1021	5	4	C	Gill	75792	42979	8.296	1.00
6A-1.432	6	1	A	Gill	19110	10566	6.418	1.00
6A-2.612	6	2	A	Intestine	24941	9817	4.082	1.00
6A-2.614	6	2	A	Gill	14363	8348	5.908	1.00
6A-3.838	6	3	A	Gill	17581	9651	5.041	1.00
6A-4.1028	6	4	A	Intestine	25225	15545	5.118	1.00
6A-4.1030	6	4	A	Gill	51257	27937	8.091	1.00
6B-1.439	6	1	B	Intestine	12303	9667	2.126	1.00
6B-1.441	6	1	B	Gill	18836	9657	6.254	1.00
6B-2.621	6	2	B	Intestine	23757	9766	3.865	1.00
6B-2.623	6	2	B	Gill	32416	19346	7.289	1.00
6B-3.847	6	3	B	Gill	17237	9563	6.139	1.00
6B-4.1037	6	4	B	Intestine	35697	20331	5.741	1.00
6B-4.1039	6	4	B	Gill	37088	21147	6.809	1.00
6C-1.448	6	1	C	Intestine	13753	6902	4.957	1.00
6C-1.450	6	1	C	Gill	14177	8385	6.675	1.00
6C-2.630	6	2	C	Intestine	24809	115	0.000	1.00

Table 2.4
(continued)

6C-2.632	6	2	C	Gill	36820	20258	5.799	1.00
6C-3.854	6	3	C	Intestine	15959	17	0.000	1.00
6C-3.856	6	3	C	Gill	65895	36694	7.379	1.00
6C-4.1046	6	4	C	Intestine	16721	11106	5.629	1.00
6C-4.1048	6	4	C	Gill	42830	24568	7.393	1.00
SE.104	Sed	0	N/A	Sediment	62834	30282	9.720	1.00
SE.105	Sed	0	N/A	Sediment	51238	29893	9.626	1.00
SE.106	Sed	0	N/A	Sediment	64483	37138	9.693	1.00
SE.1092	Sed	4	N/A	Sediment	92002	46340	7.713	1.00
SE.1093	Sed	4	N/A	Sediment	80058	43721	6.257	1.00
SE.1094	Sed	4	N/A	Sediment	68020	30128	7.668	1.00
SE.1095	Sed	4	N/A	Sediment	70775	30982	7.410	1.00
SE.1096	Sed	4	N/A	Sediment	30815	16247	7.165	1.00
SE.1097	Sed	4	N/A	Sediment	66649	27068	7.164	1.00
UB.1068	Water	4	N/A	Water	36325	17333	4.833	1.00
UB.1069	Water	4	N/A	Water	93504	50245	6.719	1.00
UB.1070	Water	4	N/A	Water	82037	40671	6.705	0.999
UB.1071	Water	4	N/A	Water	139838	67844	6.447	1.00
UB.1072	Water	4	N/A	Water	84292	47110	7.600	1.00
UB.1073	Water	4	N/A	Water	84202	46863	6.619	1.00
W1.4.158	Water	0	N/A	Water	23288	11319	5.497	1.00
W2.5.159	Water	0	N/A	Water	19843	7220	5.009	1.00
W3.6.160	Water	0	N/A	Water	42305	21207	5.189	1.00

Table 2.5 *PERMANOVA test for experimental factors and factor interactions.*

Factor	Df	Sums of Sqs	Mean Sqs	F Model	R^2	R^2 %	p-value
Oil Concentration	3	3.180	1.060	3.866	0.063	6.36%	**1.00E-04**
Bacterial Challenge	1	0.406	0.406	1.480	0.008	0.81%	0.086
Sampling Point	1	1.158	1.158	4.224	0.023	2.32%	**1.00E-04**
Matrix	3	7.977	2.659	9.697	0.159	15.95%	**1.00E-04**
Oil Concentration: Bacterial Challenge	2	1.030	0.515	1.878	0.021	2.06%	**0.004**
Oil Concentration: Sampling Point	2	0.911	0.455	1.661	0.018	1.82%	**0.012**
Oil Concentration: Matrix	6	2.129	0.355	1.294	0.043	4.26%	**0.023**
Bacterial Challenge: Sampling Point	1	0.433	0.433	1.580	0.009	0.87%	0.055
Bacterial Challenge: Matrix	3	0.970	0.323	1.179	0.019	1.94%	0.159
Sampling Point: Matrix	3	2.450	0.817	2.978	0.049	4.90%	**1.00E-04**
Oil Concentration: Bacterial Challenge: Sampling Point	2	0.553	0.277	1.009	0.011	1.11%	0.425
Oil Concentration: Bacterial Challenge: Matrix	6	1.847	0.308	1.122	0.037	3.69%	0.173
Oil Concentration: Sampling Point: Matrix	6	1.561	0.260	0.949	0.031	3.12%	0.637
Bacterial Challenge: Sampling Point: Matrix	1	0.282	0.282	1.030	0.006	0.56%	0.383
Oil Concentration: Bacterial Challenge: Sampling Point: Matrix	2	0.450	0.225	0.821	0.009	0.90%	0.780
Residuals	90	24.679	0.274	NA	0.493	49.34%	NA
Total	132	50.016	NA	NA	1		NA

PERMANOVA calculated on beta diversity based on Bray-Curtis dissimilarity of the samples. Significance indicated in bold ($\alpha < 0.05$).

Table 2.6 *Kruskal-Wallis test results based on alpha diversity of gill samples for all treatments.*

Group 1	Group 2	H	p-value	q-value
Control (n=10)	Low Oil, No Bacteria (n=11)	10.492	0.0011	**0.004**
Control (n=10)	High Oil, No Bacteria (n=11)	14.459	0.0001	**0.001**
Control (n=10)	No Oil, Bacteria (n=12)	2.504	0.1135	0.170
Control (n=10)	Low Oil, Bacteria (n=12)	12.678	0.0004	**0.002**
Control (n=10)	High Oil, Bacteria (n=12)	15.135	0.0001	**0.001**
Low Oil, No Bacteria (n=11)	High Oil, No Bacteria (n=11)	0.182	0.6695	0.670
Low Oil, No Bacteria (n=11)	No Oil, Bacteria (n=12)	6.367	0.0116	0.025
Low Oil, No Bacteria (n=11)	Low Oil, Bacteria (n=12)	0.379	0.5383	0.577
Low Oil, No Bacteria (n=11)	High Oil, Bacteria (n=12)	1.095	0.2954	0.369
High Oil, No Bacteria (n=11)	No Oil, Bacteria (n=12)	9.852	0.0017	**0.004**
High Oil, No Bacteria (n=11)	Low Oil, Bacteria (n=12)	2.004	0.1569	0.214
High Oil, No Bacteria (n=11)	High Oil, Bacteria (n=12)	0.545	0.4602	0.531
No Oil, Bacteria (n=12)	Low Oil, Bacteria (n=12)	5.880	0.0153	**0.029**
No Oil, Bacteria (n=12)	High Oil, Bacteria (n=12)	12.000	0.0005	**0.002**
Low Oil, Bacteria (n=12)	High Oil, Bacteria (n=12)	4.563	0.0327	0.054

Alpha diversity measured with Shannon index. Significance indicated in bold ($\alpha < 0.05$).

Table 2.7 *PERMANOVA test results based on beta diversity of gill samples for all treatments.*

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Control	High Oil, Bacteria	22	4.741	0.001	**0.002**
Control	High Oil, No Bacteria	21	4.662	0.001	**0.002**
Control	Low Oil, Bacteria	22	3.321	0.001	**0.002**
Control	Low Oil, No Bacteria	21	3.046	0.001	**0.002**
Control	No Oil, Bacteria	22	0.816	0.633	0.633
High Oil, Bacteria	High Oil, No Bacteria	23	1.149	0.289	0.333
High Oil, Bacteria	Low Oil, Bacteria	24	1.511	0.064	0.080
High Oil, Bacteria	Low Oil, No Bacteria	23	1.594	0.026	**0.039**
High Oil, Bacteria	No Oil, Bacteria	24	4.616	0.001	**0.002**
High Oil, No Bacteria	Low Oil, Bacteria	23	1.576	0.05	0.068
High Oil, No Bacteria	Low Oil, No Bacteria	22	1.716	0.021	**0.035**
High Oil, No Bacteria	No Oil, Bacteria	23	4.685	0.001	**0.002**
Low Oil, Bacteria	Low Oil, No Bacteria	23	1.025	0.403	0.432
Low Oil, Bacteria	No Oil, Bacteria	24	3.305	0.001	**0.002**
Low Oil, No Bacteria	No Oil, Bacteria	23	2.753	0.002	**0.004**

Beta diversity measured with Bray-Curtis dissimilarity. Significance indicated in bold ($\alpha < 0.05$).

Table 2.8 Kruskal-Wallis test results for alpha diversity of intestine samples for all treatments.

Group 1	Group 2	H	p-value	q-value
Control (n=7)	Low Oil, No Bacteria (n=10)	0.467	0.495	0.660
Control (n=7)	High Oil, No Bacteria (n=10)	1.152	0.283	0.660
Control (n=7)	No Oil, Bacteria (n=4)	2.286	0.131	0.582
Control (n=7)	Low Oil, Bacteria (n=8)	0.482	0.487	0.660
Control (n=7)	High Oil, Bacteria (n=7)	0.004	0.949	0.949
Low Oil, No Bacteria (n=10)	High Oil, No Bacteria (n=10)	0.006	0.940	0.949
Low Oil, No Bacteria (n=10)	No Oil, Bacteria (n=4)	0.720	0.396	0.660
Low Oil, No Bacteria (n=10)	Low Oil, Bacteria (n=8)	1.547	0.214	0.641
Low Oil, No Bacteria (n=10)	High Oil, Bacteria (n=7)	0.771	0.380	0.660
High Oil, No Bacteria (n=10)	No Oil, Bacteria (n=4)	0.320	0.572	0.660
High Oil, No Bacteria (n=10)	Low Oil, Bacteria (n=8)	2.021	0.155	0.582
High Oil, No Bacteria (n=10)	High Oil, Bacteria (n=7)	0.771	0.380	0.660
No Oil, Bacteria (n=4)	Low Oil, Bacteria (n=8)	2.885	0.089	0.582
No Oil, Bacteria (n=4)	High Oil, Bacteria (n=7)	2.286	0.131	0.582
Low Oil, Bacteria (n=8)	High Oil, Bacteria (n=7)	0.335	0.563	0.660

Alpha diversity measured with Shannon index. Significance indicated in bold ($\alpha < 0.05$).

Table 2.10 *PERMANOVA test results based on beta diversity of intestine samples for all treatments.*

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Control	High Oil, Bacteria	14	1.385	0.177	0.190
Control	High Oil, No Bacteria	17	2.325	0.031	0.083
Control	Low Oil, Bacteria	15	1.472	0.116	0.161
Control	Low Oil, No Bacteria	17	1.860	0.069	0.129
Control	No Oil, Bacteria	11	4.685	0.002	**0.015**
High Oil, Bacteria	High Oil, No Bacteria	17	1.465	0.152	0.175
High Oil, Bacteria	Low Oil, Bacteria	15	0.807	0.637	0.637
High Oil, Bacteria	Low Oil, No Bacteria	17	1.655	0.104	0.161
High Oil, Bacteria	No Oil, Bacteria	11	4.266	0.006	**0.023**
High Oil, No Bacteria	Low Oil, Bacteria	18	2.749	0.006	**0.023**
High Oil, No Bacteria	Low Oil, No Bacteria	20	1.632	0.118	0.161
High Oil, No Bacteria	No Oil, Bacteria	14	3.281	0.033	0.083
Low Oil, Bacteria	Low Oil, No Bacteria	18	1.830	0.045	0.096
Low Oil, Bacteria	No Oil, Bacteria	12	4.615	0.001	**0.015**
Low Oil, No Bacteria	No Oil, Bacteria	14	1.580	0.136	0.170

Significance indicated in bold ($\alpha < 0.05$).

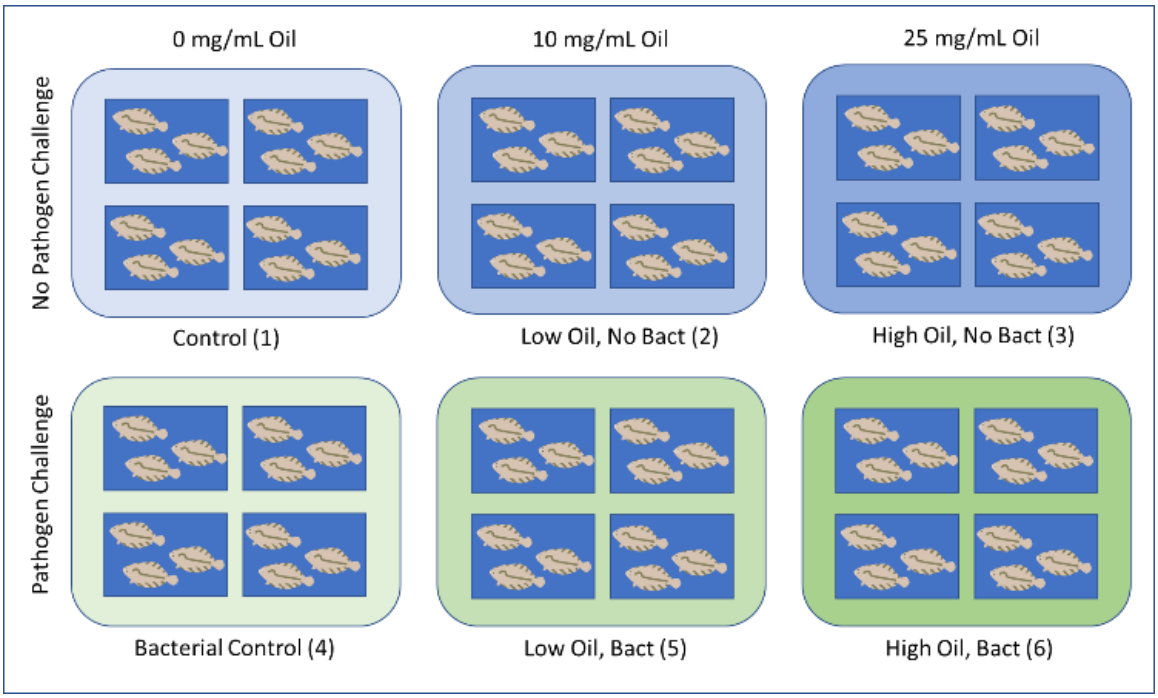


Figure 2.1 *Experimental Design*

Fully factorial experimental design for flounder exposure to three levels of oil from the *Deepwater Horizon* oil spill (0, 10, and 25 mg/mL) and a pathogenic bacterial challenge utilizing *Vibrio anguillarum*.

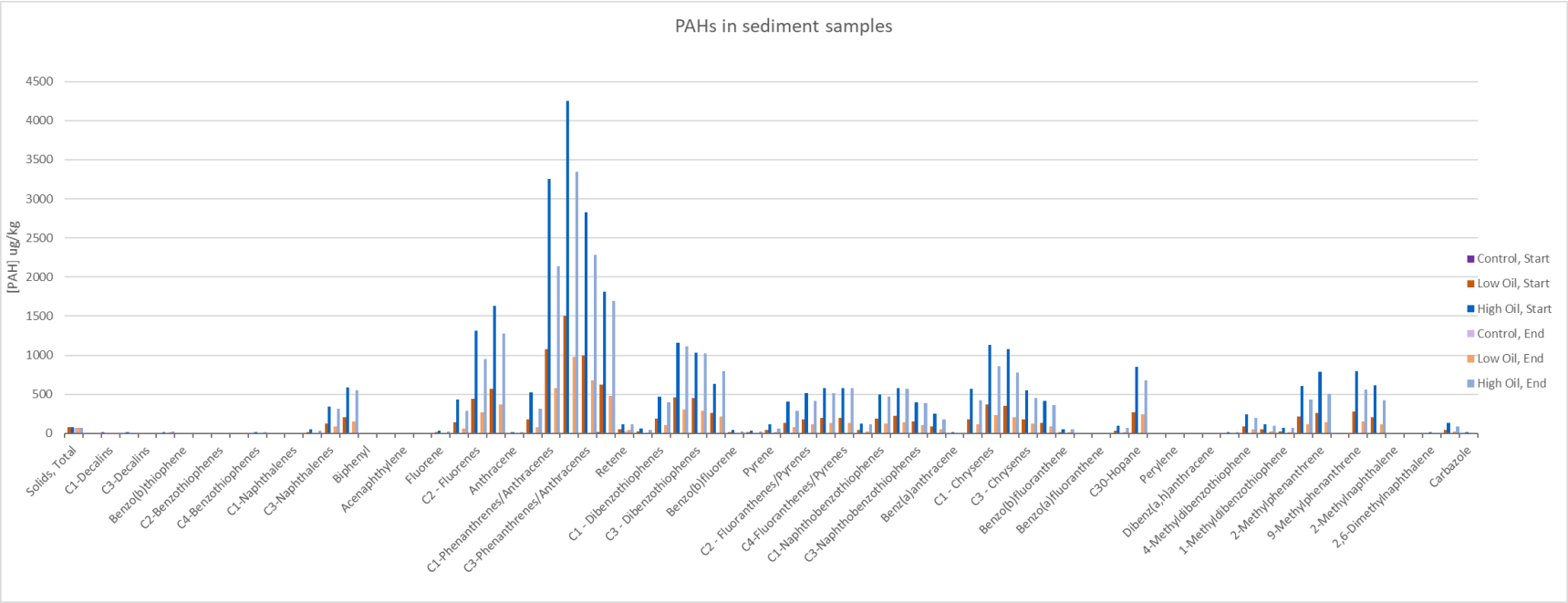


Figure 2.2 *Concentration of PAHs in sediment samples used for flounder exposure.*

Measured PAH concentrations (ug/kg) for Control, Low Oil, and High Oil treatments at the start and the end of the experiment.

