THE SUB-CHRONIC EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS ON THE SHEEPSHEAD MINNOW (*CYPRINODON VARIEGATUS*) GUT-MICROBIOME AND FORAGING BEHAVIOR

by

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Dedicated to my family, friends, and fiancé, whose unwavering love and support throughout this process gave me the strength to carry on. Thank you.

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ABSTRACT

The microbiome plays a key symbiotic role in maintaining host health and aids in acquiring nutrients, supporting development and immune function, and modulating behavior. However, more research is needed to elucidate the potential impact of environmental pollutants on host microbial communities and how microbiomes can modulate the toxicity of contaminants to the host. Through a literature review of 18 studies that assessed the impacts of various anthropogenic chemicals on fish-associated microbiomes, we found that toxicants generally decrease microbial diversity, which could lead to long-term health impacts if chronically stressed, and can increase the host's susceptibility to disease as well as the chemical resistance of certain microbes. These findings led us to explore the impacts of one of the reviewed contaminants, polycyclic aromatic hydrocarbons (PAHs), typically found in oil. The Deepwater Horizon disaster of April 2010 was the largest oil spill in U.S. history and had catastrophic effects on several ecologically important fish species in the Gulf of Mexico (GoM). This study tested the hypotheses that exposure to weathered oil would cause significant shifts in fish gut-associated microbial communities, with taxa known for hydrocarbon degradation increasing in abundance and that foraging behavior would decrease, potentially due to microbial dysbiosis via the gut-brain axis. We characterized the gut microbiome (with 16S rRNA gene sequencing) of a native GoM estuarine species, the sheepshead minnow (*Cyprinodon variegatus*). Fish were exposed to High Energy Water Accommodated Fractions (HEWAF; tPAH = 80.99 ± 12.5 µg/L) of oil over a 7-day period and whole gastrointestinal tracts were sampled for microbiome analyses. A foraging behavioral assay was used to determine feeding efficiency before and after oil exposure. The fish gut microbiome did not experience any significant changes in alpha or beta diversity but known hydrocarbon degrading taxa were noticeably present in oil-exposed communities and were absent in controls. We found the order Pseudomonadales, the family Paenibacillaceae, and *Pseudomonas pachastrellae* to be among these, with Pseudomonadales increasing in abundance. Foraging behavior was not significantly affected by oil exposure. This work highlights the need for further research to elucidate the functional metagenomic responses of the fish gut-microbiome under oil spill conditions.

CHAPTER 1. LITERATURE REVIEW OF HOST-ASSOCIATED MICROBIAL COMMUNITY RESPONSES TO ANTHROPOGENIC POLLUTANTS

1.1 Introduction

Host-associated microbiomes are the collection of symbiotic single-cellular organisms that inhabit various parts of a multi-cellular host, playing a crucial role in maintaining host homeostasis. In fact, microbes are known for their significant contributions to many aspects of host health, and recently the term "holobiont" has emerged in the field to describe a host's microbiome as an additional organ (Simon et al., 2019). They contribute towards nutrient generation, pathogen resistance, immune function and development, and can affect host behavior (Amon & Sanderson, 2017). While microbial evolution is regulated by competition between other microbes to thrive in the host environment, the host is under selective pressure to create a beneficial microbial community (Foster et al., 2017). Thus, this co-evolution can favor beneficial species that will aid in host homeostasis.

Microbiomes are dynamic communities and many factors are known to influence their structure and composition. Such factors include diet (Bolnick et al., 2014; Di Maiuta et al., 2013; Youngblut et al., 2019), age (Dulski et al., 2018; Stephens et al., 2016), habitat (Kashinskaya et al., 2015; Sullam et al., 2012), and disease (Bayha et al., 2017a; Mohammed & Arias, 2015). Exposure to environmental pollutants has also emerged as a potential important factor impacting microbiomes (Giang et al., 2018; Lloyd et al., 2016; Mohammed & Arias, 2015) . Any disturbance, also termed dysbiosis, to microbial community abundance and/or composition can alter host function and health.

Changes in microbiomes of aquatic organisms after chemical exposure is an emerging field. Anthropogenic chemicals are known to disrupt the microbiome of aquatic organisms, where they can increase host susceptibility to disease yet also aid in the biotransformation of chemicals (Bayha et al., 2017b). When microbial communities are thrown off balance, more tolerant or opportunistic microbes can colonize niches where others previously occupied, thus giving room for pathogens or other chemically resistant microbes to grow. Thus, there is a need for more research and meta-analysis into the role that the microbiome plays in chemical detoxification, disease tolerance, and elucidating what benefits it may still provide to the host when challenged with a pollutant.

This review addresses current knowledge on teleost fish microbiomes, as well as the known effects of various categories of anthropogenic pollutants on fish-associated microbiomes. Specifically, I will focus on the following questions: 1) Do chemical contaminants result in consistent effects on fish microbiomes? 2) Can the negative effects on overall microbial diversity affect resilience of fishes to environmental stress? and 3) Can selection for resistant microbes improve host fitness or increase susceptibility to pathogenic microbes? I will finish with the aims of my research and overall Thesis organization.

1.2 Teleost Fish Microbiomes

Changes in the microbiomes of aquatic organisms after chemical exposure is an emerging field. Anthropogenic chemicals are known to disrupt the microbiota of aquatic organisms, where they can increase host susceptibility to disease yet also aid in the biotransformation of chemicals (Bayha et al., 2017a). When microbial communities are thrown off balance, more tolerant or opportunistic microbes can colonize niches where others previously occupied, thus giving room. This is because these mucosal surfaces are readily colonized by bacteria as they are in direct contact with the environment when performing key functions on creating a barrier to pathogens; respiration and waste exchange; and nutrient acquisition and synthesis, respectively. Microbes inhabit specific tissues, creating pockets of unique communities that are distinct from one another and the environment. In general, fish microbiome studies report data typically from the skin, gills, and gastrointestinal tract, though the latter has been studied more extensively studied. For example, one study examining gill and gut microbial communities of reef fish across 15 families found unique communities within the same host (Pratte et al., 2018). However, there was also distinct variation between individuals of the same species, suggesting host-specific factors for community determination (Pratte et al., 2018). The microbiomes associated with these tissues can also interact with one another. For instance, dysbiosis to gut bacteria can be outwardly reflected in the skin microbiota, potentially acting as a bioindicator of disease (Legrand et al., 2018).

Microbiota inhabiting external organs like the skin mucosal layer and the gills tend to be dominated by aerobes with overall lower diversity compared to the gut (Merrifield & Rodiles, 2015a; Sullam et al., 2012; A. R. Wang et al., 2018). Since fish are in intimate contact with water, their health and associated microbiomes are directly influenced by fluctuations in the physiochemical properties of water and anything that is dissolved in it. The skin and gill

microbiome is distinct from that of the gut and is generally colonized by members of the Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla in both fresh and saltwater fish, though some exceptions exist such as Cyanobacteria being present only in gills from marine fish and skin from freshwater fish (**Table 1.1**) (Merrifield & Rodiles, 2015a). Cyanobacteria, or "blue-green algae," are a common free-living phylum in both fresh and saltwater habitats, so this discrepancy could possibly be a characteristic of the more transient nature of a free-living microbe colonizing the external microbiome of a fish. One study examining the skin microbiota of over 100 marine fish across six species found dominance of Proteobacteria, Firmicutes, and Actinobacteria with *Aeribacillus* occurring in all species, though other bacteria taxa showed more species specificity (A. Larsen et al., 2013). Characterizing the external microbiome of fish has important implications, as dysbiosis to the gills or skin can lead to the colonization of opportunistic pathogens like *Vibrio anguillarum* which can then spread to other organs (Weber et al., 2010).

While mammalian gut communities are dominated by Bacteroidetes and Firmicutes, the fish gut consists predominantly of Proteobacteria, and this phylum combined with Bacteroidetes and Firmicutes make up 90% of the bacterial communities located within the gut (Ghanbari et al., 2015a; Lozupone et al., 2012). In general, intestinal microbiomes of saltwater fish largely consist of members of the genera (including symbols for gram negative and positive) *Aeromonas* (-), *Alcaligenes* (-), *Alteromonas* (-), *Carnobacterium* (+), *Flavobacterium* (-), *Micrococcus* (+), *Moraxella* (-), *Pseudomonas* (-), and *Vibrio* (-), with dominant levels of *Vibrio*, *Photobacterium* (-), and *Clostridium* (+), while the genera *Acinetobacter* (-), *Aeromonas* (-), *Flavobacterium* (-), *Lactococcus* (+), *Pseudomonas* (-), *Bacteroides* (-), *Clostridium* (+), and *Fusobacterium* (-) make up the freshwater fish intestinal microbiota (Egerton et al., 2018; Pérez et al., 2010). These taxa are quite similar between the two habitats and growing evidence suggests that a core microbiome may exist for certain species, although the differences between fresh and saltwater gut microbiomes should be explored further (Merrifield & Rodiles, 2015b). Gut bacteria from both saltwater and freshwater fish are mostly gram negative, indicating the higher potential for the development of resistance as their complex cell walls contain an outer layer that gram-positive bacteria lack, making them less permeable to certain antimicrobials or other toxicants (Exner et al., 2017; Silhavy et al., 2010). This adds to evidence that the mucosal lining of the gut is one of the main interactive sites between commensal and potentially pathogenic microorganisms (Pérez

et al., 2010). In sum, the fish gut microbiome remains important to study for its role in antimicrobial resistance and disease.