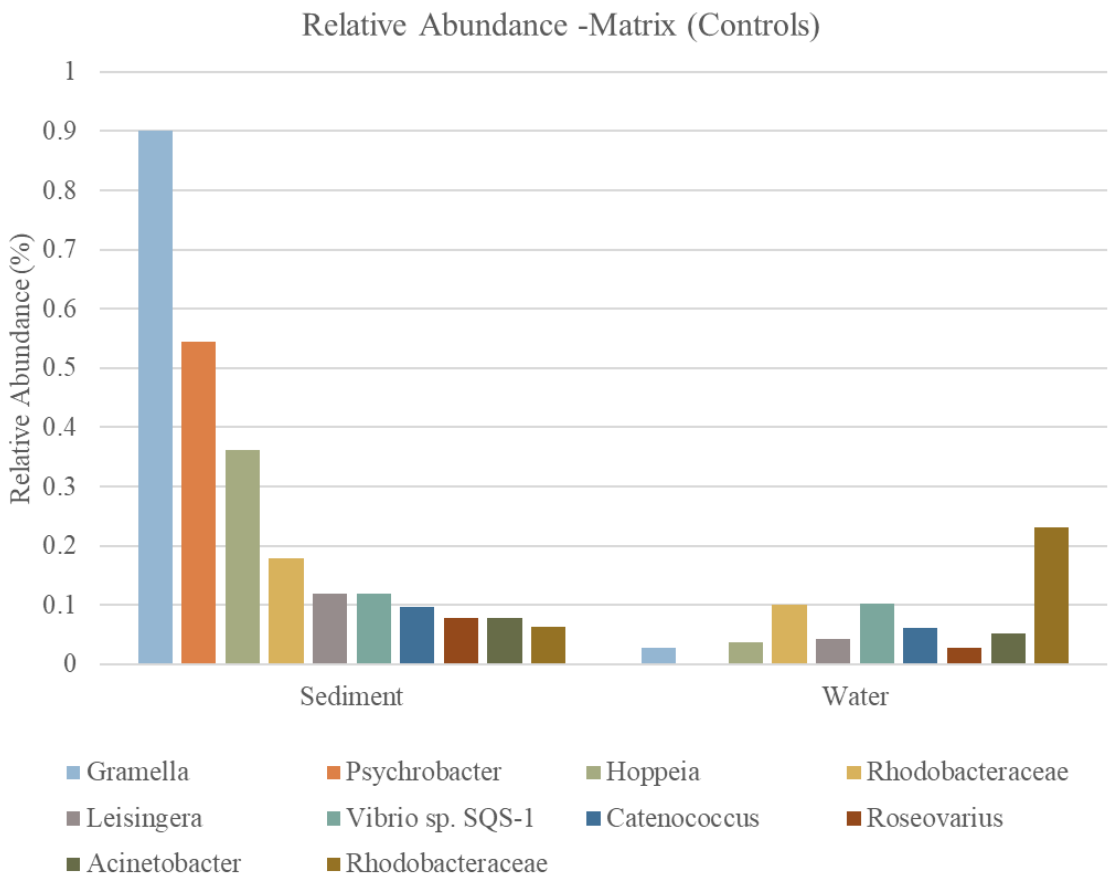
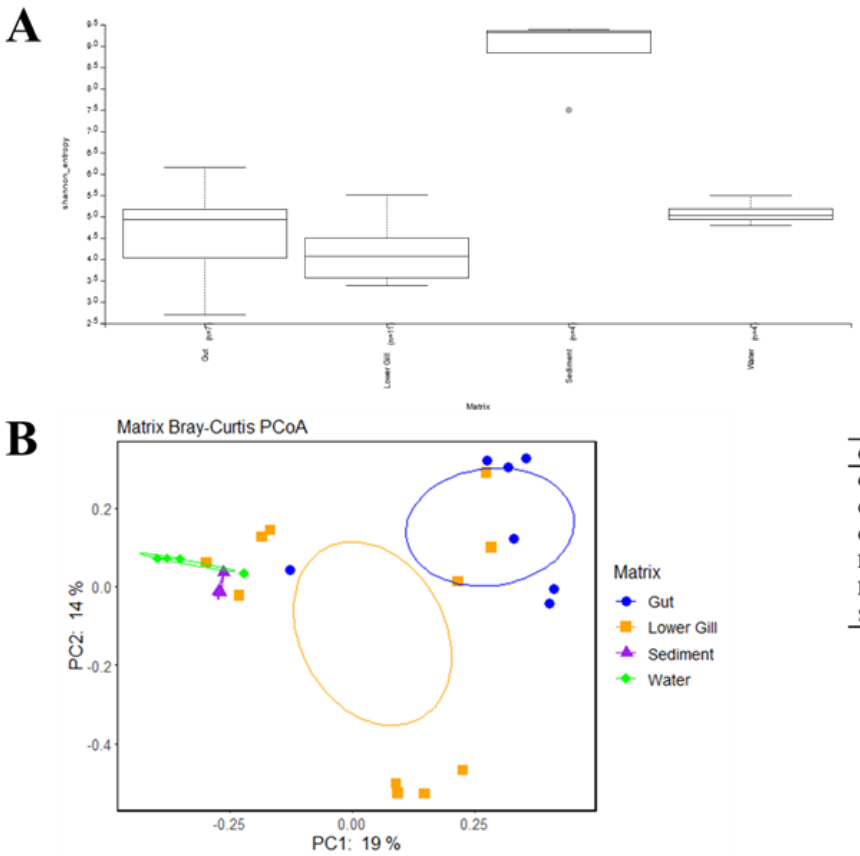


Figure 2.3 *Relative abundance of prominent taxa in sediment and water samples.*

Relative abundance of the 10 most prevalent taxa in the sediment and water samples, showing combined abundances from all six treatment tanks.

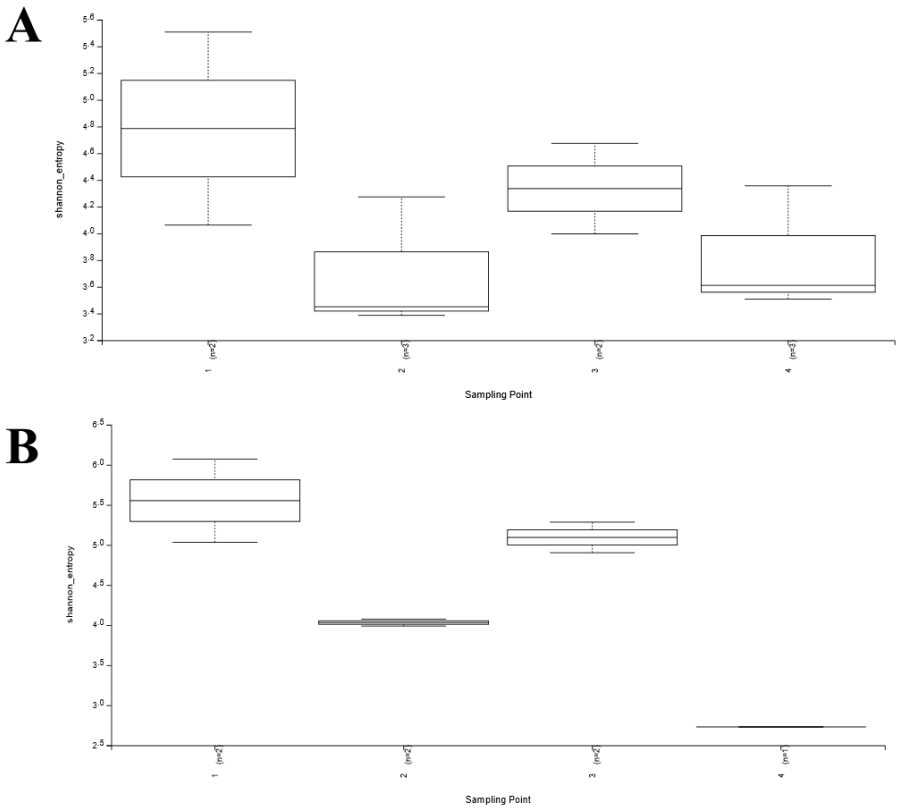


Group 1	Group 2	H	p-value	q-value
Gut (n=7)	Lower Gill (n=11)	1.085	0.298	0.357
Gut (n=7)	Sediment (n=4)	7.000	**0.008**	**0.024**
Gut (n=7)	Water (n=4)	0.571	0.450	0.450
Lower Gill (n=11)	Sediment (n=4)	8.250	**0.004**	**0.024**
Lower Gill (n=11)	Water (n=4)	4.364	**0.037**	0.056
Sediment (n=4)	Water (n=4)	5.333	**0.021**	**0.042**

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Gut	Lower Gill	18	2.814	**0.006**	**0.007**
Gut	Sediment	11	4.414	**0.002**	**0.006**
Gut	Water	11	5.322	**0.005**	**0.007**
Lower Gill	Sediment	15	2.628	**0.004**	**0.007**
Lower Gill	Water	15	3.032	**0.001**	**0.006**
Sediment	Water	8	2.322	**0.030**	**0.030**

Figure 2.4 *Diversity analysis for all sample matrices.*

Diversity analysis based on sample matrix (A) alpha diversity box plots and Kruskal-Wallis test results based on Shannon diversity index, (B) beta diversity PCoA and PERMANOVA test results based on Bray-Curtis dissimilarity, ellipses indicate 95% confidence. Significance indicated in bold ($\alpha < 0.05$).

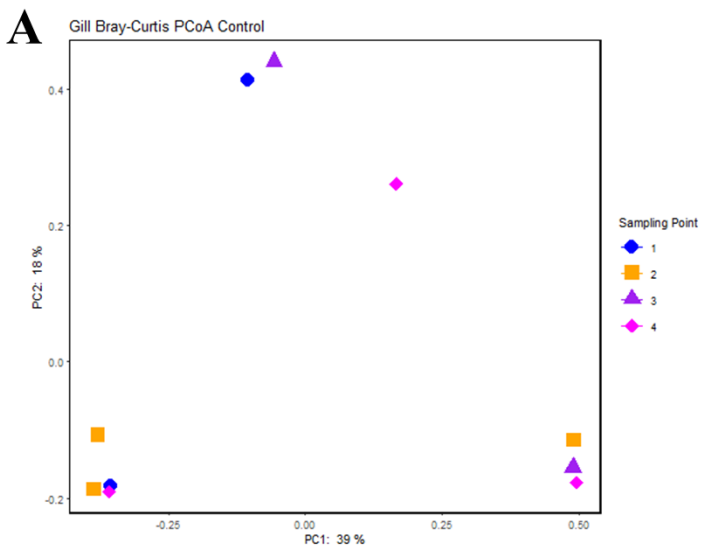


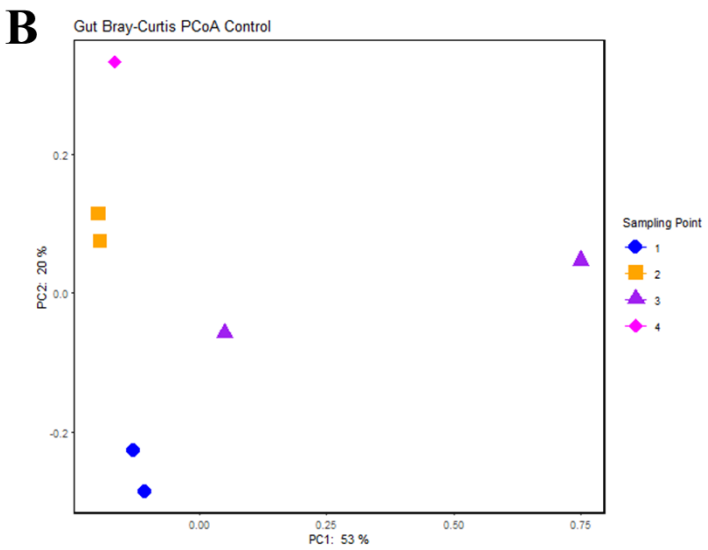
Group 1	Group 2	H	p-value	q-value
1 (n=2)	2 (n=3)	1.333	0.248	0.330
1 (n=2)	3 (n=2)	0.600	0.439	0.439
1 (n=2)	4 (n=3)	1.333	0.248	0.330
2 (n=3)	3 (n=2)	1.333	0.248	0.330
2 (n=3)	4 (n=3)	1.190	0.275	0.330
3 (n=2)	4 (n=3)	1.333	0.248	0.330

Group 1	Group 2	H	p-value	q-value
1 (n=2)	2 (n=2)	2.4	0.121	0.265
1 (n=2)	3 (n=2)	0.6	0.439	0.439
1 (n=2)	4 (n=1)	1.5	0.221	0.265
2 (n=2)	3 (n=2)	2.4	0.121	0.265
2 (n=2)	4 (n=1)	1.5	0.221	0.265
3 (n=2)	4 (n=1)	1.5	0.221	0.265

Figure 2.5 *Alpha diversity analysis for sampling timepoints.*

Box plots and Kruskal-Wallis test results for four sampling timepoints for (A) gill samples and (B) intestine samples in the Control treatment.





Group 1	Group 2	Sample size (n)	pseudo-F	p-value	q-value
1	2	5	0.805	0.614	0.699
1	3	4	0.969	0.699	0.699
1	4	5	0.986	0.521	0.699
2	3	5	1.005	0.524	0.699
2	4	6	0.990	0.497	0.699
3	4	5	0.904	0.616	0.699

Group 1	Group 2	Sample size (n)	pseudo-F	p-value	q-value
1	2	4	1.799	0.326	0.440
1	3	4	1.724	0.342	0.440
1	4	3	2.118	0.319	0.440
2	3	4	2.038	0.367	0.440
2	4	3	1.262	0.301	0.440
3	4	3	0.981	0.672	0.672

Figure 2.6 *Beta diversity analysis for sampling timepoints.*

PCoA and PERMANOVA test results for four sampling timepoints for (A) gill samples and (B) intestine samples in the Control treatment.

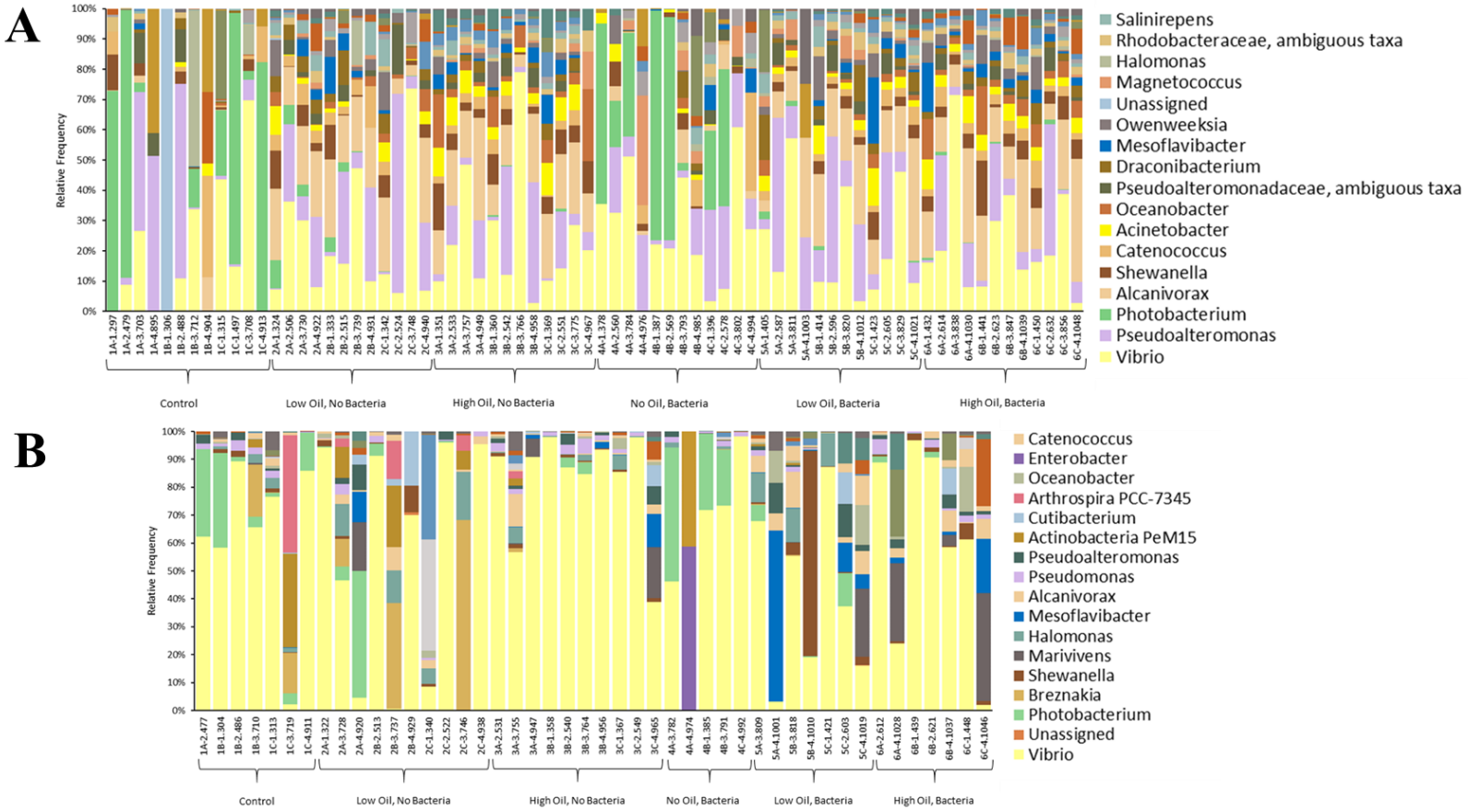
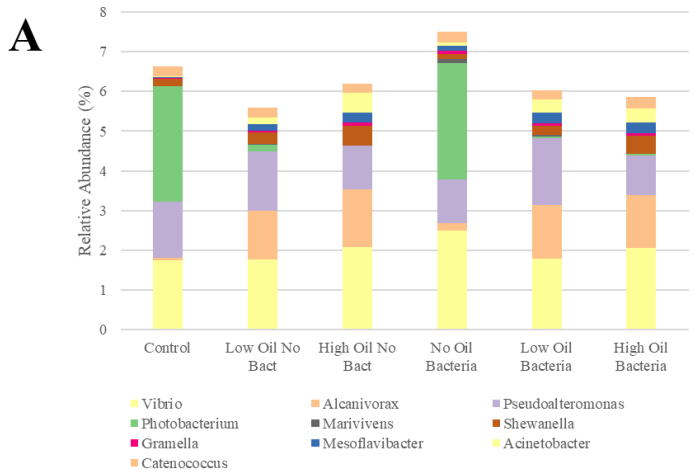
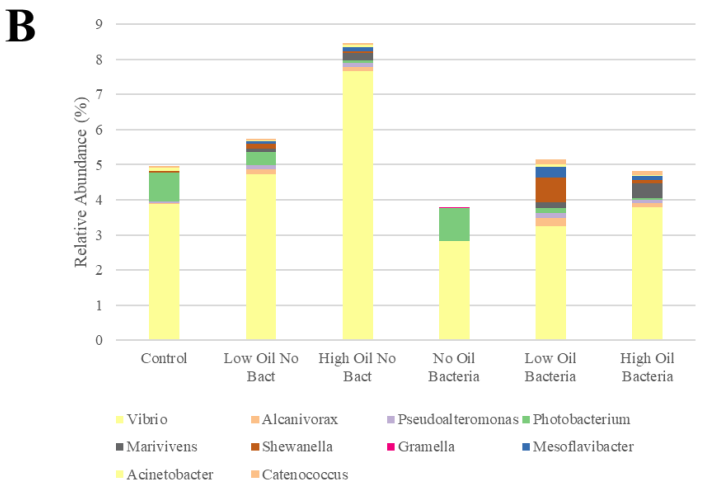