The field of microbiome research has seen a push in recent years towards Next-Generation Sequencing (NGS) methods. Studies are now primarily using sequencing through the hypervariable regions of the 16S rRNA gene, which identifies overall bacterial community compositions and clusters similar DNA sequences to form Operational Taxonomic Units (OTUs) that are further annotated and assigned to bacterial taxa (Nguyen et al., 2016). The limitation to this approach is that it does not identify any functional roles of bacteria, which is where metagenomic sequencing steps in to fill this gap. Metabolic pathways are generally conserved across bacterial taxa, and it may become more important to identify what causes shifts in microbiome functionality, although there will likely be some exceptions where there are irreplaceable bacterial taxa that execute core functions (Merrifield & Rodiles, 2015b). Both methods are important to characterize the microbiomes of healthy fish to serve as a baseline for comparison in toxicology studies.

1.3 Fish Microbiome Responses to Anthropogenic Contaminants

A broad array of studies have been conducted evaluating the effects of environmental contaminants on fish-associated microbiomes. Results show intriguing changes in patterns of microbial communities, including development of resistance and increased prevalence of pathogenic bacteria which can negatively impact host health (**Table 1.2**). These patterns were consistently observed across a wide range of contaminant classes (antibiotics, heavy metals, nanoparticles, pesticides, oil, and others).

1.3.1 Do chemical contaminants result in consistent effects on microbiomes?

Across all contaminants reviewed here, there was an overall pattern of diminishing microbial diversity as a result of chemical exposure. The only instances of increased diversity were seen in zebrafish exposed to polystyrene microplastics and copper nanoparticles (CuNPs) which was explained due to the large reduction in a common beneficial species that allowed for previously occupied niches to be filled (Y. Jin et al., 2018; Merrifield et al., 2013). Only one study, which orally exposed zebrafish to 0.1 μg/kg triclosan, analyzed interaction and connectivity

networks between microbes, which in this study increased as a result of the decreased number of communities (Gaulke et al., 2016). However, shifts in community structure were not consistent which may indicate that essential core functions of the microbiome were conserved for host health. Increases or decreases towards specific microbial taxa as a result of chemical contamination can be difficult to parse as various OTUs can fill similar functional niches in a host as discussed previously. Additionally, a decrease in overall diversity as a result of chemical contamination can lower a fish's ability to fight off pathogens, especially when this occurs on the skin or gills.

Although the main finding so far is that toxicants generally decrease microbial diversity, the question of whether contaminants result in consistent effects on fish microbiomes has several caveats that should be explored further. First, differences in the response of fish microbiomes to chemical exposure can vary based on specific toxicity mechanisms of chemicals as well as differences in environmental conditions. In addition, only one study exists for each class of contaminant reviewed which raises the need for more research to be conducted before we can begin to make comparisons. However, for chemicals in which there were two or more studies (i.e., polycyclic aromatic hydrocarbons or PAHs), interesting patterns in microbial resistance and pathogen prevalence were seen and are discussed in the following sections.

1.3.2 Can a decrease in microbial diversity affect resilience to environmental stress?

Decreased microbial diversity in fish as a result of contaminant exposure generally leads to inhibited host resilience when potentially faced with other environmental stressors. Increased disease susceptibility of fish after exposure to bactericidal agents or PAHs are good examples of this (Bayha et al., 2017; Mohammed & Arias, 2015). Decreases in beneficial bacteria tied to important functional roles were also noted along with systemic issues in the fish host. For example, in zebrafish orally exposed to copper and silver nanoparticles (0.5 g/kg), *Cetobacterium somerae* was notably reduced to undetectable levels (Merrifield et al., 2013). This beneficial gut species is common in fish and is involved in vitamin B12 production for the host, where decreases could potentially result in negative long-term health effects for the host (Tsuchiya et al., 2008). A three-month long aquatic co-exposure study to bisphenol A (BPA; 2.0×10^3, and 2.0×10^4 µg/L) and titanium dioxide nanoparticles (1.0×10^5 µg/ L) on zebrafish induced oxidative stress and gut inflammation to the host, depleting numbers of *Hyphomicrobium*, a species important for normal metabolic function in the gut (L. G. Chen, Guo, et al., 2018). The Firmicutes/Bacteroidetes ratio

in goldfish exposed to pentachlorophenol (0-100 μg/L) for 28 days was reduced as a result of exposure and positively correlated with the reduction in body and liver weight of fish (Kan et al., 2015). PAHs also induced changes in gill OTUs associated with host metabolism, inflammation, and disease in southern flounder exposed for 32 days (0.054 μg tPAH50/kg in sediment), where fish also exhibited increased mortality with exposure duration and concentration as well as reduced growth and lamellar epithelial proliferation in the gills (Nancy J. Brown-Peterson et al., 2015a). Although no other suboptimal environmental conditions were tested in conjunction with contaminants in these studies, long-term health impacts could present themselves in hosts if chronically stressed by non-lethal levels of a contaminant which could reduce their ability to survive in other adverse conditions such as those linked to climate change (ocean acidification, hypoxia, warming temperatures, etc.).

1.3.3 Can selection for resistant microbes improve host fitness and/or increase susceptibility to pathogenic microbes?

The most significant trend found across studies was an increased in microbial resistance. In general, resistance is characterized by a more active response which utilizes defense mutations capable of modifying the toxicant with continued or enhanced bacterial growth, while tolerance refers to bacteria that slow down growth and metabolism rates in order to survive the exposure conditions (Trastoy et al., 2018). Microbes are capable of modifying antimicrobials and toxicants in a variety of ways, such as producing enzymes that add chemical moieties to toxicants that increase their catabolism ad excretion (Hoffman, 2001; J.-S. Seo et al., 2009). Many studies within this review somewhat vaguely classified microbial resistance as taxa that increased in abundance and/or were known to be active in the degradation of a chemical (Gaulke et al., 2016).

Many of the resistant taxa identified in this literature search are known for their roles in chemical degradation, which could reduce the total amount of a chemical bioaccumulated in a fish and therefore improve host fitness. PAHs notably induced the increase in abundance of many hydrocarbon-degrading taxa (**Table 1.2**). Antibiotics and bactericidal agents also showed increased resistance, as well as mercury, which increased the prevalence of antibiotic-resistant microbes via co-resistance and horizontal gene transfer (Lloyd et al., 2016; Meredith et al., 2012a). If resistant taxa become more abundant as a response to contaminated areas, they could be out-competing and filling niches previously occupied by other species which could have potential

health implications for the host. The growing number of antibiotic resistant microbes is highly concerning for public health as well as aquaculture yields, and more research is needed to determine what other anthropogenic pollutants may be selectively pressuring microbes towards antibiotic resistance.

Although several studies detected increases in pathogenic taxa as a result of contaminant exposure, only two studies directly tested for disease susceptibility and reported an increase in both microbial dysbiosis and host mortality when pathogens were co-exposed with potassium permanganate and PAHs, respectively (Bayha et al., 2017a; Mohammed & Arias, 2015). Overall, shifts towards more resistant taxa indicate the loss of other species that are able to out-compete pathogenic bacteria. This creates a sort of double-edged sword where resistant bacteria may help the host reduce the total body burden of chemical contamination yet can also increase the prevalence of disease and host mortality.

1.4 Thesis Outline

The objective of my Thesis is to investigate the effects of PAHs from weathered oil on the sheepshead minnow (*Cyprinodon variegatus*) gut-microbiota. *I hypothesize that oil exposure will alter the species composition of the gut microbiota to favor functional niches capable of metabolizing PAHs.* To my knowledge, this is the first study using sheepshead minnows that has examined changes in microbial community structure after exposure to PAHs. The organization of my thesis is as follows:

- Chapter 1: General introduction.
- Chapter 2: "*Effects of Weathered Oil Exposure on Fish Gut Microbiome Composition and Feeding Behavior*". In this first data chapter, I will highlight the taxonomic shifts in the composition of the gut-microbiota via 16S rRNA sequencing as well as shifts in foraging behavior in response to an environmentally relevant concentration of weathered oil from the DWH oil spill in the Gulf of Mexico. I predict that significant shifts on the gut microbiome will occur, with communities shifting towards taxa that are known for hydrocarbon degradation. I also predict that fish foraging will decrease, potentially as a result of microbial dysbiosis via the gut-brain axis.
- Chapter 3: Summary of major findings and discussion of future directions and implications of this work.

Table 1.1. Summary of major bacterial phyla and associated orders and genera (with plus and minus indicating gram positive and gram negative) within the three main organs for marine and freshwater fish microbiome studies.