Figure 2.7 *Taxonomic bar plots.*

Taxonomic bar plots for flounder (A) gill and (B) intestine microbiome samples across all treatments.



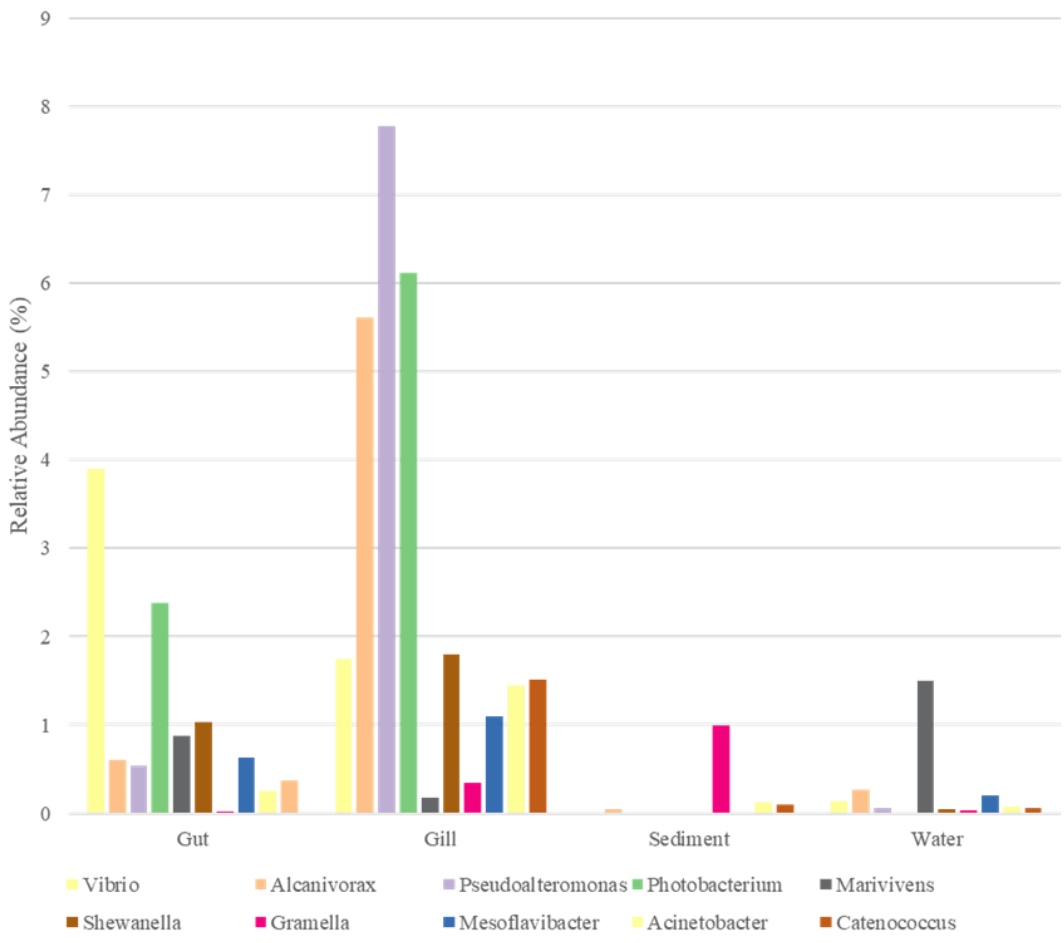


	Control (%)	Low Oil No Bacteria (%)	High Oil No Bacteria (%)	No Oil Bacteria (%)	Low Oil Bacteria (%)	High Oil Bacteria (%)
Vibrio	1.751	1.771	2.085	2.490	1.778	2.056
Alcanivorax	0.054	1.228	1.457	0.195	1.353	1.327
Pseudoalteromonas	1.413	1.485	1.083	1.089	1.698	1.005
Photobacterium	2.909	0.179	0.014	2.942	0.040	0.032
Marivivens	0.000	0.022	0.004	0.111	0.030	0.003
Shewanella	0.196	0.289	0.495	0.117	0.244	0.458
Gramella	0.021	0.028	0.080	0.083	0.062	0.070
Mesoflavibacter	0.018	0.170	0.250	0.128	0.259	0.268
Acinetobacter	0.025	0.165	0.497	0.087	0.325	0.355
Catenococcus	0.237	0.264	0.219	0.269	0.233	0.289

	Control (%)	Low Oil No Bacteria (%)	High Oil No Bacteria (%)	No Oil Bacteria (%)	Low Oil Bacteria (%)	High Oil Bacteria (%)
Vibrio	3.894	4.735	7.671	2.814	3.259	3.799
Alcanivorax	0.001	0.144	0.122	0.000	0.220	0.115
Pseudoalteromonas	0.058	0.106	0.116	0.022	0.154	0.089
Photobacterium	0.825	0.375	0.071	0.927	0.144	0.041
Marivivens	0.004	0.109	0.200	0.002	0.144	0.418
Shewanella	0.043	0.131	0.050	0.000	0.704	0.105
Gramella	0.009	0.000	0.002	0.001	0.009	0.003
Mesoflavibacter	0.000	0.070	0.123	0.000	0.311	0.120
Acinetobacter	0.071	0.025	0.066	0.000	0.066	0.027
Catenococcus	0.052	0.038	0.037	0.000	0.137	0.110

Figure 2.8 *Analysis of most abundant taxa by treatment.*

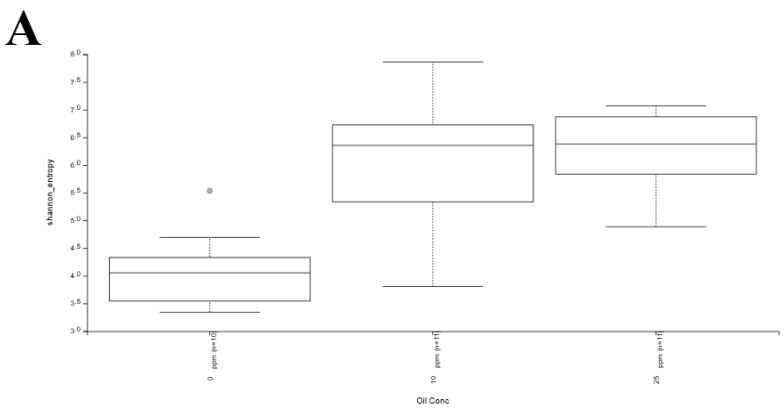
Relative abundance for 10 most prevalent taxa across all samples for (A) gill and (B) intestine samples for all treatments.

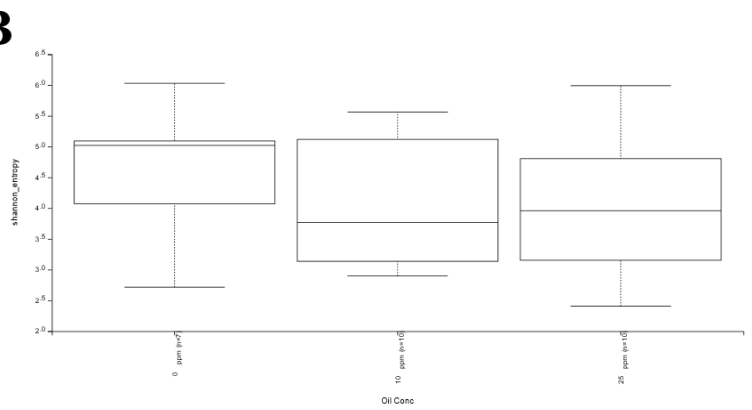


	Gut (%)	Gill (%)	Sediment (%)	Water (%)
Vibrio	3.894	1.752	0.004	0.139
Alcanivorax	0.601	5.613	0.049	0.270
Pseudoalteromonas	0.545	7.774	0.001	0.054
Photobacterium	2.382	6.117	0.008	0.003
Marivivens	0.878	0.171	0.002	1.505
Shewanella	1.034	1.799	0.003	0.050
Gramella	0.025	0.344	0.988	0.032
Mesoflavibacter	0.625	1.094	0.008	0.204
Acinetobacter	0.255	1.453	0.129	0.079
Catenococcus	0.373	1.513	0.097	0.062

Figure 2.9 *Analysis of most abundant taxa by matrix.*

Relative abundance of 10 most prevalent taxa across all samples for all four sample matrices.



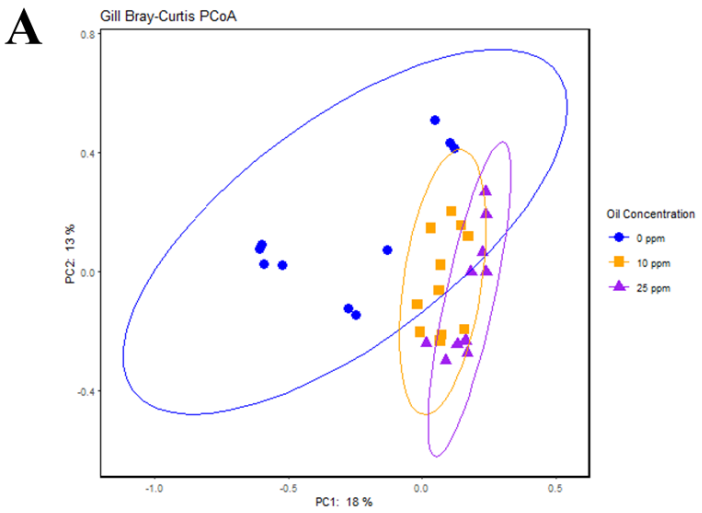


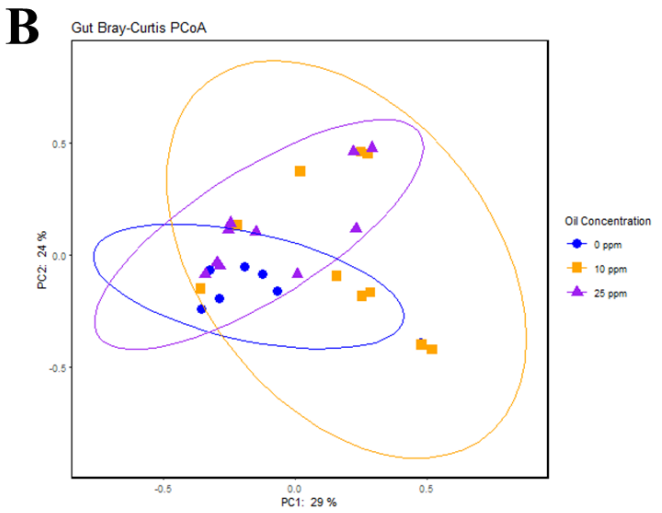
Group 1	Group 2	H	p-value	q-value
0 mg/kg (n=10)	10 mg/kg (n=11)	10.4926	**0.0012**	**0.0018**
0 mg/kg (n=10)	25 mg/kg (n=11)	14.4595	**0.0001**	**0.0004**
10 mg/kg (n=11)	25 mg/kg (n=11)	0.1822	0.6695	0.6695

Group 1	Group 2	H	p-value	q-value
0 ppm (n=7)	10 ppm (n=10)	0.771	0.380	0.570
0 ppm (n=7)	25 ppm (n=10)	1.371	0.242	0.570
10 ppm (n=10)	25 ppm (n=10)	0.006	0.940	0.940

Figure 2.10 *Alpha diversity analysis following oil exposure.*

Alpha diversity box plots and Kruskal-Wallis test results for oiled treatments for (A) gill microbiome and (B) intestine microbiome. Significance indicated in bold ($\alpha < 0.05$).



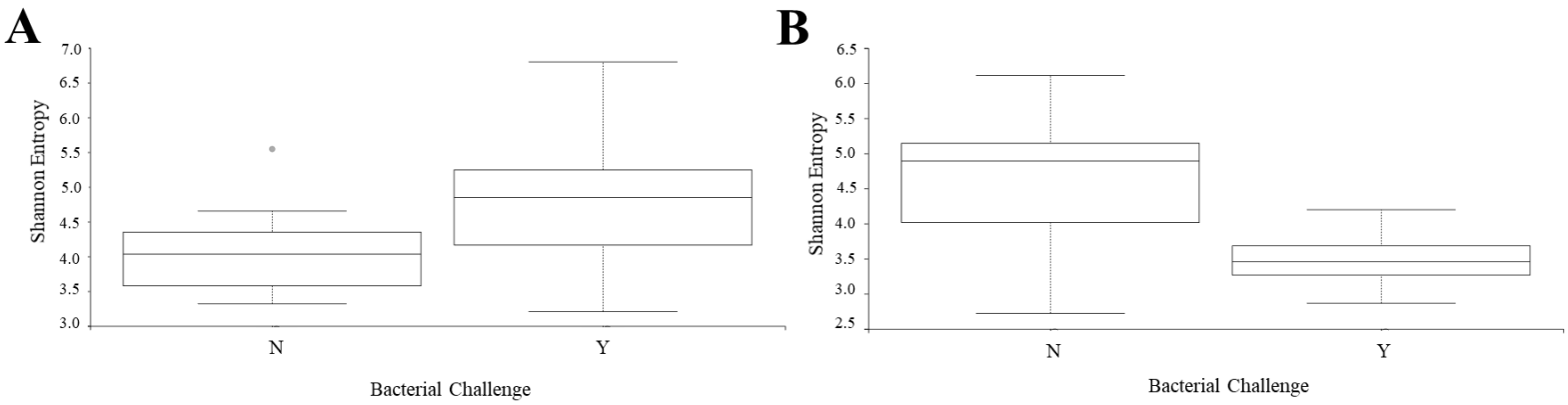


Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
0 mg/kg	10 mg/kg	21	3.003	**0.001**	**0.002**
0 mg/kg	25 mg/kg	21	4.507	**0.001**	**0.002**
10 mg/kg	25 mg/kg	22	1.718	**0.034**	**0.034**

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
0 mg/kg	10 mg/kg	17	1.809	0.076	0.114
0 mg/kg	25 mg/kg	17	2.347	**0.018**	0.054
10 mg/kg	25 mg/kg	20	1.651	0.12	0.12

Figure 2.11 *Beta diversity analysis following oil exposure.*

Beta diversity PCoA and PERMANOVA test results for oiled treatments for (A) gill microbiome and (B) intestine microbiome, ellipses indicate 95% confidence. Significance indicated in bold ($\alpha < 0.05$).

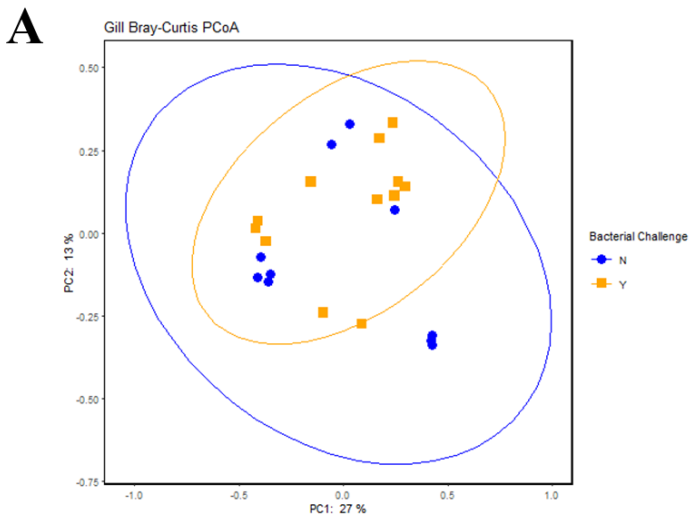


Group 1	Group 2	H	p-value	q-value
N (n=10)	Y (n=12)	2.504	0.114	0.114

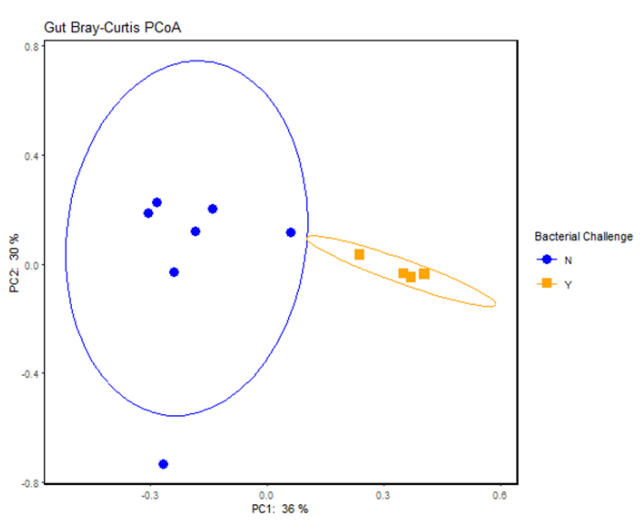
Group 1	Group 2	H	p-value	q-value
N (n=7)	Y (n=4)	2.286	0.131	0.131

Figure 2.12 *Alpha diversity analysis following pathogenic bacteria challenge.*

Alpha diversity box plots and Kruskal-Wallis test results for pathogenic bacteria exposure for (A) gill microbiome and (B) intestine microbiome. “N” indicates the No Bacteria Challenge treatment, “Y” indicates the Bacteria Challenge treatment. Significance indicated in bold ($\alpha < 0.05$).



Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
N	Y	22	0.836	0.591	0.591

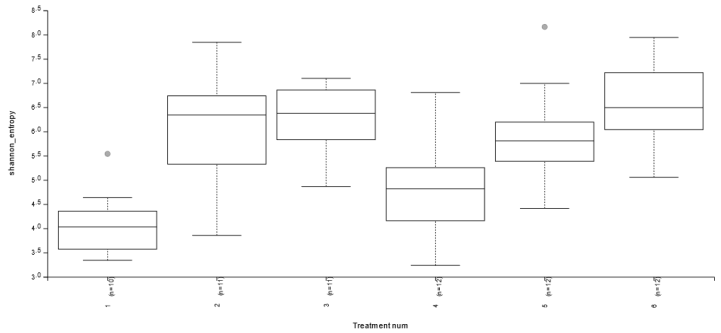


Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
N	Y	11	4.439	**0.003**	**0.003**

Figure 2.13 *Beta diversity analysis following pathogenic bacteria challenge.*

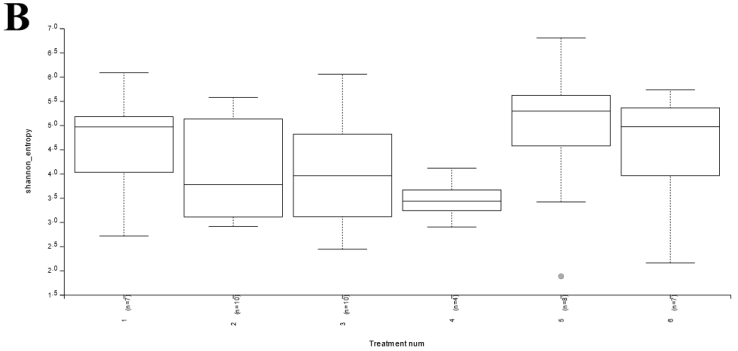
Beta diversity PCoA and PERMANOVA test results for pathogenic bacteria exposure for (A) gill microbiome and (B) intestine microbiome, ellipses indicate 95% confidence. “N indicates the No Bacteria Challenge treatment, “Y” indicates the Bacteria Challenge treatment. Significance indicated in bold ($\alpha < 0.05$).

A



Group 1	Group 2	H	p-value	q-value
Control (n=10)	Low Oil, No Bacteria (n=11)	10.49256	**0.001199**	**0.003596**
Control (n=10)	High Oil, No Bacteria (n=11)	14.4595	**0.000143**	**0.001074**
Control (n=10)	No Oil, Bacteria (n=12)	2.504348	0.113532	0.170299
Control (n=10)	Low Oil, Bacteria (n=12)	12.67826	**0.00037**	**0.00185**
Control (n=10)	High Oil, Bacteria (n=12)	15.13478	**0.0001**	**0.001074**
Low Oil, No Bacteria (n=11)	High Oil, No Bacteria (n=11)	0.182178	0.669509	0.669509
Low Oil, No Bacteria (n=11)	No Oil, Bacteria (n=12)	6.367424	*0.011623*	*0.024907*
Low Oil, No Bacteria (n=11)	Low Oil, Bacteria (n=12)	0.378788	0.538253	0.576699
Low Oil, No Bacteria (n=11)	High Oil, Bacteria (n=12)	1.094697	0.295433	0.369291
High Oil, No Bacteria (n=11)	No Oil, Bacteria (n=12)	9.852273	**0.001696**	**0.004241**
High Oil, No Bacteria (n=11)	Low Oil, Bacteria (n=12)	2.003788	0.156907	0.213964
High Oil, No Bacteria (n=11)	High Oil, Bacteria (n=12)	0.545455	0.460181	0.530978
No Oil, Bacteria (n=12)	Low Oil, Bacteria (n=12)	5.88	**0.015314**	**0.028713**
No Oil, Bacteria (n=12)	High Oil, Bacteria (n=12)	12	**0.000532**	**0.001995**
Low Oil, Bacteria (n=12)	High Oil, Bacteria (n=12)	4.563333	0.032663	0.054439

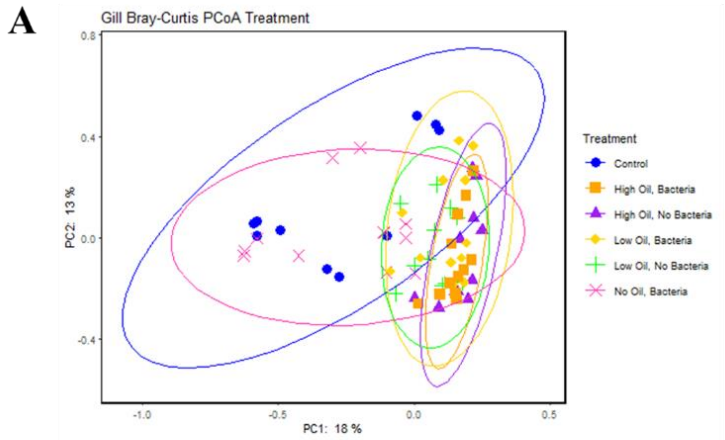
B

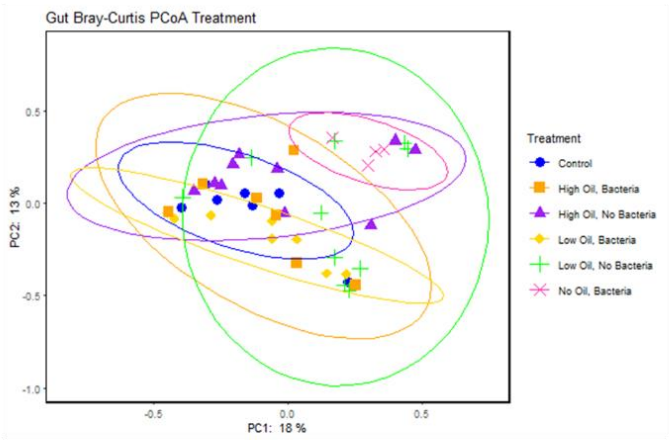


Group 1	Group 2	H	p-value	q-value
Control (n=7)	Low Oil, No Bacteria (n=10)	0.467	0.495	0.660
Control (n=7)	High Oil, No Bacteria (n=10)	1.152	0.283	0.660
Control (n=7)	No Oil, Bacteria (n=4)	2.286	0.131	0.582
Control (n=7)	Low Oil, Bacteria (n=8)	0.482	0.487	0.660
Control (n=7)	High Oil, Bacteria (n=7)	0.004	0.949	0.949
Low Oil, No Bacteria (n=10)	High Oil, No Bacteria (n=10)	0.006	0.940	0.949
Low Oil, No Bacteria (n=10)	No Oil, Bacteria (n=4)	0.720	0.396	0.660
Low Oil, No Bacteria (n=10)	Low Oil, Bacteria (n=8)	1.547	0.214	0.641
Low Oil, No Bacteria (n=10)	High Oil, Bacteria (n=7)	0.771	0.380	0.660
High Oil, No Bacteria (n=10)	No Oil, Bacteria (n=4)	0.320	0.572	0.660
High Oil, No Bacteria (n=10)	Low Oil, Bacteria (n=8)	2.021	0.155	0.582
High Oil, No Bacteria (n=10)	High Oil, Bacteria (n=7)	0.771	0.380	0.660
No Oil, Bacteria (n=4)	Low Oil, Bacteria (n=8)	2.885	0.089	0.582
No Oil, Bacteria (n=4)	High Oil, Bacteria (n=7)	2.286	0.131	0.582
Low Oil, Bacteria (n=8)	High Oil, Bacteria (n=7)	0.335	0.563	0.660

Figure 2.14 *Alpha diversity analysis following oil exposure and pathogenic bacteria challenge.*

Alpha diversity box plots and Kruskal-Wallis test results for all treatments for (A) gill microbiome and (B) intestine microbiome. Significance indicated in bold ($\alpha < 0.05$).





Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Control	High Oil, Bacteria	22	4.741	**0.001**	**0.002**
Control	High Oil, No Bacteria	21	4.662	**0.001**	**0.002**
Control	Low Oil, Bacteria	22	3.321	**0.001**	**0.002**
Control	Low Oil, No Bacteria	21	3.046	**0.001**	**0.002**
Control	No Oil, Bacteria	22	0.816	0.633	0.633
High Oil, Bacteria	High Oil, No Bacteria	23	1.149	0.289	0.333
High Oil, Bacteria	Low Oil, Bacteria	24	1.511	0.064	0.080
High Oil, Bacteria	Low Oil, No Bacteria	23	1.594	**0.026**	**0.039**
High Oil, Bacteria	No Oil, Bacteria	24	4.616	**0.001**	**0.002**
High Oil, No Bacteria	Low Oil, Bacteria	23	1.576	0.05	0.068
High Oil, No Bacteria	Low Oil, No Bacteria	22	1.716	**0.021**	**0.035**
High Oil, No Bacteria	No Oil, Bacteria	23	4.685	**0.001**	**0.002**
Low Oil, Bacteria	Low Oil, No Bacteria	23	1.025	0.403	0.432
Low Oil, Bacteria	No Oil, Bacteria	24	3.305	**0.001**	**0.002**
Low Oil, No Bacteria	No Oil, Bacteria	23	2.753	**0.002**	**0.004**

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Control	High Oil, Bacteria	14	1.385	0.177	0.190
Control	High Oil, No Bacteria	17	2.325	0.031	0.083
Control	Low Oil, Bacteria	15	1.472	0.116	0.161
Control	Low Oil, No Bacteria	17	1.860	0.069	0.129
Control	No Oil, Bacteria	11	4.685	**0.002**	**0.015**
High Oil, Bacteria	High Oil, No Bacteria	17	1.465	0.152	0.175
High Oil, Bacteria	Low Oil, Bacteria	15	0.807	0.637	0.637
High Oil, Bacteria	Low Oil, No Bacteria	17	1.655	0.104	0.161
High Oil, Bacteria	No Oil, Bacteria	11	4.266	**0.006**	**0.023**
High Oil, No Bacteria	Low Oil, Bacteria	18	2.749	**0.006**	**0.023**
High Oil, No Bacteria	Low Oil, No Bacteria	20	1.632	0.118	0.161
High Oil, No Bacteria	No Oil, Bacteria	14	3.281	**0.033**	0.083
Low Oil, Bacteria	Low Oil, No Bacteria	18	1.830	**0.045**	0.096
Low Oil, Bacteria	No Oil, Bacteria	12	4.615	**0.001**	**0.015**
Low Oil, No Bacteria	No Oil, Bacteria	14	1.580	0.136	0.170

Figure 2.15 *Beta diversity analysis following oil exposure and pathogenic bacteria challenge.*

Beta diversity PCoA and PERMANOVA test results for all treatments for (A) gill microbiome and (B) intestine microbiome, ellipses indicate 95% confidence. Significance indicated in bold ($\alpha < 0.05$).

CHAPTER III – THE INFLUENCE OF PAH CONTAMINANT LOADS AND REEF LOCATION ON EASTERN OYSTER DIGESTIVE GLAND MICROBIOME

Introduction

The Eastern oyster (*Crassostrea virginica*) is one of the most valued shellfish species in the Atlantic and Gulf of Mexico waters of the U.S., in 2016 US$90,399,000 in landings was recorded across the Gulf (National Marine Fisheries Service 2018). This species ranges from Canada to Argentina in areas of low salinity, intertidal zones (Breitburg, Coen, Luckenbach, and Mann 2000). Oysters are a reef building species that provide habitat for a diverse array of epibenthic invertebrates and fishes (Breitburg, Coen, Luckenbach, and Mann 2000, Coen et al. 2007). Oyster reefs also serve as marsh sediment stabilization, reducing erosion of marshes and other coastline habitats (Grabowski et al. 2012). The oysters themselves improve water quality as they filter feed, removing phytoplankton and organic material from the water column at a rate of up to 50 gal per day (Breitburg, Coen, Luckenbach, Mann, et al. 2000, zu Ermgassen et al. 2013). This carbon sequestration reduces the potential scope of eutrophication processes and the hypoxia that can occur as a result (Kellogg et al. 2014).

Oyster reefs are both ecologically and economically valuable, unfortunately oyster populations have been in decline for the last century in part due to over-fishing, natural disasters such as hurricanes, and other anthropogenic impacts including oil spills (Jackson et al. 2001, Coen et al. 2007, La Peyre et al. 2014, Posadas et al. 2017, Mississippi Department of Marine Resources 2019). Studying the impacts these events have on oysters will assist in recovery efforts in the future. One way to do this is to

examine the microbiome of the organism, as they are often an intermediary between the environment and the organism itself (Adamovsky et al. 2018).

Microbiomes are the communities of bacteria and other microbes that live on and within organisms (Colston and Jackson 2016). They provide many benefits to the host including aiding in digestion, providing food, and fighting pathogens (Kau et al. 2011, Maltby et al. 2013, Adamovsky et al. 2018). The bacteria within the microbiome community are often able to breakdown food molecules that the host cannot, for example plant polysaccharides including cellulose (Lee et al. 2015). A classic example of microbiota providing food for the host directly is zooxanthellae providing sugars for the corals that host them (Strychar and Sammarco 2011). Commensal bacteria are able to fight off pathogens before they are able to affect the host, for example microbiome assemblages on the skin of rainbow trout are able to inhibit the growth of two pathogenic fungi (Lowrey et al. 2015). Additionally, bacteria within the microbiome aid the host through biotransformation of xenobiotic contaminants, for example a strain of *Cellulosimicrobium* has been shown to reduce chromium contamination in soils and promote the growth of plants (Chatterjee et al. 2009).

Many studies have found identified microbiomes in oysters and established that communities can vary between hemolymph, gut, and gill tissues (Chauhan et al. 2014, Pierce 2016, Ossai et al. 2017). Li and Wang (2017) cataloged microbiomes in oysters from the Puget Sound and found that Tenericutes, Chlamydiae, Proteobacteria, and Firmicutes were the dominant taxa, and a few species of the *Vibrio* genus were present in all oysters. King et al. (2012) studied oysters from two sites in Louisiana, Lake Caillou and Hackberry Bay, and characterized the contents of both the stomach and intestine. At

both sites the intestine community differed from that of the stomach and was more diverse. In addition to differing by tissue, microbiomes can also differ by location and through time. In the same study from King et al. (2012), the microbial communities were significantly different between Lake Caillou and Hackberry Bay. At two sites in the Wadden Sea differences in microbiome were most strongly attributed to environmental conditions associated with each site (Lokmer, Goedknegt, et al. 2016). As the host organism grows through its development, the microbiome has been shown to change as well, and once the host reaches adulthood the microbiome remains changing through time (King et al. 2012, Stephens et al. 2016).

Microbiome composition can be also affected by contaminants and other abiotic stressors (Wegner et al. 2013). Lokmer and Wegner (2015) tested the effects of temperature, temperature stress, and a *Vibrio* infection challenge on the hemolymph microbiome of Pacific oysters (*Crassostrea gigas*). Temperature had more of an effect than *Vibrio* infection on the composition of the microbiome in both healthy and non-healthy oysters, disrupting the structure of the community and lowering diversity. Thomas et al. (2014) described the oyster microbiome and its propensity to degrade oil. After being exposed to oil in the water column, populations of microbes in the oysters more closely resembled populations of microbes in contaminated sediment and contained taxa that are commonly found in oiled environments in the Gulf, predominantly within the *Pseudomonas* genus. Microbiome dynamics are also linked to changes in oyster disease and survival due to the response of the microbiome to external biotic and abiotic factors including changes in temperature and salinity (Lokmer and Wegner 2015).