Fish Organ	Marine Fish Taxa	Freshwater Fish Taxa	Source
Skin	• Proteobacteria ○ Pseudomonadales, Enterobacterales, Aeromonadales, Burkholderiales, Vibrionales, Rhizobiales, Rhodospirillales, Alteromonadales, Rickettsiales, Caulobacterales, Xanthomonadales, Neisseriales, Sphingomonadales • Firmicutes ○ Bacillales, Lactobacillales, Clostridiales • Bacteroidetes ○ Bacteriodales, Flavobacteriales, Cytophagales, Sphingobacteriales • Actinobacteria ○ Actinomycetales	• Proteobacteria ○ Pseudomonadales, Enterobacterales, Aeromonadales, Burkholderiales, Vibrionales, Rhizobiales, Rhodospirillales, Alteromonadales, Rickettsiales, Caulobacterales, Xanthomonadales, Neisseriales, Sphingomonadales • Firmicutes ○ Bacillales, Lactobacillales, Clostridiales • Bacteroidetes ○ Bacteriodales, Flavobacteriales, Cytophagales, Sphingobacteriales • Actinobacteria ○ Actinomycetales • Cyanobacteria	*(Merrifield & Rodiles, 2015b)*
Gills	• Proteobacteria ○ Pseudomonadales, Enterobacteriales, Aeromonadales, Vibrionales, Alteromonadales, Rhizobiales, Burkholderiales, Pasteurellales, Caulobacterales, Xanthomonadales • Firmicutes	• Proteobacteria ○ Pseudomonadales, Enterobacterales, Aeromonadales, Burkholderiales, Vibrionales, Rhizobiales, Rhodospirillales, Alteromonadales, Rickettsiales, Caulobacterales, Xanthomonadales, Neisseriales, Sphingomonadales	*(Merrifield & Rodiles, 2015b)*

Table 1.1 continued

	- Bacillales, Lactobacillales, Erysipelotrichales - Bacteroidetes - Flavobacteriales, Bacteriodales - Actinobacteria - Actinomycetales - Cyanobacteria	- Firmicutes - Bacillales, Lactobacillales, Clostridiales - Bacteroidetes - Bacteriodales, Flavobacteriales, Cytophagales, Sphingobacteriales - Actinobacteria - Actinomycetales - Cyanobacteria	
Gut	- Proteobacteria *Aeromonas* (-), *Acinetobacter* (-), *Alcaligenes* (-), *Alteromonas* (-), *Moraxella* (-), *Pseudomonas* (-), *Vibrio* (-) - Firmicutes *Carnobacterium* (+), *Clostridium* (+), *Aeribacillus* (+) - Bacteroidetes *Flavobacterium* (-) - Actinobacteria *Micrococcus* (+)	- Proteobacteria *Aeromonas* (-), *Acinetobacter* (-), *Pseudomonas* (-) - Firmicutes *Lactococcus* (+) - Bacteroidetes *Bacteroides* (-) *Flavobacterium* (-) - Fusobacteria *Fusobacterium* (-)	*(Egerton et al., 2018; A. Larsen et al., 2013; Pérez et al., 2010)*

Table 1.2. Summary of the effects of various contaminant classes on fish-associated microbiomes with all concentrations expressed as parts per billion (ppb).

Category	Contaminant	Concentration	Fish Species	Environment	Microbiome	Notable findings	Source
Antibiotics	Rifampicin	25,000 µg/L in water	Western mosquitofish (*Gambusia affinis*)	Captive	Skin mucosal and whole gastrointestinal tract	Skin cultures took 1.6 days to recover and became >70% resistant; gut cultures took 2.6 days and became >90% resistant. Rifampicin lowered diversity and selected for Comamonadaceae family in the skin but not the gut. *Variovorax*, *Vibrio*, *Pelomonas*, *Hydrogenophaga*, *Pseudomonas*, *Mitsuaria*, and the Spirochaeta family increased in skin. *Myroides* genus dominated both during treatment but declined during recovery. *Variovorax* became dominant in the gut, and *Rickettsia* cleared from both tissues.	*(Carlson et al., 2017)*
Bactericidal	Potassium permanganate	5,400 µg/L in water	Channel catfish (*Ictalurus punctatus*)	Captive	Skin (tip of the lower lobe of caudal fin) and gills (second right gill arch)	Exposure significantly decreased diversity and increased host mortality and susceptibility to *F. columnaris* disease. Loss of diversity in exposed groups (dominated by Firmicutes); loss of pathogen competitors (*Aeromonas*, *Citrobacter*, *Pseudomonas* and *Luteimonas*).	*(Mohammed & Arias, 2015)*

Table 1.2 continued

Bacteriostatic	Triclosan	0.1 and 1 µg/L in water	Fathead minnow (*Pimephales promelas*)	Captive	Whole gastrointestinal tract	Gut microbial communities were significantly changed following exposure, but differences disappeared after 2 weeks. Resistant genera included *Acidovorax*, *Hydrogenophaga*, *Thauera*, *Methylophilaceae*, and *Methylotenera* (denitrification), *Acidovorax* and *Thauera* (triclosan degradation), and CK-1C4-19 (unclassified).	*(Narrowe et al., 2015)*
		0.1 µg/kg in food	Zebrafish (*Danio rerio*)	Captive	Intestinal contents (digesta)	*Pseudomonas* and Rhodobacteraceae increased in abundance; Enterobacteriaceae, Aeromonadaceae, and *Plesiomonas* decreased in abundance. *Pseudomonas*, Rhodobacteraceae, Rhizobiales, and CK-1C4-19 could be biomarkers for pollution.	*(Gaulke et al., 2016)*
Heavy metals	Mercury (Hg)	0.0244 - 0.132 µg/kg total Hg in food	Mummichog (*Fundulus heteroclitus*)	Wild	Whole gastrointestinal tract	Gut microbiomes had increased abundance of *mer*A gene. Hg resistant bacteria from wild caught fish were more likely to be resistant to multiple antibiotics.	*(Lloyd et al., 2016)*

Table 1.2 continued

						Most abundant genera included *Vibrio*, *Photobacterium*, *Pseudomonas*, *Halomonas*, and *Propionibacterium*.	
		0.0015 µg/L in lake water; 0.0818 to 1.08 µg/L Hg in field collected fish tissues	Feral brook trout (*Salvelinus fontinalis*)	Wild	Whole Gastrointestinal Tract	*Shewanella* was the least resistant to antibiotics and *Serratia* was the most resistant. *Hafnia* resistant towards protein synthesis inhibitor antibiotics, *Carnobacterium* resistant to cell wall synthesis inhibitor antibiotics.	*(Meredith et al., 2012b)*
Nanoparticles	Metal nanoparticles (Cu and Ag)	0.5 µg/kg in food	Zebrafish (*Danio rerio*)	Captive	Whole gastrointestinal tract	Overall community dysbiosis and reduced abundance of *Cetobacterium somerae* to undetectable levels. Two unidentified bacterial clones from Firmicutes were sensitive to Cu but were present in Ag and control fish.	*(Merrifield et al., 2013)*
	Radio-labeled graphene nanoparticles	50, 75, and 250 µg/L suspensions of large (300-700 nm) and small (20-70 nm) nanoparticles in water	Zebrafish (*Danio rerio*)	Captive	Rectum content (feces)	Graphene nanoparticles accumulated in the gut, and exposure increased community diversity. Proteobacteria and *Aeromonas* decreased. Both treatments associated with pathogenic *Escherichia coli*,	*(Lu et al., 2017)*

Table 1.2 continued

						Aeromonas caviae, and *Streptococcus mutans*.	
	Silver nanoparticles	13-19 nm particles in 800 µg/L and 400 µg/L in food	Nile tilapia (*Oreochromis niloticus* L.)	Captive	Intestinal tissue	Microbial culture count showed dose-dependent decreases at 0.8 mg/L.	*(Sarkar et al., 2015)*
Nanoparticles and Bisphenol A (BPA)	Titanium dioxide nanoparticles and BPA	1.0 x 10^5 µg/ L of 9.7 nm nano-TiO_2 particles 0, 2.0 x 10^3, and 2.0 x 10^4 µg/L BPA in water	Zebrafish (*Danio rerio*)	Captive	Intestines (pooled from five individuals as a biological replicate, n = 3)	Co-exposure increased pathogenic *Lawsonia* and decreased normal metabolic *Hyphomicrobium*. A positive relationship was found between fish body weight and *Bacteroides* abundance, also associated with genera of *Anaerococcus*, *Finegoldia*, and *Peptoniphilus*.	*(L. G. Chen, Guo, et al., 2018)*
Pesticide	Pentachlorophenol (PCP)	10, 50, and 100 µg/L PCP in water	Goldfish (*Carassius auratus*)	Captive	Intestinal content (digesta)	Bacteroidetes increased, and *Bacteroides* genus had the highest abundance, which was significantly correlated with dosage and duration of exposure. Bacteroidetes/*Bacteroides* abundance and Firmicutes/Bacteroidetes ratio reduced and associated with lower body and liver weight under PCP stress.	*(Kan et al., 2015)*

Table 1.2 continued

Plastics	Polystyrene microplastics	0.5 and 50 mm-diameter polystyrene microplastics at 1.0 x 10^5 and 1.0 x 10^6 µg/ L in water	Zebrafish (*Danio rerio*)	Captive	Whole gastrointestinal tract	Exposure decreased Bacteroidetes and Proteobacteria, but increased abundance of Firmicutes. *Flavobacterium* (pathogen) increased significantly in both treatments; *Bacteroides* and *Rhodobacteria* increased slightly.	*(Y. Jin et al., 2018)*
Polycyclic Aromatic Hydrocarbons	Crude oil	0.0574 µg tPAH50/kg in sediment	Southern flounder (*Paralichthys lethostigma*)	Wild	Gill tissue (upper and lower limbs), intestinal tissue	Oil exposure increased susceptibility to disease (*Vibrio anguillarum*). Increases in *Oceanospirillales*, *Thalassolituus*, and *Alcanivorax* (known oil degraders).	*(Bayha et al., 2017a)*
		0.054 µg tPAH50/kg in sediment	Southern flounder (*Paralichthys lethostigma*)	Wild	Gill tissue (upper and lower limbs), intestinal tissue	Gammaproteobacteria, Sphingobacteria, Deltaproteobacteria, and Epsilonproteobacteria increased with oil exposure in intestines; Alphaproteobacteria and Clostridia decreased. Known oil degraders (*Alcanivorax*, *Arcobacter*, *Donghicola*, and *Acinetobacter*) and pathways related to chemical degradation increased.	*(Nancy J. Brown-Peterson et al., 2015b)*
		Field concentrations not provided	Gulf killifish (*Fundulus grandis*)	Wild	Skin mucosal layer	Beta- and Gammaproteobacteria most prevalent, specifically *Pseudomonas*.	*(A. M. Larsen et al., 2015a)*