In April of 2010, the *Deepwater Horizon* drilling rig exploded resulting in the release of more than 3.15 million barrels amount of crude oil and natural gas into the water column (McNutt et al. 2011, Sammarco et al. 2013, Beyer et al. 2016). The Gulf of Mexico has a long history of oil production and inevitably oil spills, including the two largest spills the Ixtoc oil spill in 1979 and the *Deepwater Horizon* oil spill (Overton et al. 2004, Norse and Amos 2010). In addition to manmade spill events, the Gulf is known for natural seeps that also introduce oil contamination into the environment (MacDonald et al. 2015, Kennicutt 2017).

The most toxic effects of exposure to crude oil have been attributed to polycyclic aromatic hydrocarbons (PAHs) (Beyer et al. 2016). Some of these toxic properties can be predicted by the number of rings the molecule has and their origin, e.g., pyrogenic or petrogenic sources (Wang, Liu, et al. 2014). PAHs with fewer rings are lighter and thus tend to evaporate more quickly, while petrogenic PAHs tend to be more bioavailable than pyrogenic PAHs (Thorsen et al. 2004, Liu et al. 2016). Especially of note here are the PAHs that are deposited in sediments for their proximity to benthic organisms, including oysters, as well as decreased microbial degradation rates caused by hypoxic conditions and leading to increased long-term exposure potential (Thomas et al. 2014, Wang, Liu, et al. 2014).

Exposure to oil negatively affects oyster oocyte fertilization, including impaired sperm size and increased production of reactive oxygen species, as well as embryogenesis, for example deformed cells and arrested embryos (Laramore et al. 2014, Vignier et al. 2017). Oil exposure also has negative effects on all larval stages, including impaired survivorship, growth, and development (such as convex mantles, indented shell

margins, and protruded mantles) (Vignier et al. 2015, Finch et al. 2016, Langdon et al. 2016). Oyster densities and the overall area of oyster habitat in the northern Gulf were reduced post-spill (Powers, Grabowski, et al. 2017, Powers, Rouhani, et al. 2017). Work done before the *Deepwater Horizon* spill showed both lethal and sub-lethal effects of exposure to oil such as reduced growth, increased susceptibility to disease, and impaired reproduction (La Peyre et al. 2014).

Oysters in the Mississippi Sound face many challenges that have caused their populations to decline, including overfishing or large-scale contamination events, and as a result there have been increasing efforts to restore the native reefs (Mississippi Department of Marine Resources 2019). In order to successfully accomplish this, there needs to be data to inform the best practices. My goal is two-fold (1) to characterize the microbial communities of oysters collected from the Mississippi Sound, and (2) examine if there exists a correlation between PAH concentrations in oyster tissues and the bacterial community in the intestinal gland.

Materials and Methods

Field Collection and Laboratory Processing

Oyster and sediment samples were collected from fifteen commercially harvested oyster reefs located in the Mississippi Sound (Figure 3.1). As part of a citizen scientist effort, the utilization of local commercial fishermen's boats as research vessels was implemented for sampling. Water quality parameters were collected at each oyster and sediment sampling location. After collection, samples were driven to the Gulf Coast Research Laboratory in Ocean Springs, MS to be processed as follows. The outside of the

oyster was cleaned using a wire brush and rinsed with ethanol. Using aseptic techniques, each oyster was shucked from the bill, to avoid damaging the internal organs. Three samples of the intestinal gland from each oyster were removed, each sample was then immediately placed into RNAlater® (Invitrogen™, Waltham, MA) and frozen at -80 °C for storage before analysis. The remaining tissue was removed from the shells, homogenized, and stored at -20 °C until analysis. The oyster shells were ground until 15 g of homogenized sample was produced for analysis and stored at -20 °C. Sediment, tissue, and shell samples were shipped to the Mississippi State Chemistry Laboratory for analysis of PAH content using high performance liquid chromatography (HPLC) to determine absolute concentrations of 21 PAHs. The list of PAHs assessed can be found in Table 3.1.

Binning of Oyster Reefs

For further geographic analysis of the oyster microbiome, 11 reefs were binned according to their location within the Mississippi Sound. Western reefs include St. Joe Reef, Waveland Reef, St. Stanislaus Reef, Henderson Point, Hornets Reef, Pass Marianne, and Pass Christian Reef; central reefs include Biloxi Bay Reef and Shearwater Reef; and eastern reefs include Graveline Bayou and the Pascagoula Oyster Reef Complex. There were no oysters collected from the remaining 4 reefs (Buoy Reef, Pelican Reef, Telegraph Reef, and Long Beach Reef).

DNA Sequencing and Bioinformatics Analysis

DNA was extracted using the DNeasy PowerSoil® kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then sent to GENEWIZ for Illumina MiSeq 2x250bp paired-end sequencing of the V3-V4 region of the 16S rRNA gene. The extracted DNA was amplified at the V3-V4 regions via polymerase chain reaction (PCR), barcoded for each sample, and then run through a final quality control step before paired-end sequencing. Sequences were run through QIIME2 bioinformatics software as follows. First, sequences were demultiplexed using q2-demux and trimmed and denoised for quality control using DADA2 (Callahan et al. 2016). Sequences were then run through phylogenetic analysis via q2-phylogeny to identify relationships and frequency of sequences and assigned taxonomic labels as Amplicon Sequence Variants (ASVs) based on the SILVA 99% 138 reference database using the q2-feature-classifier (Bokulich et al 2018) classify-consensus-vsearch (Quast et al. 2013, Rognes et al. 2016, Bolyen et al. 2019). Shannon diversity index was used to determine the alpha diversity of the microbial communities in conjunction with the Kruskal-Wallis statistical test. Analyzing differences between the microbial assemblages of each treatment was done with PERMANOVA based on a Bray-Curtis dissimilarity matrix (using q2-diversity) in conjunction with Principal Coordinates Analysis (PCoA) generated in R (R Core Team 2020).

Results

PAH Analysis Results

Of the 15 PAHs measured, only two returned concentrations above levels of detection in tissue – naphthalene and pyrene. Naphthalene showed an average of 7.6 mg/L across all tissue samples, with a maximum of 34.0 mg/L in a sample from Reef 2 – Henderson Point. The average concentration of pyrene across tissue samples was 11.0 mg/L, with a maximum of 31.6 mg/L in a tissue sample from Reef 6 – Shearwater Reef. The total concentrations of all PAHs in tissue samples for each reef can be found in Table 3.2.

In shell, five of the 15 PAHs returned concentrations above levels of detection – Acenaphthene, Fluorene, Naphthalene, Phenanthrene, and Pyrene. Naphthalene showed the highest concentrations across all shell samples with an average of 43.0 mg/L and a maximum of 259.04 mg/L in a shell sample from Reef 6 – Shearwater Reef. Fluorene was only identified in one shell sample from Reef 14 – Hornets Reef. The total PAH concentrations in shell samples for each reef can be found in Table 3.2.

Sediment samples showed measurable concentrations for all but one of the PAHs measured, Ideno(1,2,3-cd)pyrene. Similar to shell samples, the most abundant PAH in sediment samples was Naphthalene, with an average of 23.21 mg/L and a maximum concentration of 36.4 mg/L from Reef 13 – Graveline Bayou (Table 3.3). The second and third most abundant PAHs in sediment samples were Benzo(b)fluoranthene (average = 15.30 mg/L) and Phenanthrene (average = 13.45 mg/L), respectively.

Figure 3.2 shows the total concentration of PAHs in oyster shell and tissue samples as well as the single sediment sample for each reef sampled. Shearwater Reef

showed the highest combined levels of PAH concentrations in both shell and tissue (max = 285.01 mg/kg in shell), the effects seen in the earlier studies with oyster larvae all occurred between 0 and 500 mg/kg, thus the levels of contamination observed in this study are within the range that has been shown to have negative effects to this organism. In general, the concentration of total PAHs in the sediment samples was higher than within the oysters, the samples from Shearwater Reef were the only ones not to follow this pattern. The highest concentration of total PAHs in sediment was measured at Graveline Bayou (166.35 mg/kg).

Overall Characterization of the Oyster Digestive Gland Microbiome

A total of 66 oyster digestive gland samples were analyzed, resulting in the identification of 818,308 total sequences, which were matched to generate 1021 unique ASVs. During quality control steps only 2 samples were filtered from the data pool, the first and third oysters from Graveline Bayou (‘13-1’ & ‘13-3’) resulting in 64 samples remaining. The sample with highest number of ASVs came from Waveland Reef and contained 14,395 ASVs (‘8-8’, Table 2.5), and the average number of ASVs per sample was 1740.7. Across all oyster digestive gland samples, 571 ASVs were unassigned (Supplementary Data).

Using total ASV counts and the concentration of PAHs in respective samples it is possible to observe any overall correlations between the two factors (Figure 3.3). With respect to PAHs in oyster shell there is not a strong correlation with number of ASVs within each sample, though there may be a slight positive correlation (Figure 3.3A). In tissue samples, there is also a very slight relationship, though negative in this case,

between PAH concentration and ASV abundance (Figure 3.3B). These results suggest there is little effect of the chemical contamination on the abundance of ASVs in these oyster samples.

The taxonomic bar plot (Figure 3.4) shows the microbial make-up of each sample. Overall, the most common genera across all samples are *Sphingomonas*, *Mycoplasma*, and *Novosphingobium*. Oyster intestinal gland samples exhibit a large variation in diversity. Some samples are dominated by a single genus, for example a sample from Henderson Point (Reef 2) consists entirely of *Sphingomonas* and others exhibit much higher diversity and no single dominant group, for example sample 6 from Waveland Reef (Reef 8) has a community made up of 12.47% *Sphingomonas*, 6.33% *Chloroflexi*, 5.97% *Gemmatimonas*, etc. (Supplementary ASV Table).

Effect of PAH Contamination in Tissue on the Oyster Digestive Gland Microbiome

To look at the effect of PAH contamination on the oyster microbiome, alpha diversity measures based on Shannon index were correlated to the concentrations of total PAHs in tissue samples. A scatter plot of sample Shannon index and PAH concentration suggests no correlation in this comparison (Figure 3.5A). Spearman rho and Pearson r test statics near zero also suggest no correlation ($\rho = -0.13$ & $r = -0.12$) and non-significant p-values of the test statistics confirm this result (p-value = 0.41 & p-value = 0.45, respectively; Figure 3.5A).

Beta diversity was calculated with Bray-Curtis dissimilarity to examine differences in microbial community composition between samples. The PCoA for tissue samples suggests no correlation between PAH contamination and beta diversity (Figure

3.6A). Pearson and Spearman test statistics near zero, 0.064 and 0.044 respectively, as well as non-significant p-values of 0.186 and 0.41 confirm that there is no correlation between tissue contamination of PAHs at the observed concentrations and the community composition of the oyster digestive gland microbiome (Figure 3.6A).

Effect of PAH Contamination in Shell on the Oyster Digestive Gland Microbiome

The relationship of microbiome alpha diversity to PAHs in the shell follows the same pattern as in tissue. The scatter plot suggests no correlation of alpha diversity to PAH concentration within the shell (Figure 3.5B). This is confirmed by Spearman and Pearson test statistics near zero (0.041 & 0.006, respectively) and non-significant p-values (p-value = 0.797, p-value = 0.972; Figure 3.5B).

The PCoA for PAH concentration in shell suggests no correlation with composition as well (Figure 3.6B). Spearman and Pearson test statistics are near 0 also suggesting no correlation between PAH concentration and beta diversity (ρ = -0.035 & r = -0.035), this result is confirmed by non-significant p-values of 0.5 and 0.538, respectively (Figure 3.6B).

Effect of PAH Contamination in Nearby Sediment on the Oyster Digestive Gland Microbiome

Total PAH concentrations in the sediment were on a similar scale to concentrations in the tissues but show a slightly stronger correlation with microbial structure. The alpha diversity scatter plot suggests no correlation between sediment PAH contamination and the Shannon entropy of the oyster digestive gland microbiome (Figure

3.5C). Spearman and Pearson test statistics are near 0, which is in agreement with the scatterplot, and non-significant p-values confirm that there is no correlation with alpha diversity (Figure 3.5C). The PCoA for PAHs in nearby sediments shows slight grouping of samples by PAH concentration (Figure 3.5C). Spearman and Pearson test statistics are near 0 however, suggesting very little correlation ($\rho = 0.076$, $r = 0.023$). The Pearson r had a non-significant p-value = 0.52, while the p-value associated with Spearman ρ was 0.04, confirming the non-monotonic result of the Spearman ρ value and thus no correlation between sediment associated PAHs and oyster digestive gland microbiome beta diversity (Figure 3.5C).

PERMAOVA analysis for the beta diversity of oyster digestive glands and the primary variables (Tissue-associated PAH concentration, Shell-associated PAH concentration, Sediment-associated PAH concentration, and Reef Name) that may be responsible for the observed variation in community structure was also performed (Figure 3.5). The PERMANOVA showed that only Reef accounted for a significant percent of variation (p-value = 0.018).

Effect of Reef and Location on the Oyster Digestive Gland Microbiome

Only one pair of reefs showed a significant difference in alpha diversity from each other, Henderson Point and Pass Marianne (p-value = 0.043; Figure 3.6). Alpha diversity was higher at reefs such as Hornets Reef, Waveland Reef, and Pass Marianne, and lower at Graveline Bayou, Shearwater, and St. Joe (Figure 3.6A). Non-significant PERMANOVA results confirm that there is also no correlation with beta diversity (Figure 3.6B). Only one pair of reefs were significantly different from each other in

community composition, Henderson Point and Waveland Reef (p-value = 0.05; Figure 3.6B). Interestingly, these are not the same reefs that differed in alpha diversity. Overall, the reef these oysters come from shows very little effect on microbiome diversity.

In order to further examine the geographic influence on microbial community the study reefs were binned by region, reefs from St. Joe to Pass Christian were designated 'western', reefs from Biloxi Bay to Shearwater Reef were designated 'central', and reefs from Graveline Bayou to the Pascagoula Oyster Reef Complex were designated 'eastern'. Examining microbial community diversity at this coarser geographic level shows no statistical significance between regions of the Mississippi Sound for either alpha diversity (Figure 3.7A) or composition (Figure 3.7B).

Discussion

PAHs in Oyster Tissue, Shell, and Nearby Sediments

Shell can act as a long-term reservoir, or a record of exposure, while tissue is evidence of more recent exposure due to natural detoxification processes that reduce the concentration of xenobiotics within the organism (Kovacs et al. 2010, Carmichael et al. 2012). This expected pattern was observed in the oysters used in this project, 5 of the measured PAHs were observed in shell samples while tissue samples only returned 2 measurable PAHs. The average concentration for each PAH is higher in shell than in tissue, which may indicate more long-term exposure of these oysters to PAH contamination.

Despite the *Deepwater Horizon* oil spill occurring more than five years before sampling, the presence of PAHs in the Gulf is still expected due to burial in the sediment

as well as many sources of continuous contamination such as natural seeps, continued drilling, and heavy boating traffic and even run-off from terrestrial sources (Wang, Liu, et al. 2014, Beyer et al. 2016, Kennicutt 2017). Here, there were many more measured PAHs present in the sediment than either the shell or the tissue, interestingly concentrations of the PAHs in the sediment are similar to that in the shell. This may be indication of the oysters incorporating large relative quantities of PAHs into their shells. Carmichael et al. (2012) used stable isotopes δ13C and δ15N to measure the quantity of oil-derived elements that were assimilated into oyster shells following the *Deepwater Horizon* oil spill and found no evidence of this taking place, so this may not be reason for the observed similar PAH concentrations in oyster shells and nearby sediments. More simply, this pattern may be a result of sampling bias, if the chosen PAHs are not as representative of all the PAHs present in the sediment as previously thought there may be large concentrations of PAHs present that were not measured.

Overall Comparison of the Oyster Microbiome

The result of a negative correlation between increasing PAH concentration and decreasing ASV count suggests that there may be an effect of PAH exposure on the diversity of the oyster digestive gland microbiome. Microbiomes have been shown to be affected by exposure to PAHs for example, the introduction of crude oil into beach sands following the *Deepwater Horizon* oil spill, the abundance of bacteria in oil sand increased by two orders of magnitude despite diversity decreasing to half of the non-oiled sand (Huettel et al. 2018). More analysis was necessary to dig into this negative correlation

and discern whether or not PAHs in the oyster shell and tissue had an effect on the digestive gland microbiome.

The oyster microbiome samples had a large variation in diversity, with some samples only containing one taxa and others having no dominant taxa but were made up of many different taxa. Overall, the most abundant genera were *Sphingomonas*, *Mycoplasma*, and *Novosphingobium*. Previous studies have found these three genera present in large abundances in other oyster microbiomes, suggesting an affinity of these taxa to form associations with oysters (King et al. 2012, Wegner et al. 2013, Lokmer, Goedknegt, et al. 2016).

Effect of PAH Contamination in Oyster Tissue and Shell on the Digestive Gland Microbiome

Negative Pearson and Spearman test statistics may at first appear to show a slight negative correlation between the microbiome alpha diversity and PAH concentration in the tissue samples that would be consistent with the ASV-PAH regression, however non-significant p-values refute any statistical correlation. There was no correlation between tissue-associated PAH contamination and beta diversity either. Similarly, PAH contamination in shells showed no correlation to alpha or beta diversity.

At the concentrations of PAH observed, there is little evidence of effect of tissue contamination on microbiome in wild oysters caught in the Mississippi Sound, suggesting the measured PAH concentrations could be too low to have an effect on the microbiome. McConkey et al. (1997) found the EC50 of phenanthrene to bacteria to be 10.8 ppm under visible light conditions, whereas the highest ΣPAH concentration measured in this

study was 285 ppb (0.285 ppm) in the shell, 2 orders of magnitude lower. In the previous chapter, the flounder microbiome was affected at sediment PAH concentrations of 10 mg/kg (10,000 ppb) whereas the highest measured PAHs in this study were 166 ppb, again 2 orders of magnitude lower. This further supports the proposition that the concentration of oyster-associated PAHs observed in this study are low enough to not be of concern to the homeostasis of the Eastern oyster digestive gland microbiome.

Effect of PAH Contamination in Nearby Sediments on the Oyster Digestive Gland Microbiome

Alpha diversity showed no correlation with sediment PAHs; however, the Spearman test statistic was significant for beta diversity of these samples, indicating a positive correlation with sediment PAHs. Sediment PAH contamination differed at each sampling location (min = 20 ppb, max = 166 ppb) which may suggest that an unknown geographic variable is potentially responsible for the singular significant Spearman test statistic and required further analysis. In the PERMANOVA test, the interaction of sediment PAHs and Reef had a high R^2 and was nearly statistically significant which may be an indication of biological significance that is not detected by statistical tests. Both Lokmer et al. (2016) and King et al. (2012) found significant location effect on the composition of the oyster microbiome, with location having a stronger impact than temporal changes, for example.

Effect of Reef and Location on the Oyster Digestive Gland Microbiome

To test the hypothesis that location may have had and influence, the reef each sample was collected from was used to analyze the diversity of the samples. Contradictory to previous research, location had very little effect on either the alpha or beta diversity of the oyster microbiome (King et al. 2012, Trabal Fernández et al. 2014, Lokmer, Kuenzel, et al. 2016). Both diversity metrics showed significant differences between only one pair of reefs each, Henderson Point & Pass Marianne and Henderson Point and Waveland respectively. Differences in spatial scale may be responsible for this inconsistency. All reefs for this study were within the Mississippi Sound, a singular body of water, while the spatial extent of the other studies was much larger. These results taken together suggest something other than PAH contamination or geography is responsible for differences in the microbiome of these oysters, it may be a result of large individual variation, or the sampling size was too small to allow for the detection of patterns within the variation.

Conclusions

The primary members observed in the oyster digestive gland microbiome were *Sphingomonas*, *Mycoplasma*, and *Novosphingobium*, however the samples exhibited a large variation in diversity. When comparing the overall effect of PAH loads on the total number of ASVs within the sample, there was a slight negative relationship for both shell and tissue loads, suggesting microbial diversity may be inhibited by PAH contamination. Shannon alpha diversity and Bray-Curtis beta diversity metrics were used to look farther into the effect of PAH on the microbiome. There was a similar negative relationship

within the alpha diversity of tissue-associated PAHs, however the beta diversity was not affected by the PAH loads for these samples. There was no significant relationship observed for shell-associated PAHs in either alpha or beta diversity, as well as for both metrics according to PAH contamination in nearby sediments. Using location as the independent factor, only one pair of reefs differed from each other (Henderson Point and Waveland) and binning into three regions (west, central, and east) also showed no significant differences in alpha and beta diversity. These results suggest something besides PAH contamination is the primary factor affecting the microbiome of the wild oysters, such as the very high individual variability or small sample size.

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Table 3.1 *List of PAHs analyzed in sediment, shell, and tissue samples collected from oysters from the Mississippi Sound.*

Compound Name
Dibenz(a,h)anthracene/Benzo(g,h,i)perylene
Acenaphthene
Anthracene
Benzo(a)anthracene
Benzo(a)pyrene
Benzo(b)fluoranthene
Benzo(k)fluoranthene
Fluoranthene
Fluorene
Indeno(1,2,3-cd) pyrene
Naphthalene
Phenanthrene
Pyrene

Table 3.2 *Measured concentrations of PAHs present in oyster tissue and shell samples combined for each reef (mg/L).*

	PAHs within Tissue Samples		PAHs within Shell Samples				
Reef	Naphthalene (mg/L)	Pyrene (mg/L)	Acenaphthene (mg/L)	Fluorene (mg/L)	Naphthalene (mg/L)	Phenanthrene (mg/L)	Pyrene (mg/L)
St. Stanislaus	62.7	0	36.96	0	19.8	3.88	18.8
Henderson Point	60	0	40.91	0	3.33	0	11.7
Buoy Reef	3.53	51	0	0	36.1	10.8	0
Shearwater Reef	0	104.6	0	0	803.51	42.78	0
St. Joe Reef	0	84.75	0	0	52.3	0	0
Waveland Reef	0	51.15	20.09	0	655.94	99.06	0
Pass Marianne	0	0	24.61	0	0	0	18.7
Pass Christian	0	0	4.51	0	0	0	70.43
Graveline Bayou	0	0	0	0	249.84	173	0
Hornets Reef	10.6	39.56	0	70	15.6	0	0
Pascagoula Oyster Reef Complex	0	0	0	0	162.41	90.49	0

Table 3.3 *Measured concentrations of relevant PAHs in all sediment samples (mg/L).*

PAH Name	Total	Average
Dibenz(a,h)anthracene/Benzo(g,h,i)perylene	46.89	11.72
Acenaphthene	32.20	10.73
Anthracene	1.77	0.89
Benzo(a)anthracene	26.08	6.52
Benzo(a)pyrene	32.54	6.51
Benzo(b)fluoranthene	214.15	15.30
Benzo(k)fluoranthene	16.24	3.25
Chrysene	42.30	6.04
Fluoranthene	54.47	7.78
Fluorene	6.60	2.20
Indeno(1,2,3-cd)pyrene	0.0	0.0
Naphthalene	371.30	23.21
Phenanthrene	188.32	13.45
Pyrene	76.92	10.99
ΣPAH	**329.99**	**36.67**

Table 3.4 *Descriptive statistics for all oyster digestive gland microbiome samples, DNA sequencing, and diversity analysis.*

Sample	Reef Name	Reef Region	Sequence Count	ASV Count	Shannon Diversity	Good's Coverage	Chao1
1-1	St. Stanislas Reef	West	127	14	-0.0	1.0	1.0
1-2	St. Stanislas Reef	West	1307	626	3.362	1.0	14.0
1-3	St. Stanislas Reef	West	2589	526	2.853	1.0	10.0
1-4	St. Stanislas Reef	West	102	13	-0.0	1.0	1.0
1-5	St. Stanislas Reef	West	74	15	-0.0	1.0	1.0
1-6	St. Stanislas Reef	West	109	25	-0.0	1.0	1.0
2-1	Henderson Point Reef	West	277	59	-0.0	1.0	1.0
2-2	Henderson Point Reef	West	357	25	-0.0	1.0	1.0
2-3	Henderson Point Reef	West	619	145	1.528	1.0	4.0
2-5	Henderson Point Reef	West	471	167	2.390	1.0	6.0
2-6	Henderson Point Reef	West	586	186	2.148	1.0	6.0
2-7	Henderson Point Reef	West	1000	443	2.568	1.0	7.0
5-1	Biloxi Bay	Central	3030	981	2.970	1.0	11.0
5-2	Biloxi Bay	Central	951	286	0.795	1.0	3.0
5-3	Biloxi Bay	Central	1093	154	1.309	1.0	3.0
5-4	Biloxi Bay	Central	250	72	1.861	1.0	4.0
5-5	Biloxi Bay	Central	2962	787	2.679	1.0	12.0
5-6	Biloxi Bay	Central	3503	898	3.537	1.0	17.0
6-1	Shearwater Reef	Central	410	64	0.758	1.0	2.0
6-2	Shearwater Reef	Central	881	176	2.528	1.0	6.0
6-3	Shearwater Reef	Central	421	107	1.966	1.0	4.0
6-5	Shearwater Reef	Central	242	16	-0.0	1.0	1.0
6-6	Shearwater Reef	Central	459	66	1.547	1.0	3.0
6-7	Shearwater Reef	Central	704	245	1.991	1.0	5.0
7-1	St. Joe Reef	West	1498	132	1.755	1.0	4.0
7-2	St. Joe Reef	West	493	181	2.228	1.0	6.0
7-3	St. Joe Reef	West	278	69	1.394	1.0	3.0
7-4	St. Joe Reef	West	108	17	-0.0	1.0	1.0
7-5	St. Joe Reef	West	240	26	1.0	1.0	2.0
7-6	St. Joe Reef	West	1469	570	2.318	1.0	11.0
8-1	Waveland Reef	West	2520	1002	2.104	1.0	7.0
8-2	Waveland Reef	West	2599	304	1.747	1.0	5.0
8-3	Waveland Reef	West	42538	5352	4.989	1.0	48.0
8-6	Waveland Reef	West	65438	11563	5.860	1.0	76.0
8-8	Waveland Reef	West	76926	14359	6.313	1.0	116.0
8-9	Waveland Reef	West	75950	8413	5.670	1.0	75.0
9-2	Pass Marianne Reef	West	171	44	-0.0	1.0	1.0
9-3	Pass Marianne Reef	West	67995	5867	5.157	1.0	53.0

Table 3.4 cont.

9-4	Pass Marianne Reef	West	1852	625	2.836	1.0	10.0
9-5	Pass Marianne Reef	West	173	25	-0.0	1.0	1.0
9-6	Pass Marianne Reef	West	48924	5868	4.960	1.0	55.0
9-7	Pass Marianne Reef	West	678	198	2.366	1.0	7.0
12-1	Pass Christian Reef	West	1381	515	2.003	1.0	7.0
12-2	Pass Christian Reef	West	5395	1785	3.700	1.0	23.0
12-3	Pass Christian Reef	West	870	243	1.490	1.0	5.0
12-4	Pass Christian Reef	West	1082	361	1.191	1.0	4.0
12-5	Pass Christian Reef	West	953	441	2.670	1.0	8.0
12-6	Pass Christian Reef	West	35457	8400	5.763	1.0	87.0
13-4	Graveline Bayou	East	773	126	1.902	1.0	4.0
13-5	Graveline Bayou	East	754	58	-0.0	1.0	1.0
13-7	Graveline Bayou	East	1794	1054	2.460	1.0	9.0
13-8	Graveline Bayou	East	332	28	-0.0	1.0	1.0
14-1	Hornets Reef	West	383	159	-0.0	1.0	1.0
14-3	Hornets Reef	West	76659	6983	5.275	0.999	59.0
14-4	Hornets Reef	West	66629	10807	6.051	0.999	95.0
14-5	Hornets Reef	West	75100	7791	5.677	0.999	71.0
14-6	Hornets Reef	West	557	126	2.479	1.0	6.0
14-7	Hornets Reef	West	1107	201	1.937	1.0	4.0
16-5	Pascagoula Oyster Reef Complex	East	38229	5009	4.928	1.0	50.0
16-6	Pascagoula Oyster Reef Complex	East	271	103	1.402	1.0	3.0
16-8	Pascagoula Oyster Reef Complex	East	46370	6099	5.100	1.0	60.0
16-9	Pascagoula Oyster Reef Complex	East	1994	915	2.371	1.0	8.0
16-11	Pascagoula Oyster Reef Complex	East	49127	2908	4.780	1.0	45.0

Table 3.5 *PERMANOVA test results for all experimental factors.*

	Df	Sums of Sqs	Mean Sqs	F Model	R^2	p-value
Tissue PAH	1	0.504	0.504	1.030	0.022	0.224
Shell PAH	1	0.513	0.513	1.049	0.022	0.142
Sediment PAH	1	0.533	0.533	1.089	0.023	0.061
Reef Name	10	5.119	0.512	1.046	0.224	**0.018**
Tissue PAH: Shell PAH	1	0.504	0.504	1.030	0.022	0.159
Tissue PAH: Sediment PAH	1	0.473	0.473	0.966	0.021	0.793
Shell PAH: Sediment PAH	1	0.470	0.470	0.960	0.021	0.828
Tissue PAH: Reef Name	5	2.505	0.501	1.024	0.110	0.118
Shell PAH: Reef Name	7	3.441	0.492	1.005	0.150	0.381
Tissue PAH: Shell PAH: Sediment PAH	1	0.492	0.492	1.006	0.022	0.409
Residuals	17	8.316	0.489	NA	0.364	NA
Total	46	22.870	NA	NA	1.000	NA

PERMANOVA test based on Bray-Curtis dissimilarity beta diversity metric of oyster digestive gland microbiome for PAH concentration measured in tissue, shell, and sediment, as well as the reef from which the samples were collected. Significance indicated in bold ($\alpha < 0.05$).

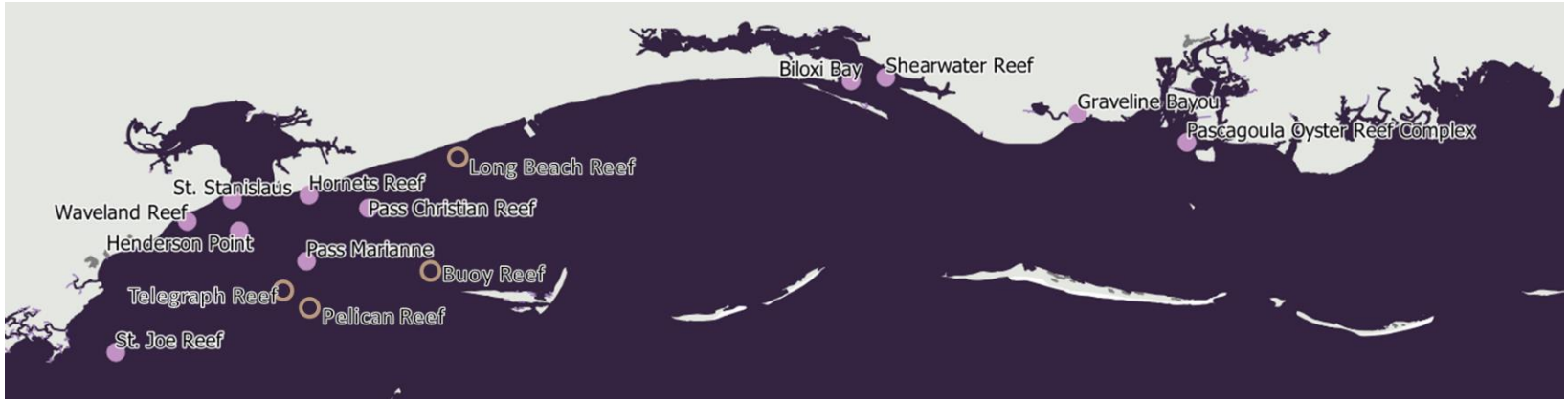


Figure 3.1 *Oyster reef sampling locations.*

Locations with viable samples shown as solid circles (●), locations with no live oysters shown as open circles (○). Sampling took place between July and December of 2017.

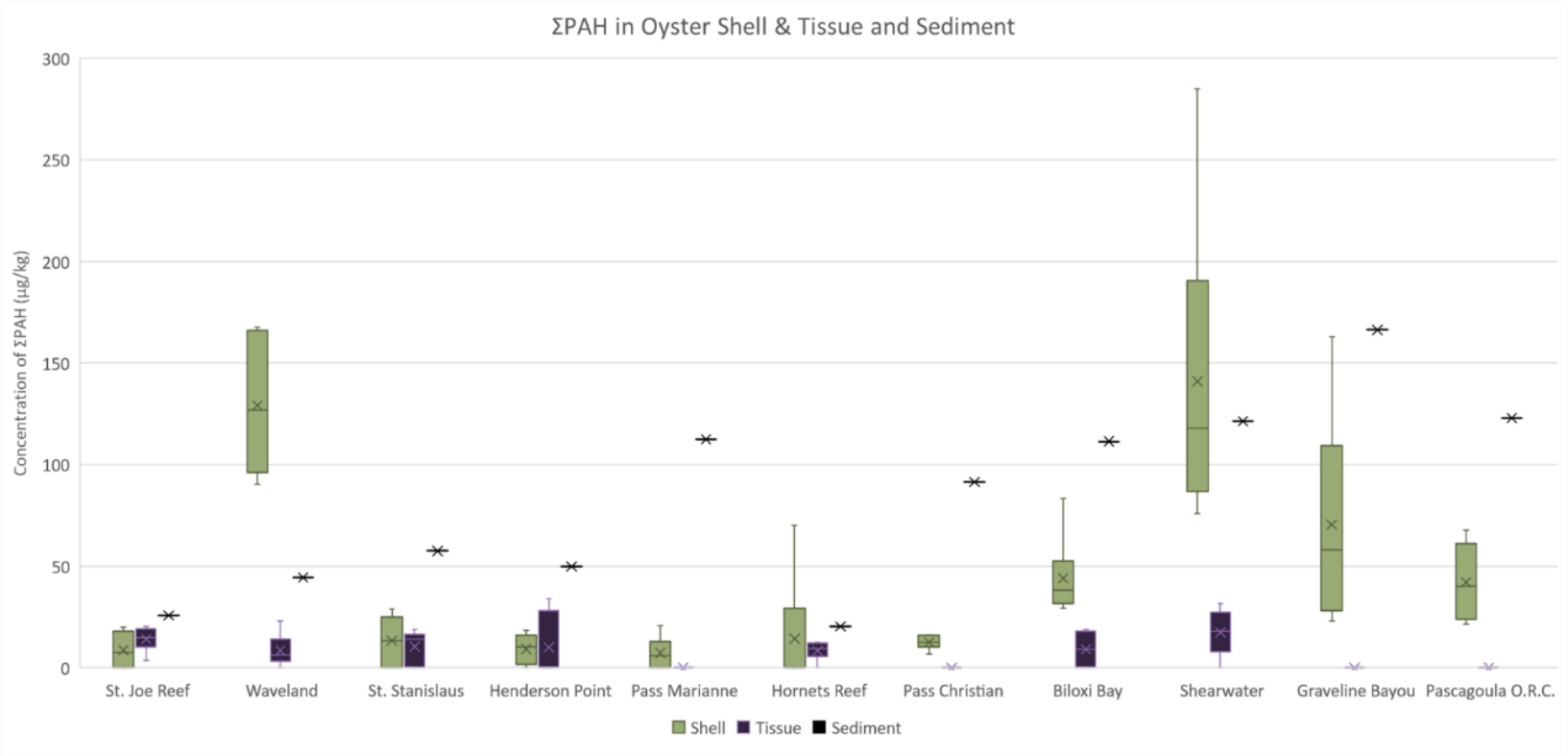


Figure 3.2 *Total PAH concentration measured in experimental samples.*

Total PAH concentrations (µg/kg) measured in oyster tissue and shell as well as the single sediment sample for each reef.