Table 1.2 continued

						Seasonality influenced structure, no significant difference in communities from oil/non-oil sites. *Pseudomonas*, *Acinetobacter*, *Acidovorax*, *Vibrio*, and *Clostridium* more abundant on skin than in water.	
		10, 50, 100 µg/L concentrations of oil in water (16 EPA PAH concentration of Troll C crude oil = 1.6 x 10^6)	Atlantic cod (*Gadus morhua*)	Wild	Whole gastrointestinal tract	Higher levels of exposure associated with decreased gut microbial diversity. Bacteroidetes, Firmicutes, *Porphyromonadaceae*, *Rikenella*, *Ruminococcaceae*, *Alistipes*, and *Clostridiales* decreased in high oil concentrations. *Deferribacterales* increased in abundance and is a possible oil degrader.	*(Bagi et al., 2018a)*
Other/ Mixture	"Model pollutants"	0.42 µg/ L atrazine 0.43 µg/L estradiol 0.65 µg/L and 0.23 µg/L PCBs in water	Zebrafish (*Danio rerio*)	Captive	Intestinal tissue (pooled from ten individuals as a biological replicate, n = 3)	Atrazine had little effect; Estradiol increased abundances of *Streptococcus*, *Acinetobacter*, and *Capnocytophaga* via ER signaling. PCB groups showed increased *Aeromonas* reproduction which was positively associated with oxidative damage; Intestinal permeability regulated by interactions between *Aeromonas*,	*(L. G. Chen, Zhang, et al., 2018)*

Table 1.2 continued

						Mannheimia and *Blastococcus* via AhR signaling.	
	Sewage plant effluent	Concentrations (not provided) of 155 pharmaceutical and personal care products and 16 perfluorinated compounds found via passive sampling	Brown trout (*Salmo trutta* L.)	Wild	Intestinal contents (digesta)	Two bacterial taxa associated with effluent (unclassified *Nakamurellaceae* and *Oscillatoriales*) found in significantly high levels in intestinal microbiota in trout sampled downstream from the sewage plant.	*(Giang et al., 2018)*

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CHAPTER 2. EFFECTS OF WEATHERED OIL EXPOSURE ON FISH GUT MICROBIOME COMPOSITION AND FEEDING BEHAVIOR

2.1 Abstract

The microbiome plays a key symbiotic role in maintaining host health and aids in acquiring nutrients, supporting development and immune function, and modulating behavior. The Deepwater Horizon disaster of April 2010 was the largest oil spill in U.S. history and had catastrophic effects on several ecologically important fish species in the Gulf of Mexico (GoM). This study tested the hypotheses that exposure to weathered oil would cause significant shifts in fish gut-associated microbial communities with taxa known for hydrocarbon degradation increasing in abundance and that foraging behavior would decrease, potentially due to microbial dysbiosis via the gut-brain axis. We characterized the gut microbiome (16S rRNA) of a native GoM estuarine species, the sheepshead minnow (*Cyprinodon variegatus*). Fish were exposed to oil in High Energy Water Accommodated Fractions (HEWAF; tPAH = 80.99 ± 12.5 µg/L) over a 7-day period and whole gastrointestinal tracts were sampled for microbiome analyses. A foraging behavioral assay was used to determine feeding efficiency before and after oil exposure. The fish gut microbiome did not experience any significant changes in alpha or beta diversity, yet the gut selected some microbes from the environment, resulting in a less diverse community than the water environment. We also noticed that the fish gut microbiome may change over time, when comparing our baseline community samples to those of control or oil-exposed fish in the experiment. Our most significant finding was that known hydrocarbon degrading taxa were discriminant of oil-exposed communities and were absent in the control. From our LEfSe analyses, we found the order Pseudomonadales, the family Paenibacillaceae, and *Pseudomonas pachastrellae* to be among these, with Pseudomonadales increasing in abundance. However, none of the most abundant taxa had any significant shifts as a result of oil exposure, with only rare taxa having significant shifts in abundance from control to oil treatments. Potential low bioavailability of the oil may have been a factor in our observation of minor shifts in taxa. In the foraging study, we found that behavior was not significantly affected by oil exposure. This work highlights the need for further research to elucidate the functional metagenomic responses of the fish gut-microbiome under oil spill conditions.

2.2 Introduction

The Deepwater Horizon (DWH) oil spill in 2010 caused an estimated total of 4.4 million barrels of crude oil to be spilled into the Gulf of Mexico (GoM) and the coastal waters of Louisiana, Mississippi, Alabama, and Florida over the course of approximately three months (Crone & Tolstoy, 2010; Zukunft, 2010). The sheer magnitude of the spill made it the largest in U.S. history (Y. Liu et al., 2011). Since then, a multitude of research has been conducted to elucidate the short and long-term effects on the environment and aquatic organisms impacted by the spill. Much of this has focused on polycyclic aromatic hydrocarbons (PAHs), which are the main toxic component of oil. PAHs consist of a wide variety of highly volatile and environmentally persistent chemicals, generally composed of at least 2 benzene rings with hydrophobic and lipophilic double bonds. Due to their lipophilic nature, they can be readily absorbed from an animal's gastrointestinal tract and bio-accumulate in tissues to higher concentrations than the surrounding environment (Abdel-Shafy & Mansour, 2016a). PAHs differ in molecular weight (MW), which directly impacts their volatility, toxicity, and water solubility. Higher MW compounds consist of 3 to 4 fused benzene rings (typically found in weathered surface slick oil) whereas lower MW compounds are comprised of only 2 fused rings (typically found in source oil) (Tarr et al., 2016). Lower MW PAHs tend to be more volatile, water soluble, and less toxic than heavier PAHs which are more environmentally persistent (Alegbeleye et al., 2017b). The slick oil that reached the coastal surface waters in the GoM went through environmental weathering (via evaporation, dissolution, photo- and biodegradation) which removed lighter oil components and could have caused an increase in toxicity to aquatic organisms (Carls & Meador, 2009; Esbaugh et al., 2016; Geier et al., 2018; O'Shaughnessy et al., 2018). For this reason, it is imperative to explore the various biological endpoints of weathered oil toxicity.

Investigating the impacts of toxicants on the microbiome of aquatic organisms is a relatively new area of research, yet it is rapidly increasing in interest among ecotoxicologists. As most microbial species are extremely difficult to culture, modern genomic practices have been incorporated to determine microbial community composition by sequencing amplification products from the hypervariable regions of the 16S rRNA gene (Ghanbari et al., 2015b). Existing studies on fish microbiomes have largely used this method to determine changes in microbial taxa abundance and composition in response to a toxicant, with several exhibiting decreased diversity and overall dysbiosis (an imbalance of microbial taxa) in response to anthropogenic pollutants

(**Table 1.1**). However, it is possible for the microbiome to aide the host in abating toxicant exposure.

The host-associated microbiome plays an important role in mediating the effects of toxicity, especially through the detoxification of chemicals (Adamovsky et al., 2018a; Bagi et al., 2018b). Previous studies have found that some microbes are capable of PAH degradation. It is well known that some taxa within marine microbial communities can degrade oil (Baelum et al., 2012; Gutierrez et al., 2013; King et al., 2014; Looper et al., 2013); however, not enough attention has been given to the effects of oil on the gut microbiome of fish inhabiting areas impacted by oil spills (Bayha et al., 2017b; N J Brown-Peterson et al., 2015; A. M. Larsen et al., 2015b). Among the studies that have examined the impacts of oil on the fish gut microbiome, some of the same taxa of known hydrocarbon-degrading microbes found to increase in abundance in the Gulf waters impacted by the DWH oil spill also increased their numbers in the gut. In the two southern flounder (*Paralichthys lethostigma*) studies, these include members of the class Gammaproteobacteria, including Operational Taxonomic Units (OTUs) with genera from *Alcanivorax, Oceanospirillales, Acinetobacter*, and *Donghicola* (Bayha et al., 2017b; Nancy J. Brown-Peterson et al., 2015c). *Deferribacterales* were detected in Atlantic cod (*Gadus morhua*) exposed to oil which is an OTU of a recently discovered phylum that is capable of degrading PAHs in the gut microbiome (Bagi et al., 2018b). Though these oil-degrading microbes belong to diverse groups, they all perform a similar functional niche.

Beyond host-associated microbial dysbiosis, several physiological effects of oil exposure in fish have been reported through altered behavioral responses. Previous studies have found that oil can increase mortality by predation caused by a reduction in shoaling, sheltering, and increased risk-taking (Johansen et al., 2017), as well as impaired feeding rates, decreased total food consumption, and irregular swimming patterns (Guven et al., 2018; Rowsey et al., 2019). These behaviors contribute to decreased survivorship and lower reproductive fitness of the individual. Reproduction has a high energy cost, so fish must maintain adequate levels of nutrients during this time. If exposure to oil impairs their ability to forage, their reproductive fitness could be greatly reduced and may negatively affect population growth. However, impaired foraging ability due to oil exposure may be mediated by the microbiome.