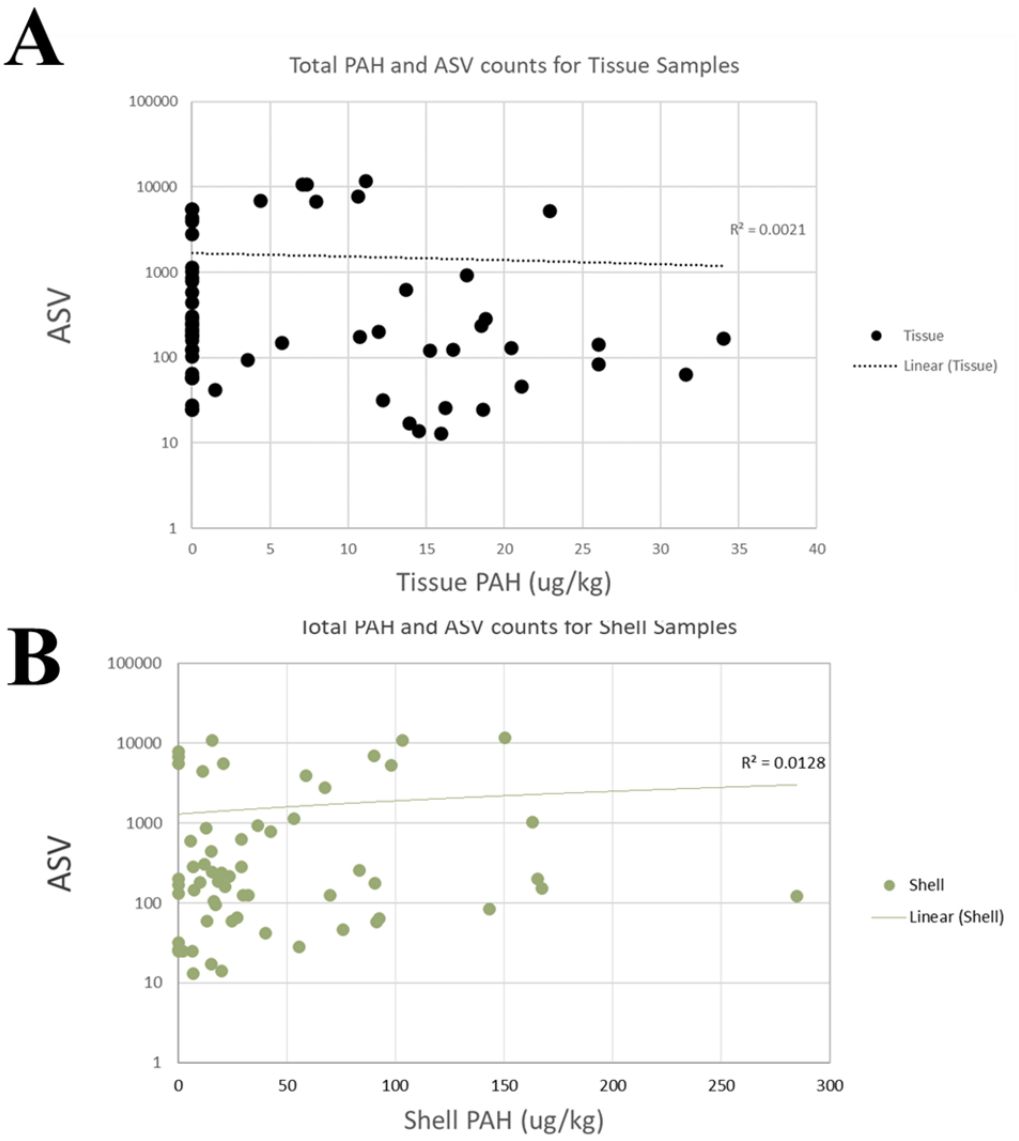


Figure 3.3 *ASV and PAH Regression Plot.*

Correlation analysis between PAH concentration within (A) shell and (B) tissue and the total number of ASVs per sample.

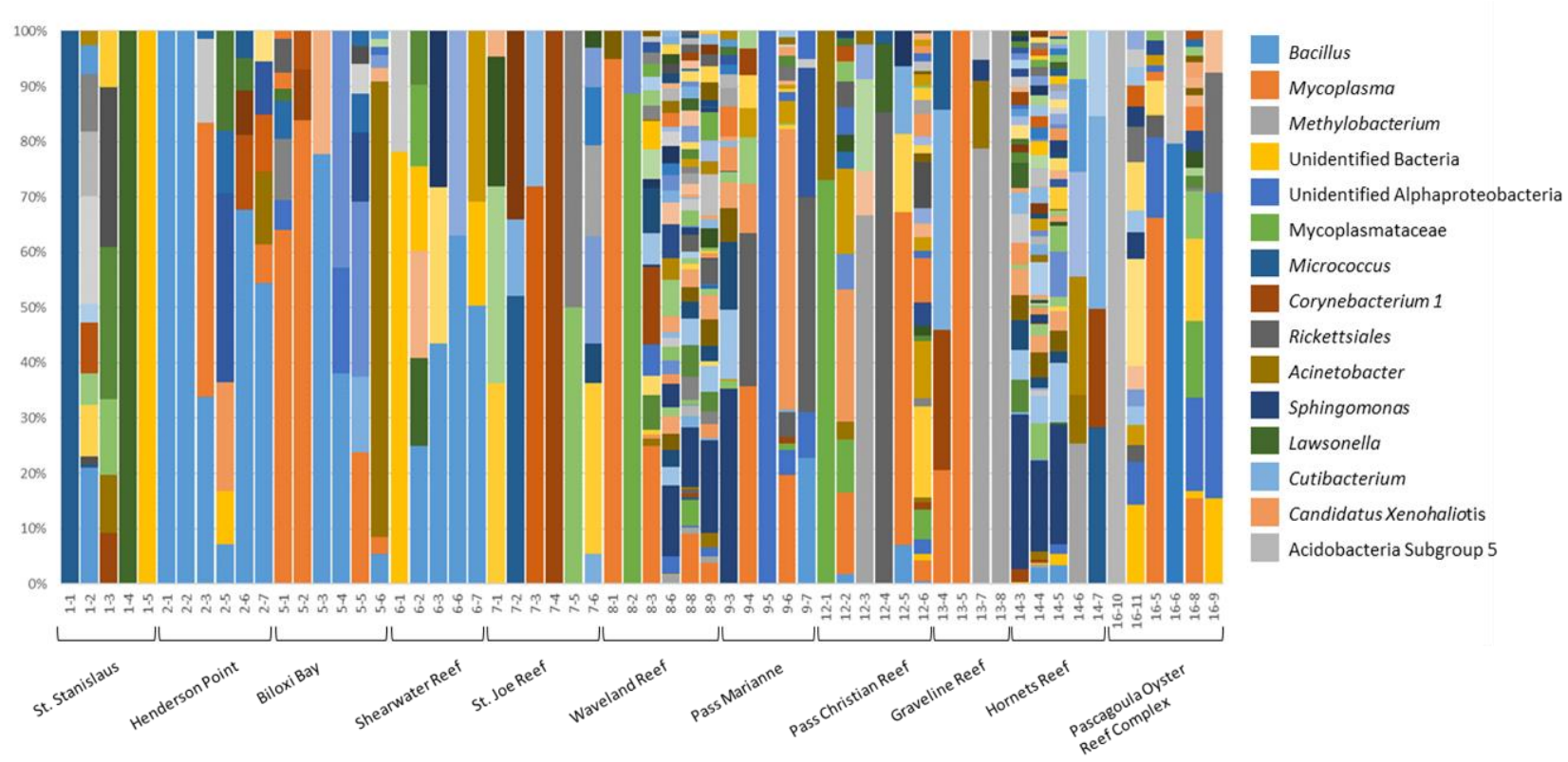
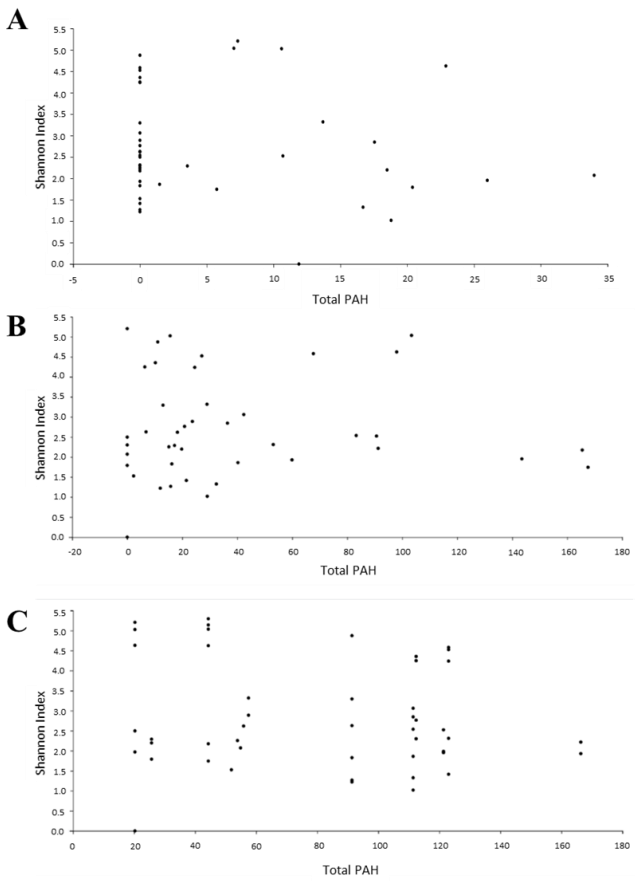


Figure 3.4 *Taxonomic bar plots for all oyster digestive gland microbiome samples.*



Alpha Diversity	Spearman ρ	Pearson r
Test statistic	-0.129	-0.121
P-value	0.414	0.446
Sample size	42	42

Beta Diversity	Spearman ρ	Pearson r
Test Statistic	0.044	0.064
p-value	0.41	0.186
Sample size	46	46

Alpha Diversity	Spearman ρ	Pearson r
Test statistic	0.041	0.006
P-value	0.797	0.972
Sample size	42	42

Beta Diversity	Spearman ρ	Pearson r
Test Statistic	-0.035	-0.035
p-value	0.5	0.538
Sample size	46	46

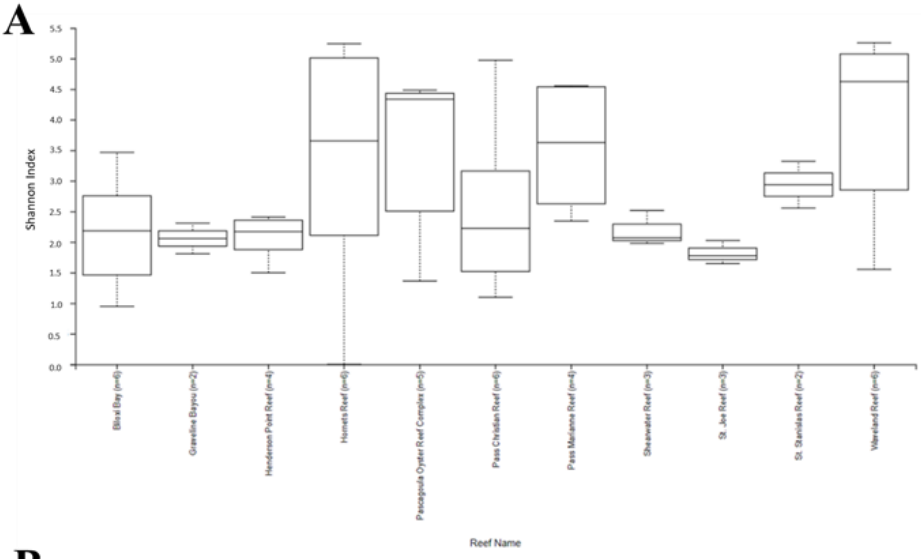
Alpha Diversity	Spearman ρ	Pearson r
Test statistic	-0.099	-0.145
P-value	0.508	0.331
Sample size	47	47

Beta Diversity	Spearman ρ	Pearson r
Test Statistic	0.076	0.023
p-value	0.04	0.52
Sample size	46	46

Figure 3.5 *Microbiome diversity and PAH contamination analysis for oyster digestive gland samples.*

Alpha diversity box plots, Kruskal-Wallis test results, and PERMANOVA test results for the correlation between PAH concentration and diversity for (A) PAH contamination in oyster tissue, (B) PAH contamination in oyster shell, and (C) PAH contamination in nearby sediments. Significance indicated in bold ($\alpha < 0.05$).