The microbiome plays an important role in moderating host energy homeostasis, and the various metabolites produced from microbes can mediate behavior via the gut-brain axis (Butt &

Volkoff, 2019; van de Wouw et al., 2017). The process of metabolites mediating behavior is highly complex and involves numerous cellular and molecular pathways. One of the most widely studied microbial metabolites are Short-Chain Fatty Acids (SCFAs), which are capable of modifying the functionality of enterocytes in the gut from microbial fermentation of dietary nutrients (Butt & Volkoff, 2019; van de Wouw et al., 2017). They provide a variety of benefits to the intestinal tract, acting as an energy source for colon epithelial cells, decreasing colon pH to facilitate mineral absorption and decrease ammonia intake, producing anti-inflammatory effects, and promoting satiety, among many others effects (Rivière et al., 2016). SCFAs, produced after microbial fermentation, reach the brain either directly through vagus nerves or indirectly via receptors on enteroendocrine cells, releasing appetite-regulating hormones and peptides into circulation and ultimately affecting long-term energy homeostasis and eating behaviors (van de Wouw et al., 2017). However, the mechanisms behind the functions of the gut-brain axis and molecular signaling by SCFAs are still poorly understood (Adamovsky et al., 2018b; Butt & Volkoff, 2019; van de Wouw et al., 2017). In another possible avenue, microbes can influence the hypothalamic-pituitary-adrenal (HPA) axis via the production of endocrine hormones such as catecholamines and γ-aminobutyric acid, where gut dysbiosis could induce an altered stress response and thus decrease some behaviors such as foraging, although stress hormones themselves are capable of inducing microbial dysbiosis (Butt & Volkoff, 2019; Sudo, 2014). Gut dysbiosis induced by pollutants is mediated through an increase in the stress hormone cortisol produced by the hypothalamic-pituitary-adrenal (HPA) axis, thus potentially affecting foraging behavior (Butt & Volkoff, 2019). PAHs, in particular, can also cause gut epithelial inflammation, possibly decreasing nutrient uptake (Ribière et al., 2016). Although microbial alterations to stress responses or bacterial SCFAs could be likely contributors toward changes in host behavior as a result of pollutant exposure, there are many other factors (such as direct impacts of stress hormones or other host systemic alterations) that could result in changes in feeding behavior as it is challenging to separate host-derived cellular and molecular responses to stimuli from specifically microbial-derived responses.

In this second chapter, laboratory simulated oil spill conditions were predicted to induce dysbiosis of the fish gut microbiome whereby members of known oil-degrading taxa would increase in abundance in the gut. The hypothesis that oil exposure will alter the species composition of the gut microbiota to favor functional niches capable of metabolizing PAHs was

tested. In a separate experiment with identical exposure conditions, foraging was predicted to become less efficient in response to weathered oil exposure, testing the hypothesis that reduced foraging ability due to oil exposure could be caused in part by microbiome dysbiosis as described above.

2.3 Materials and Methods

2.3.1 Animal Model and Colony Maintenance

The sheepshead minnow (*Cyprinodon variegatus*) was chosen as the model species in this study due to its frequent use in toxicity studies, ease of culturing in captivity, and environmental relevance in regard to the DWH oil spill. It is an omnivorous estuarine species native to coastal waters of the Atlantic ranging in distribution from the GoM as far south as Venezuela (Hubbs et al., 2008). Estuarine fish are known for their ability to tolerate a wide range of environmental extremes including fluctuations in temperature and salinity (Caffrey et al., 2014; EPA, 2002), but sheepshead minnows in particular can be reproductively and developmentally sensitive to environmental pollutants such as oil when compared to other estuarine species that have developed a higher tolerance to pollutants (Hamilton et al., 2017; Jasperse et al., 2019b, 2019a; Osterberg et al., 2018; Reid et al., 2016; Simning et al., 2019). Only females were used in this study as males tended to exhibit more frantic and aggressive behaviors when transferred to smaller enclosures, and sex-specific differences in foraging behavior or gut microbial responses was not an endpoint being measured in either study.

Adult female fish (120 days post-hatch, dph) were ordered from Aquatic BioSystems (Fort Collins, Colorado) and acclimated and maintained in a recirculating tank system for at least one month prior to the start of experiments. This system was built in an environmentally controlled room with temperatures maintained at 25 ± 1℃ and a 16L:8D photoperiod. The tank system comprised eight 39 L and six 70 L tanks, all maintained at 25 ± 1℃, 25 ppt salinity, 7-9 mg/L dissolved oxygen (DO), and 7.5 - 8.5 pH. Fish were fed twice daily with Aquatic BioSystems' custom made low-soy dried brine shrimp flakes from Brine Shrimp Direct (Ogden, Utah).

2.3.2 Experimental Design

Both studies were a fully factorial design ran in three separate experimental runs with five replicates per treatment per run (**Fig. 2.1**). A total of 15 fish were exposed to weathered oil from the surface (OFS, see more on source of oil below) diluted in artificial saltwater, or High Energy Water Accommodated Fractions (HEWAF), at a nominal concentration of 5% HEWAF exposure dose for 7 days. The same number of fish were kept as controls. Sample size was calculated in G*Power (Faul et al., 2009), conducted with an a priori test within F test family, using the Linear multiple regression: Fixed model, R^2 deviation from zero test. Microbiome changes and foraging behavior were used as the response variables for each respective study. Five extra fish from the holding tank system were sacrificed to establish a baseline gut microbiome and five water samples were collected from the tank system to determine the environmental microbiome. All microbiome experiments were performed from August 6th through August 26th, 2019, and the foraging study was conducted between September 20th and October 10th, 2019.

Figure 2.1 Experimental design for both the foraging and microbiome studies. Microbiome study fish were fed only flake food and foraging study fish were supplemented with *Daphnia magna* on days one and seven.

We used slick A oil collected on July 29, 2010 from surface skimmers on barge number CTC02404 and provided to us by British Petroleum (Sandoval et al., 2017). This nominal concentration of 5% was chosen based upon a previous study that found transcriptional changes in sheepshead minnows exposed to low concentrations of slick A oil (approximately 1%) from the DWH spill (Jones et al., 2017). As no other studies have examined microbiome effects in this species as a result of weathered oil exposure to our knowledge, we increased the concentration slightly in hopes of inducing microbial dysbiosis.

The experimental units were 1.5 L closed-system glass jars (Specialty Bottle, Seattle, WA) with aluminum foil covers, containing artificial saltwater at 25 ppt salinity maintained in incubators (Powers Scientific, Inc., Pipersville, PA) with controlled temperature (25 ± 1 °C) and photoperiod (16L:8D). All water quality parameters were controlled and tested daily from the tank system to ensure that all were within the standard ranges (**Table S-2.1**). Water changes were performed every 24 hours to maintain the oil concentration in the treatment group and to maintain good water quality. Response variables were microbial composition and total amount of prey captured by the fish for the microbiome and foraging studies, respectively, with HEWAF as the predictor variable for both experiments.

2.3.3 Experimental Runs

High Energy Water Accommodated Fraction (HEWAF) Preparation

We prepared the HEWAF by mixing 1 g of OFS with 1 L of artificial saltwater in a Waring model CB15 commercial blender (Waring Lab, Stamford, CT) on the high setting for 30 seconds. Once blended, the mixture was allowed to settle in a 1 L glass separatory funnel for at least one hour. The bottom 800 mL were drained for immediate use in exposures.

Measurement of total PAHs using Fluorometry and GC/MS

As most PAHs fluoresce, we indirectly measured total PAH concentrations from water in the oil treatment group using a Turner Designs 10-AU fluorometer (Turner Designs, San Jose, CA). Fluorescence spectroscopy is a cost-effective way to estimate total PAHs in an aqueous solution, and it is imperative to capture these measurements throughout the course of an exposure due to the tendency of lower molecular weight PAHs to dissipate (Alegbeleye et al., 2017c; Williams & Bridges, 1964). Our protocol was adapted from an established procedure (Greer et al.,

2012). Water samples from the oil treatment units (1 mL from each of the five units) were taken in triplicate in scintillation vials mixed with equal parts 100% ethanol at 0 and 24 hours for immediate fluorescence analysis. Serial dilutions of the HEWAF stock, water, and ethanol were prepared each day along with the experimental samples to create a standard curve in which to quantify the total PAHs of the samples from the linear regression equation. Our standard curve contained concentrations of 100, 75, 50, 25, 5, 2.5, and 1% HEWAF. All samples were then sonicated in a water bath for 3 minutes to reduce PAH adhesion to the container before being transferred to 1.5 mL Eppendorf tubes to be centrifuged at 10,000 RPM for 10 minutes to remove salt particles. Avoiding the salt pellet at the bottom of the tube, a 4.5 mL aliquot of each sample was then transferred to a quartz cuvette for analysis in the fluorometer. To identify and quantify PAH analytes and tPAHs, we collected three-1 L samples of HEWAF stock (one from each experimental run), and sent them to ALS Environmental (Kelso, WA) for Gas Chromatography/Mass Spectrometry (GC/MS) analysis.

To estimate the total number of PAHs from each of the fluorescence readings, we used the linear regression equation from each standard curve with the raw fluorescence value represented as full scale units (FSU) used as the y value to solve for x. The x value was then divided by 100 and multiplied by the total PAH (µg/L) value obtained from ALS Environmental (**Table 2.2**). The sample from the first experimental run was outside of the temperature range when received by ALS and was thus excluded from calculations as volatiles could have been lost at warmer temperatures, reducing the measurable tPAH. The sample from the third experimental run was created after the jar of OFS was accidentally left out at room temperature for approximately two days, and it is unknown how much tPAH was lost through volatility during that time. The sample from the second experimental run had the highest tPAH, yet the lowest FSU values. This may be due to the fact that it was a weaker HEWAF resulting in low fluorescence yet had the highest tPAH as it was the only sample not subject to degradation in some form. The FSU values for the first and third samples were extremely close, with the combined standard curve data having an R^2 value still above 0.99. Using the equation from the combined standard curves, the fluorescence for the second sample suggests that it was approximately 84% the strength of the other two samples. If this were of the same strength as the other samples, the tPAH would have been 3489.097 ug/L. Thus, we used this value as the 100% reference point for all HEWAFs prepared prior to the third sample, and 2777.99 ug/L as the 100% reference point for all HEWAFs prepared on or after the

third sample for both the microbiome and foraging studies as this sample accounts for the loss of tPAHs through volatility after being at room temperature.

DNA Extractions

On day seven of each experimental run, all fish were euthanized in 250 mg/L buffered tricaine methanesulfonate (MS-222) in RO water and dissected to then immediately extract DNA from their whole gastrointestinal tract (GI) plus digesta. Fish microbial communities can be found in both the digesta and mucosal lining of the intestines, so both were included as this study is not focused on differentiating the two (Gajardo et al., 2016). All equipment was autoclaved prior to dissections, trays and surrounding areas were cleaned with 100% ethanol, and sterile gloves were worn and replaced for each individual fish to reduce potential contamination. Fish were blotted dry with Kimwipes before being weighed and measured for total body length. We then removed the complete GI tract and placed it in a sterile 1.5 mL microcentrifuge tube for DNA extraction using the Qiagen DNEasy Blood and Tissue Kit, Spin Column Protocol (Qiagen, Venlo, Netherlands). Water samples (100 mL each) were vacuum filtered with a 0.45 um PES vacuum filtration assembly (Cole-Parmer, Vernon Hills, IL) and DNA was extracted using the Qiagen DNEasy PowerWater Kit (Qiagen, Venlo, Netherlands) and quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE, USA). All DNA extracts were stored at -20 °C until ready for shipping to CD Genomics (Shirley, NY) for library preparation and sequencing.

Foraging Behavior Trials

We conducted behavioral trials for each fish on days one and seven of each experimental run. These trials were adapted from previous studies that determined prey capture rate of adult and larval mummichogs (*Fundulus heteroclitus*) and sheepshead minnows in response to PAH contamination (Jasperse et al., 2019b; Judith S. Weis et al., 2001; Judith S Weis et al., 2003). Fish were fed flake food throughout the duration of the experiment and supplemented with *Daphnia magna* as the live prey species on days one and seven, and all fish were fasted for at least 24 hours prior to the start of feeding trials. *D. magna* were chosen as the prey species due to their higher salinity tolerance and ease of visually counting due to their larger size (Ebert, 2005). Trials were

conducted inside the incubator under the same exposure conditions described earlier. Fish were first moved to a separate 3 L beaker filled with 1.5 L of clean water from the tank system and allowed to acclimate for 10 minutes. After acclimation, 10 *D. magna* were added to the beaker and a GoPro Hero3+ camera (GoPro, San Mateo, CA) that was mounted directly above began to record visual data for 3 minutes. These videos were later analyzed to count the number of *D. magna* consumed. All fish were euthanized in 250 mg/L buffered MS-222 in RO water at the conclusion of each experimental run.

2.3.4 Bioinformatics and Statistical Analyses

Oil Exposures

To assess the variability in tPAH results across experimental runs as well as between initial and final fluorescence measurements, one-way ANOVAs along with t-tests assuming unequal variances were conducted.

Microbiome Study

To characterize microbial taxonomic composition, all samples were sequenced at CD Genomics using the 16S rRNA gene amplification method, targeting the V3/V4 region of the gene. Amplification products were sequenced on the Illumina MiSeq platform (300bp paired-end raw reads). Reads were assigned to samples according to their assigned barcodes, truncated, and merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). Raw tags underwent quality filtering performed under the split_libraries_fastq.py -q 19 filtering parameter in Trimmomatic v0.33 (http://www.usadellab.org/cms/?page=trimmomatic), with all other parameters set to default. To acquire OTUs, tags with 97% similarity were clustered with UCLUST in QIIME (version 1.8.0) and the resulting OTUs were annotated based on the Silva taxonomic database (Caporaso et al., 2010; Edgar, 2010). Abundance information for each taxonomic level was determined using QIIME.

Several downstream statistical measures were used to analyze the taxonomic characterization samples between treatment groups. The size of a microbial sequence library can vary between samples and can therefore be an important driver of alpha diversity results. As such, we normalized the varying number of reads across samples at 44,012 sequences. Rarefaction

curves, which plot the observed number of species per number of reads, were used to determine if the communities were sufficiently sampled at this sequencing depth (Weiss et al., 2017). Chao1 and ACE species richness metrics as well as Shannon, Simpson, and Phylogenetic Diversity (PD) Whole Tree diversity indices were calculated with Mothur (version 1.30) and used to describe microbial alpha diversity which was compared between treatments using a one-way ANOVA followed by Tukey's HSD. Non-Metric Multidimensional Scaling (NMDS) ordinations were created in RStudio (Version 3.6.1, RStudio, Inc.) to visualize sample clustering based on Bray-Curtis dissimilarity matrices, with PERMANOVA tests conducted to determine if the communities in each treatment were significantly different from one another. Relative abundance bar graphs were created in Microsoft Excel to describe overall taxonomic characterizations of samples and treatments. METASTATs analysis, a non-parametric method that uses t-tests with sample permutation, was used to determine if any of the top ten most abundant taxa were differentially represented between treatments (White et al., 2009). Significance for METASTATs results was determined using adjusted *p*-values (*q*) at a 0.05 cutoff. Finally, we used Linear Discriminant Analysis (LDA) Effect Size (LEfSe) to determine discriminant taxa across all treatment groups as well as between only control and oil treatments. LEfSe is a statistical algorithm that first detects statistically different features between taxa in various treatment groups (using the Kruskal-Wallis rank-sum test), then couples this with tests to determine if these differences are consistent with previous biological knowledge (using the Wilcoxon rank-sum test), and finally uses LDA to estimate the effect sizes of these features (Segata et al., 2011). Essentially, this method gives a more robust estimation of taxa that possess differentially abundant characteristics with respect to an environmental or experimental group. In our analysis, we included any taxa that had an LDA score > 2.

Foraging Study

All statistical analyses were conducted in RStudio (Version 3.6.1, RStudio, Inc.). We checked the normality of the data using Shapiro-Wilk's test and the homogeneity of variances using the Fligner-Killeen test. Since the amount of prey captures are not true count data due to the limit being 10 *D. magna* per trial, we determined statistical significance using a binomial general linear model (GLM) for proportional data.

2.4 Results

2.4.1 Oil Exposures

Water quality conditions remained relatively constant throughout both studies (**Table S-2.1**). Mean (± SEM) temperature was 25.44 ± 0.05 °C, salinity 24.85 ± 0.11ppt, pH 7.98 ± 0.01, DO 8.52 ± 0.15 mg/L, and total ammonia > 0.1 mg/L. Nitrates and nitrites were mostly stable at 0 mg/L with one slight spike during the latter part of experiment 2 of the foraging study (0.21 ± 0.12 mg/L and 0.01 ± 0.01 mg/L, respectively). All fish were within the weight and total body length parameters to classify as adults, and all DNA yields were of sufficient quantity (**Table S-2.2**).

The fit of calculated tPAH values and raw fluorescence units for each experimental run in both studies were assessed using linear regressions (**Fig. 2.2**). Regressions for both experiments across all runs were of good fit, with R^2 values > 0.97.

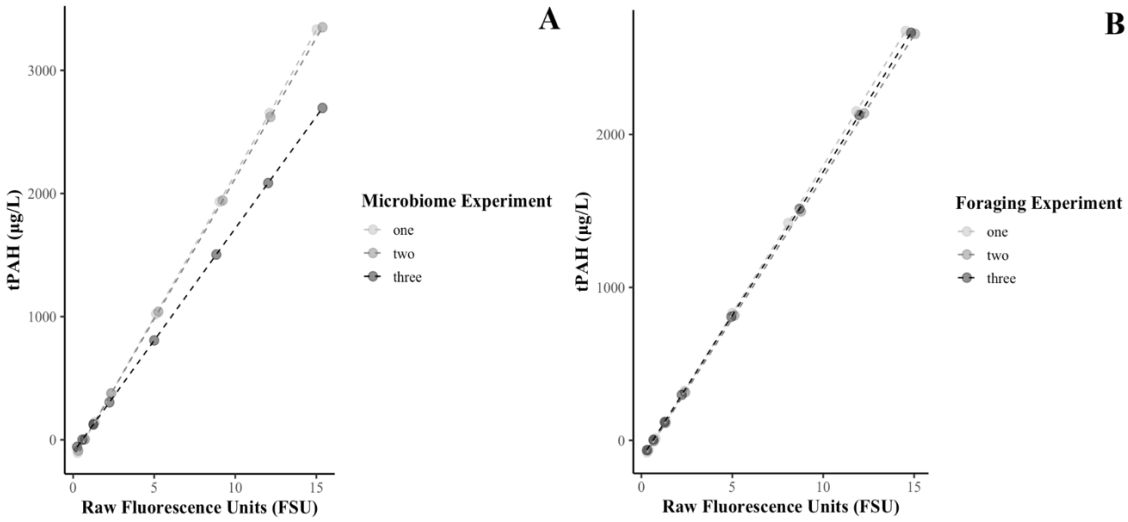


Figure 2.2 Linear regressions of raw fluorescence units (FSU) and total PAHs (µg/L) per experimental run for the microbiome (A; Adjusted R^2 = 0.9799) and foraging (B; Adjusted R^2 = 0.9993) studies.