A



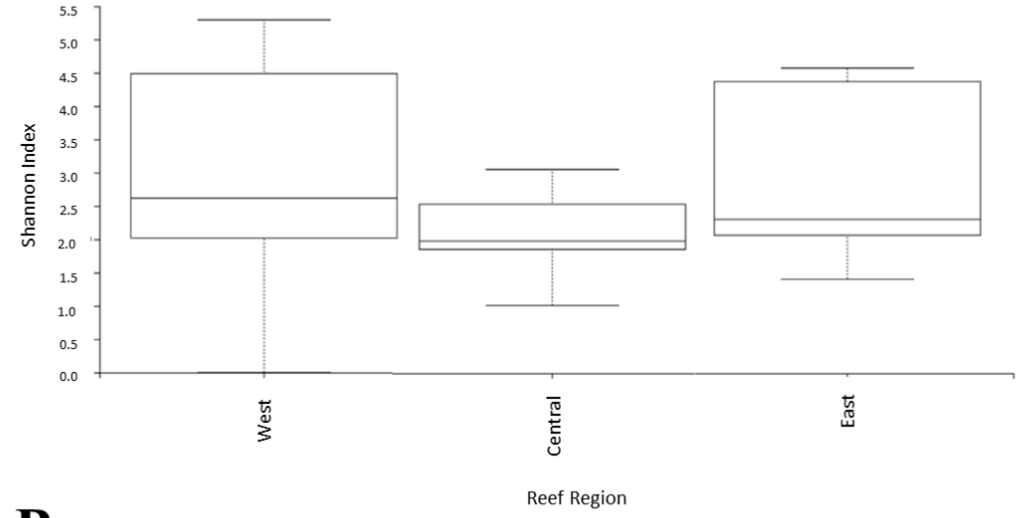
B

		Kruskal-Wallis Results			PERMANOVA Results		
Group 1	Group 2	H	p-value	q-value	pseudo-F	p-value	q-value
Biloxi Bay (n=6)	Graveline Bayou (n=2)	0.111	0.739	0.84	1	1	1
Biloxi Bay (n=6)	Henderson Point Reef (n=4)	0.045	0.831	0.863	1	1	1
Biloxi Bay (n=6)	Hornets Reef (n=6)	0.923	0.337	0.639	1.188	0.19	0.898
Biloxi Bay (n=6)	Pascagoula Oyster Reef Complex (n=5)	2.133	0.144	0.514	1	1	1
Biloxi Bay (n=6)	Pass Christian Reef (n=6)	0.103	0.749	0.84	1.011	0.471	1
Biloxi Bay (n=6)	Pass Marianne Reef (n=4)	2.227	0.136	0.514	1	1	1
Biloxi Bay (n=6)	Shearwater Reef (n=3)	0.067	0.796	0.863	1	1	1
Biloxi Bay (n=6)	St. Joe Reef (n=3)	0.267	0.606	0.84	1	1	1
Biloxi Bay (n=6)	St. Stanislas Reef (n=2)	1	0.317	0.623	1	1	1
Biloxi Bay (n=6)	Waveland Reef (n=6)	3.103	0.078	0.514	1.088	0.106	0.752
Graveline Bayou (n=2)	Henderson Point Reef (n=4)	0.214	0.643	0.84	1	1	1
Graveline Bayou (n=2)	Hornets Reef (n=6)	1	0.317	0.623	1.133	0.384	1
Graveline Bayou (n=2)	Pascagoula Oyster Reef Complex (n=5)	1.35	0.245	0.621	1	1	1
Graveline Bayou (n=2)	Pass Christian Reef (n=6)	0.111	0.739	0.84	1.008	0.552	1
Graveline Bayou (n=2)	Pass Marianne Reef (n=4)	3.429	0.064	0.514	1	1	1
Graveline Bayou (n=2)	Shearwater Reef (n=3)	0.333	0.564	0.84	1	1	1
Graveline Bayou (n=2)	St. Joe Reef (n=3)	1.333	0.248	0.62	1	1	1
Graveline Bayou (n=2)	St. Stanislas Reef (n=2)	2.4	0.121	0.514	1	1	1
Graveline Bayou (n=2)	Waveland Reef (n=6)	1	0.317	0.623	1.107	0.123	0.752
Henderson Point Reef (n=4)	Hornets Reef (n=6)	1.136	0.286	0.623	1.166	0.196	0.898
Henderson Point Reef (n=4)	Pascagoula Oyster Reef Complex (n=5)	2.16	0.142	0.514	1	1	1
Henderson Point Reef (n=4)	Pass Christian Reef (n=6)	0.045	0.831	0.863	1.01	0.482	1
Henderson Point Reef (n=4)	Pass Marianne Reef (n=4)	4.083	0.043	0.514	1	1	1
Henderson Point Reef (n=4)	Shearwater Reef (n=3)	0.125	0.724	0.84	1	1	1
Henderson Point Reef (n=4)	St. Joe Reef (n=3)	0.5	0.48	0.776	1	1	1
Henderson Point Reef (n=4)	St. Stanislas Reef (n=2)	3.429	0.064	0.514	1	1	1
Henderson Point Reef (n=4)	Waveland Reef (n=6)	2.227	0.136	0.514	1.134	**0.05**	0.752
Hornets Reef (n=6)	Pascagoula Oyster Reef Complex (n=5)	0.133	0.715	0.84	1.178	0.167	0.898
Hornets Reef (n=6)	Pass Christian Reef (n=6)	0.641	0.423	0.775	1.2	0.101	0.752
Hornets Reef (n=6)	Pass Marianne Reef (n=4)	0.045	0.831	0.863	0.907	0.842	1
Hornets Reef (n=6)	Shearwater Reef (n=3)	0.6	0.439	0.775	1.151	0.234	0.919
Hornets Reef (n=6)	St. Joe Reef (n=3)	1.667	0.197	0.553	1.151	0.232	0.919
Hornets Reef (n=6)	St. Stanislas Reef (n=2)	0	1	1	1.133	0.378	1
Hornets Reef (n=6)	Waveland Reef (n=6)	0.102	0.749	0.84	1	0.389	1
Pascagoula Oyster Reef Complex (n=5)	Pass Christian Reef (n=6)	0.533	0.465	0.775	1.01	0.45	1
Pascagoula Oyster Reef Complex (n=5)	Pass Marianne Reef (n=4)	0.54	0.462	0.775	1	1	1
Pascagoula Oyster Reef Complex (n=5)	Shearwater Reef (n=3)	1.089	0.297	0.623	1	1	1
Pascagoula Oyster Reef Complex (n=5)	St. Joe Reef (n=3)	1.8	0.18	0.553	1	1	1
Pascagoula Oyster Reef Complex (n=5)	St. Stanislas Reef (n=2)	0.15	0.699	0.84	1	1	1
Pascagoula Oyster Reef Complex (n=5)	Waveland Reef (n=6)	1.633	0.201	0.553	1.123	0.096	0.752
Pass Christian Reef (n=6)	Pass Marianne Reef (n=4)	1.136	0.286	0.623	1.01	0.485	1
Pass Christian Reef (n=6)	Shearwater Reef (n=3)	0	1	1	1.01	0.504	1
Pass Christian Reef (n=6)	St. Joe Reef (n=3)	0.267	0.606	0.84	1.009	0.465	1
Pass Christian Reef (n=6)	St. Stanislas Reef (n=2)	0.111	0.739	0.84	1.008	0.563	1
Pass Christian Reef (n=6)	Waveland Reef (n=6)	2.077	0.15	0.514	1.103	0.102	0.752
Pass Marianne Reef (n=4)	Shearwater Reef (n=3)	3.125	0.077	0.514	0.977	1	1
Pass Marianne Reef (n=4)	St. Joe Reef (n=3)	4.5	**0.034**	0.514	1	1	1
Pass Marianne Reef (n=4)	St. Stanislas Reef (n=2)	0.214	0.643	0.84	1	1	1
Pass Marianne Reef (n=4)	Waveland Reef (n=6)	0.182	0.67	0.84	0.959	0.59	1
Shearwater Reef (n=3)	St. Joe Reef (n=3)	2.333	0.127	0.514	1	1	1
Shearwater Reef (n=3)	St. Stanislas Reef (n=2)	3	0.083	0.514	1	1	1
Shearwater Reef (n=3)	Waveland Reef (n=6)	1.667	0.197	0.553	1.122	0.065	0.752
St. Joe Reef (n=3)	St. Stanislas Reef (n=2)	3	0.083	0.514	1	1	1
St. Joe Reef (n=3)	Waveland Reef (n=6)	2.4	0.121	0.514	1.122	0.065	0.752
St. Stanislas Reef (n=2)	Waveland Reef (n=6)	0.444	0.505	0.794	1.107	0.123	0.752

Figure 3.6 *Diversity analysis of oyster microbiome by reef.*

(A) Alpha diversity box plots and (B) Kruskal-Wallis and PERMANOVA test results for oyster microbiome analysis by sampled reef. Significance indicated in bold ($\alpha < 0.05$).

A



B

		Kruskal-Wallis Results			PERMANOVA Results		
Group 1	Group 2	H	p-value	q-value	pseudo-F	p-value	q-value
Central (n=9)	East (n=7)	1.235	0.266	0.4	1	1	1
Central (n=9)	West (n=31)	2.268	0.132	0.396	1.068	0.137	0.206
East (n=7)	West (n=31)	0.06	0.807	0.807	1.072	0.11	0.206

Figure 3.7 *Diversity analysis of oyster microbiome by region.*

(A) Alpha diversity box plots and (B) Kruskal-Wallis and PERMANOVA test results for oyster microbiome analysis generated by binning sampling reefs into three regions based on their locations within the Mississippi Sound. Significance indicated in bold ($\alpha < 0.05$).

CHAPTER IV – CONCLUSIONS

Microbiomes are commensal, symbiotic, and pathogenic microbial communities that can be made up of bacteria, archaea, fungi, protozoa, and viruses (Adamovsky et al. 2018, Evariste et al. 2019). Microbiota produce many metabolites, peptides, and proteins that interact with the host organism and improve host fitness through the regulation of nutrient absorption and energy metabolism (Krajmalnik-Brown et al. 2012, Nicholson et al. 2012, Rowland et al. 2018). The microbiome also interacts closely with the host immune system, from the beginning of development into adulthood the microbiome improves infectious disease prevention capabilities of the host by fighting off and outcompeting pathogens as well as providing nutrients and molecular signals that improve the hosts' immune defenses (Hooper et al. 2012, Belkaid and Hand 2014, Takiishi et al. 2017, Ubeda et al. 2017). Microbiomes also provide protection for the host against environmental pollutants (Evariste et al. 2019). Through biotransformation the microbes alter or metabolize environmental pollutants thus reducing toxicity before the pollutant can enter the host and cause harm (Van de Wiele et al. 2005, Diaz-Bone and Van De Wiele 2010, Haiser and Turnbaugh 2013, Claus et al. 2016). Disruption of the microbiome can affect these benefits and therefore have a negative impact on the health of the host, examining stresses on the microbiome and how it responds can help with environmental impact studies of pollution and aid in conservation of ecosystems at risk.

This research sought to examine how oil affects the microbiome of two benthic, estuarine species, southern flounder (*Paralichthys lethostigma*) and eastern oysters (*Crassostrea virginica*). Previous studies have found that exposure to oil can alter the richness and composition of the microbiome. The addition of hydrocarbons to the water

column resulted in enrichment of *Oceanospirales* spp., 85% - 90% of the sequences were *Oceanospirales* at the oiled location and 5% of sequences at the non-oiled control location were *Oceanospirales* (Hazen et al. 2010). Almost 2 months after the spill occurred the dominance of *Oceanospirales* was succeeded by *Colwellia* and *Cycloclastus*, indicating a slow return to normal diversity following the initial disruption caused by the spill (Valentine et al. 2010, Redmond and Valentine 2012, Gutierrez et al. 2013). In sediments, the introduction of crude oil also caused increases in abundance of oil-degrading bacteria such as *Alcanivorax*, *Marinobacter*, and Rhodobacteraceae at the expense of the diversity of the indigenous bacteria (Kostka et al. 2011). Gammaproteobacterium and *Colwellia* also dominated microbial communities in sediments contaminated with hydrocarbons following the *Deepwater Horizon* oil spill (Mason et al. 2014). Microbiomes associated with animal species are also disrupted following exposure to crude oil. The microbiome of the larvae of a reef sponge (*Rhopaloeides odorabile*) exhibited decreased biodiversity in treatments containing crude oil, including a decrease in the abundance of an important ammonia-oxidizing symbiont (Luter et al. 2019). The zebrafish (*Danio rerio*) microbiome increased in both alpha and beta diversity when exposed to crude oil which was primarily a result of increased abundance of potential oil degraders including *Vibrio*, *Flavobacterium*, and *Pseudomonas*, and a decrease in abundance of beneficial bacteria such as *Cetobacterium* and *Lactobacillus* (González-Penagos et al. 2020).

Southern Flounder

PAHs and oil contamination are known have negative effects on organisms, including depression of the immune system. The first part of this research sought to investigate the interaction of oil contamination on flounder's response to a challenge by a known pathogenic bacteria, *Vibrio anguillarum*, utilizing concentrations of oil at 0 mg/kg, 10 mg/kg, & 25 mg/kg and a bacterial bath of $5.30 \text{ X } 10^5 \pm 1.81 \text{ X } 10^5$ CFU/mL. Indeed, this study saw changes in the flounder microbiome that resulted from these exposures, however the responses differed between gill and intestine microbiome. The gill microbiome was more sensitive to oil exposure than that of the intestine, while the intestine microbiome responded more strongly to the pathogen challenge than the gill. When the combination of oil exposures and pathogen challenge was examined, the resulting interaction accounted for a small percent of the total variation (2%) captured in this study, while microbiome location and the concentration of oil responsible for a majority of the variation (22%). This suggests that oil does not have a major effect on flounders' microbiome response to a pathogenic bacterial challenge at these concentrations and this timescale.

Previous studies have also sought to examine the effect of oil and pathogen exposure on southern flounder. Brown-Peterson et al. (2015) conducted a 32-day exposure of juvenile southern flounder utilizing similarly oiled sediments at five concentrations, 0.04–395mg/kg tPAH50 (sum of 50 individual PAH concentration measurements). The flounder experienced increased mortality with increasing concentrations and time, at and above 8 mg/kg tPAH50 growth was inhibited, histological impacts in tissues were observed, altered gene expression was measured, and

microbial assemblages exhibited distinct shifts with exposure. Chronic exposure to oiled sediments negatively affected flounder at multiple biological levels (Brown-Peterson et al 2015). A follow-up study from Bayha et al. (2017) introduced a pathogen challenge to the oil exposure, using a *Vibrio anguillarum* concentration of $9.03 \text{ X } 10^5 \pm 5.09 \text{ X } 10^4$ CFU/mL and an oil concentration of 57.4 mg/kg tPAH50, after a 7-day oil exposure the flounder were placed in the bacteria bath for one hour then returned to their exposure tanks, two flounder were sampled 24 hours after the pathogen challenge and the remaining fish were observed for 48 hours following the pathogen challenge. Flounder in the treatment with both oil and pathogen experienced 94.4% mortality within 48 hours of the pathogen challenge, while flounder in the pathogen-only treatment exhibited mortality of less than 10%. Exposure to both pathogen and oil resulted in distinct gill and intestine microbiome communities (including much higher incidence of *Vibrio anguillarum*), and oil exposure alone resulted in reduced expression of Immunoglobulin M and downregulation of transcriptome response especially in genes related to immune function. Again, exposure to oiled sediments resulted in impaired immune function which led to increases of bacterial infection (Bayha et al 2017).

In the present research, such a strong oil and pathogen response was not observed which suggests there is a lower limit to concentrations of oil and pathogen that will cause an effect, and it appears to be between 25 mg/kg and 57.4 mg/kg of tPAH in the sediment and between $5.30 \text{ X } 10^5 \pm 1.81 \text{ X } 10^5$ CFU/mL and $9.03 \text{ X } 10^5 \pm 5.09 \text{ X } 10^4$ CFU/mL of *V. anguillarum*. Focusing on oil exposure only, the results from this study are consistent with the previous research such that concentrations near 10 mg/kg tPAH in sediment does capture changes in flounder microbiome. Changes in microbiome composition affect the

benefits provided to the flounder, such as producing nutrients and improving immune response which, in turn, impact the health of the fish and chronic exposure via long-lasting contamination in sediments may result in ecosystem wide impacts into the future.

Eastern Oyster

The second part of this research aimed to examine how long-term exposure to PAHs affects the microbiome of eastern oysters in the Mississippi Sound. The wild oysters' microbiome seemed unaffected by the contamination of PAHs within tissues or in nearby sediments. There are many potential reasons for this result: it may be because the oysters did not experience contamination levels high enough to cause changes in the microbiome, or, due to the long history of oil production in the area, both the oysters and their microbiome are capable of quickly responding to oil exposure and, five years after the spill in 2015, what was seen here was the tail-end of the disturbance where the signal has faded into the background noise of individual variation, or the sampling size was too small to capture the effect of these PAH concentrations' effect. Unfortunately, there have been very few studies looking at the effects of oil on the oyster microbiome using culture independent techniques. Thomas et al. (2014) utilized mesocosms spiked with oil collected during the *Deepwater Horizon* oil spill and inoculated with samples from oyster tissues to examine the potential for the oyster microbiome to degrade hydrocarbons and found that *Pseudomonas* was the primary member in the oil-enriched communities. Other studies performed on the oyster microbiome to date have primarily focused on characterization of communities of wild oysters collected from different locations, or during incidence of disease (King et al. 2012, Chauhan et al. 2014, Pierce et al. 2016,

Ossai et al. 2017). The present study contributes to this small field of oyster microbiome toxicology by providing an initial insight into the long-term effects of oil exposure on the oyster microbiome. And, fortunately for the health of oysters and thus the services provided to the ecosystem by their reefs in the Mississippi Sound, it appears that PAH contamination at the concentrations observed here may not impact the microbiome community of the eastern oyster.

Responses of Flounder and Oysters to PAH Contamination

Southern flounder and eastern oysters are both benthic, estuarine species found in the same geographic regions which suggests they should be exposed to similar conditions (Coen and Luckenbach 2000, Roberts 2012, Corey et al. 2017). However, oysters are sessile organisms; they settle onto a surface and typically remain there through their lifetime unless they are moved by some external force (Grabowski et al. 2012). Oysters are also epibenthic, they remain above the sediment, which may reduce their exposure to contaminants buried in sediments until the sediment is disturbed (Coen et al. 2007). Flounder are motile fish that can swim away from stresses if possible, which can limit their exposure to contaminants, however common behaviors such as burial within the sediment can put them in direct contact with any harmful contaminants that have been sequestered therein (Roberts 2012).

The flounder intestine microbiome was dominated by *Vibrio*, constituting 25% of the community composition, with the next most abundant taxa being *Photobacterium* which accounted for 2.4% of the community. The microbiome of the oyster digestive gland differed from the flounder microbiome. Some samples showed similar dominance

by a singular taxon such as *Sphingomonas* and *Mycoplasma*, however many samples were characterized by no single majority taxa and instead were made up of small proportions of many different bacteria, including *Chloroflexi*, *Gemmatimonas*, and *Novosphingobium*. There were taxa that appeared in both flounder and oyster microbiomes including *Ilumatobacter*, *Actinomyces*, *Mycobacterium*, and *Corynebacterium*, however they account for a very small percentage of each community.

Sediment concentrations are not consistent across both chapters of this research, the maximum tPAH concentration measured at any of the reefs was 166 ug/kg, whereas the concentrations of PAHs were measured at 11.61 mg/kg for Low Oil treatment, and 32.96 mg/kg for High Oil treatment, 3 orders of magnitude greater. This difference may be the secondary reason for the pattern of results seen in these two chapters following differences driven by the organisms themselves, the flounder microbiome was shown to be affected by the oil contamination in the sediments, while PAH contamination did not significantly affect the oyster microbiome. Getting the full picture of the impacts of a large-scale event such as the *Deepwater Horizon* oil spill is complicated, even two benthic, estuarine species (which one might expect to be similarly affected) exhibit differences in their responses to such disturbances.

Future Directions

Continued study of the effects of oil and PAH contamination are useful for identifying our human impact on the planet and are especially important in heavily commercial and contaminated areas like the northern Gulf of Mexico where there is a long history of heavy industry, a high population, and large fisheries. This study found

the flounder gill microbiome to be more sensitive to contaminated sediments, but that is not the only route of exposure for flounder and other fish in these waters. Examining a food-borne route of exposure may more significantly affect the microbiome of the digestive and immune systems. Additionally, the lesions observed on wild flounder that in-part prompted these studies suggest an impaired immune response occurring on the skin, thus looking at the skin microbiome and other immune system markers may aid in discovering the cause of the lesions.

Chapter 3 covered an observational field study of the oyster digestive gland microbiome which comes with its own pitfalls in experimental design. The next steps would be to perform experiments in the laboratory setting, using this and other similar field studies to achieve environmentally relevant contamination loads and observe the effect on the microbiome while being able to control for some of those unknown environmental factors.

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