All estimated tPAH concentrations were calculated based upon standard curves created for each HEWAF made as described previously. The mean (± SEM) tPAH measurements for the initial concentration (0 hours) calculated from fluorescence results across all experimental runs were 228.53 ± 10.52 µg/L for the microbiome study, 197.18 ± 8.38 µg/L for the foraging study, and 212.86 ± 7.14 µg/L across both studies. After 24 hours, concentrations had decreased to 20.53

± 21.61 µg/L for the microbiome study, 44.32 ± 10.48 µg/L for the foraging study, and 32.43 ± 12.01 µg/L across both studies. The estimated 12-hour tPAH values were calculated as the geometric mean of 0- and 24-hour samples (Microbiome: 68.5 ± 3.89 µg/L; Foraging: 93.49 ± 2.03 µg/L; Both studies: 80.99 ± 12.5 µg/L). As PAHs dissipated quickly, the 12-hour tPAH values were interpreted as our ultimate tPAH concentration across both studies.

For the microbiome study, means were significantly different for the initial 0-hour values across all experimental runs ($F(2, 15) = 3.68$, $p = 0.05$), with the variation coming from experiments 2 and 3 ($t(10) = 2.53$, $p = 0.03$), as experiment 1 was not significantly different ($t(10) = -1.97$, $p = 0.08$; $t(10) = 0.56$, $p = 0.58$) (**Table S-2.2**). No variation was found in the final values across all experimental runs ($F(2, 15) = 1.99$, $p = 0.17$). All means were significantly different from initial to final tPAH measurements for all experimental runs ($t(6) = 4.51$, $p < 0.01$; $t(10) = 7.65$, $p < 0.001$; $t(9) = 6.58$, $p < 0.001$) (**Fig. 2.2-A**). No significant differences were found between means across the three experimental runs for initial and final results in the foraging study ($F(2, 15) = 1.27$, $p = 0.31$; $F(2, 15) = 2.57$, $p = 0.11$) (**Table S-2.2**), but means were significantly different from initial to final measurements in all experimental runs ($t(7) = 7.33$, $p < 0.001$; $t(9) = 6.98$, $p < 0.001$; $t(6) = 6.61$, $p < 0.001$) (**Fig. 2.2-B**).

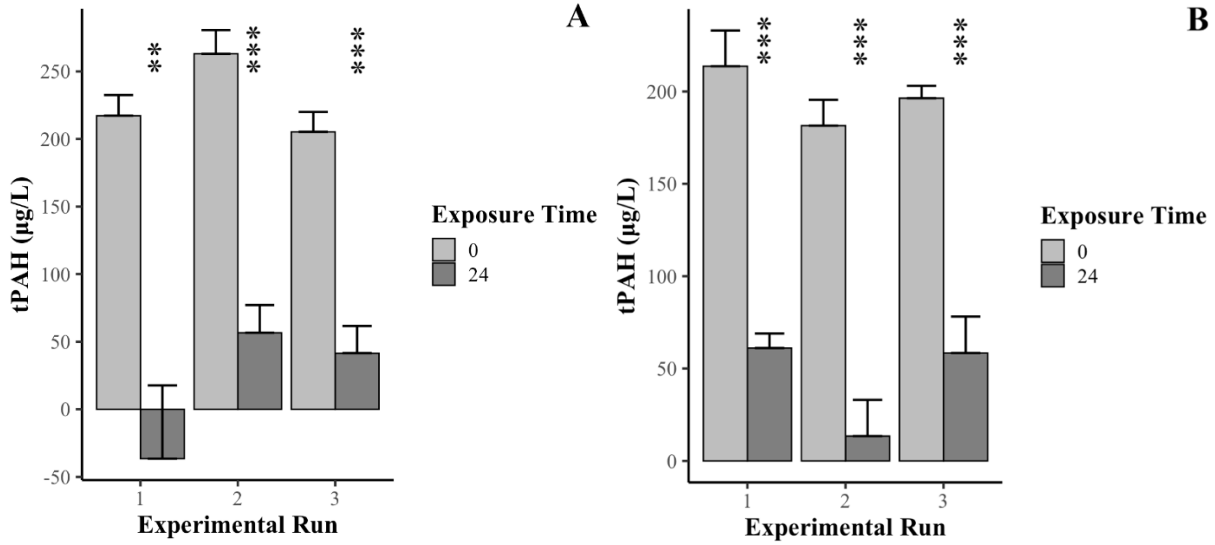


Figure 2.3 Mean ± SEM of total PAH concentrations measured by fluorescence at 0 and 24 hours after oil exposure in the microbiome (A) and foraging (B) studies. Stars indicate significant differences between 0- and 24-hour values (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Overall, the GC/MS results showed that the weathered oil was mainly composed of 3- and 4-ring PAHs at 61% and 21% respectively; 2-ring PAHs representing 18%; and 5-ring < 1% (**Fig. 2.3**). The full GC/MS results are displayed in **Table 2.2**.

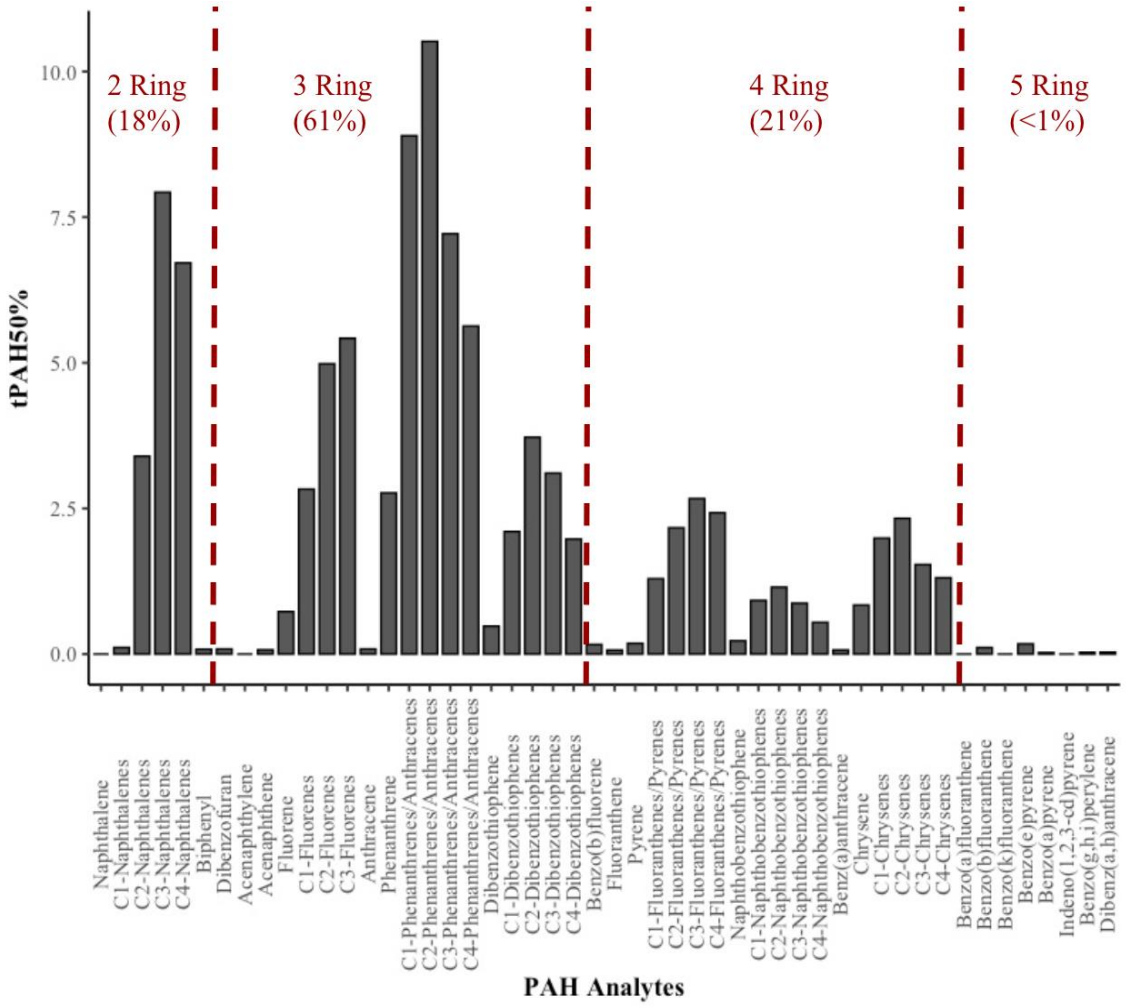


Figure 2.4 Relative abundance of a selection of 50 common PAH analytes found within each of the three 100% HEWAF samples.

Table 2.1 GC/MS results of PAH analytes and other aromatic compounds from 100% HEWAF stock solutions prepared in each experiment. Concentrations are in µg/L, analyzed using method 8270D SIM (EPA, 1998). ND = not detected. Method Reporting Limit displayed for each experiment.

Analyte Name	Experiment 1	Experiment 2	Experiment 3	Method Reporting Limit
C1-Decalins	ND	ND	0.32	0.2 / 0.39 / 0.2
C2-Decalins	0.63	1.1	0.83	0.2 / 0.39 / 0.2
C3-Decalins	5.5	11	10	0.2 / 0.39 / 0.2
C4-Decalins	29	53	47	0.2 / 0.39 / 0.2
C1-Benzothiophenes	0.36	0.76	0.61	0.2 / 0.39 / 0.2
C2-Benzothiophenes	1.2	2.2	1.8	0.2 / 0.39 / 0.2
C3-Benzothiophenes	2.1	4	3.4	0.2 / 0.39 / 0.2
C4-Benzothiophenes	4.6	7.5	5.9	0.2 / 0.39 / 0.2
C1-Naphthalenes	1.6	2.8	2.6	0.2 / 0.39 / 0.2
C2-Naphthalenes	48	86	76	0.2 / 0.39 / 0.2
C3-Naphthalenes	110	200	180	0.2 / 0.39 / 0.2
C4-Naphthalenes	95	170	150	0.2 / 0.39 / 0.2
Biphenyl	1.2	2	1.9	0.2 / 0.39 / 0.2
Dibenzofuran	1.2	2.2	2	0.2 / 0.39 / 0.2
Acenaphthene	1	1.9	1.7	0.2 / 0.39 / 0.2
Fluorene	10	18	17	0.2 / 0.39 / 0.2
C1-Fluorene	39	70	66	0.2 / 0.39 / 0.2
C2-Fluorene	68	120	120	0.2 / 0.39 / 0.2
C3-Fluorene	75	130	130	0.2 / 0.39 / 0.2
Anthracene	1.2	2.2	2	0.2 / 0.39 / 0.2
Phenanthrene	38	69	64	0.2 / 0.39 / 0.2
C1-Phenanthrene/Anthracene	120	220	210	0.2 / 0.39 / 0.2
C2-Phenanthrene/Anthracene	140	260	250	0.2 / 0.39 / 0.2
C3-Phenanthrene/Anthracene	96	180	170	0.2 / 0.39 / 0.2
C4-Phenanthrene/Anthracene	78	140	130	0.2 / 0.39 / 0.2
Retene	3.2	5.4	5.1	0.48 / 0.96 / 0.48
Dibenzothiophene	6.6	12	11	0.2 / 0.39 / 0.2

Table 2.1 continued

C1-Dibenzothiophene	29	52	49	0.2 / 0.39 / 0.2
C2-Dibenzothiophene	51	94	85	0.2 / 0.39 / 0.2
C3-Dibenzothiophene	41	75	76	0.2 / 0.39 / 0.2
C4-Dibenzothiophene	25	51	46	0.2 / 0.39 / 0.2
Benzo(b)fluorene	2.3	3.9	4	0.2 / 0.39 / 0.2
Fluoranthene	0.96	1.7	1.6	0.2 / 0.39 / 0.2
Pyrene	2.6	4.4	4.4	0.2 / 0.39 / 0.2
C1-Fluoranthene/Pyrene	17	31	32	0.2 / 0.39 / 0.2
C2-Fluoranthene/Pyrene	29	53	52	0.2 / 0.39 / 0.2
C3-Fluoranthene/Pyrene	37	65	63	0.2 / 0.39 / 0.2
C4-Fluoranthene/Pyrene	32	60	58	0.2 / 0.39 / 0.2
Naphthobenzothiophene	3.2	5.5	5.3	0.2 / 0.39 / 0.2
C1- Naphthobenzothiophene	13	23	21	0.2 / 0.39 / 0.2
C2- Naphthobenzothiophene	16	28	27	0.2 / 0.39 / 0.2
C3- Naphthobenzothiophene	12	22	20	0.2 / 0.39 / 0.2
C4- Naphthobenzothiophene	7.6	13	13	0.2 / 0.39 / 0.2
Benz(a)anthracene	0.97	1.7	1.7	0.2 / 0.39 / 0.2
Chrysene	12	20	20	0.2 / 0.39 / 0.2
C1-Chrysene	28	48	47	0.2 / 0.39 / 0.2
C2-Chrysene	32	57	55	0.2 / 0.39 / 0.2
C3-Chrysene	22	38	35	0.2 / 0.39 / 0.2
C4-Chrysene	18	32	31	0.2 / 0.39 / 0.2
Benzo(b)fluoranthene	1.5	2.8	2.6	0.2 / 0.39 / 0.2
Benzo(e)pyrene	2.4	4.3	4.1	0.2 / 0.39 / 0.2
C30-Hopane	16	29	28	0.2 / 0.39 / 0.2
Benzo(a)pyrene	0.35	0.65	0.59	0.2 / 0.39 / 0.2
Dibenz(a,h)anthracene	0.43	0.81	0.78	0.2 / 0.39 / 0.2
Benzo(g,h,i)perylene	0.44	0.62	0.72	0.2 / 0.39 / 0.2
4-Methyldibenzothiophene	14	26	24	0.2 / 0.39 / 0.2
2-Methyldibenzothiophene	7.1	13	12	0.2 / 0.39 / 0.2

Table 2.1 continued

1-Methyldibenzothiophene	4.7	8.5	8.1	0.2 / 0.39 / 0.2
3-Methylphenanthrene	27	49	46	0.2 / 0.39 / 0.2
2-Methylphenanthrene	32	57	53	0.2 / 0.39 / 0.2
2-Methylanthracene	0.56	1.1	0.94	0.2 / 0.39 / 0.2
9-Methylphenanthrene	31	57	57	0.2 / 0.39 / 0.2
1-Methylphenanthrene	25	46	43	0.2 / 0.39 / 0.2
2-Methylnaphthalene	1	1.8	1.7	0.2 / 0.39 / 0.2
1-Methylnaphthalene	1.3	2.2	2.1	0.2 / 0.39 / 0.2
2,6-Dimethylnaphthalene	19	34	30	0.2 / 0.39 / 0.2
2,3,5-Trimethylnaphthalene	34	62	56	0.2 / 0.39 / 0.2
Carbazole	0.64	1.2	1.2	0.2 / 0.39 / 0.2
ΣPAH	1626.44	2947.24	2777.99	

2.4.2 16S rRNA Sequencing

2.4.2.1 Alpha Diversity

The results of our rarefaction analysis, or the relationship between observed number of species and sequencing depth, standardized all the samples at 44,012 reads. Good's coverage was used to estimate the percent of the total number species represented per sample, which was > 99% for all samples, indicating that < 1% of the reads in a sample were representative of only a singleton OTU (**Table S-4**). Hence, the sequencing quantity was sufficient for all of the samples (**Fig. 2.9**). The average (± SEM) observed number of species across treatment groups was 301 ± 9 for baseline, 235 ± 15 for control, 248 ± 18 for oil, and 351 ± 28 for water (**Table S-4**). The rarefied OTU tables were used to calculate the rest of our analyses.

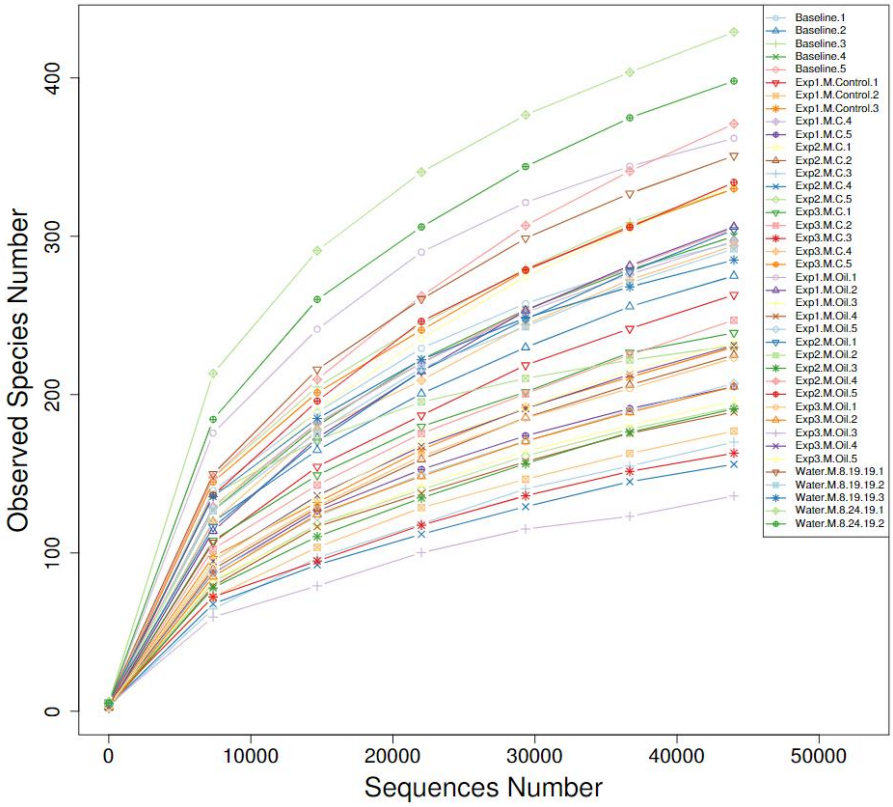


Figure 2.5 Rarefaction curve representing the number of species per number of sequences generated to judge the sequencing sufficiency of each sample. A shallower slope indicates that a reasonable number of individual samples have been taken, while a steeper slope indicates that the sequencing.

Water associated communities have higher alpha diversity metrics than host-associated communities

We used five different metrics to calculate species richness and alpha diversity within each of the various treatment groups (**Fig. 2.10 A-D**). The average (± SEM) species richness and diversity values are outlined in **Table S-4**. We did not find statistical differences between any treatment groups for species richness metrics or PD Whole Tree ($p > 0.05$), yet significant differences between the water-associated and gut-associated microbiomes for both Shannon and Simpson indices were noted ($p < 0.05$; **Table S.5**). These results confirm that the water had an overall higher diversity than the gut-associated microbiomes in regard to the Shannon index, but the lower Simpson index indicates that the water communities may have lower evenness than the gut communities. Although Shannon and Simpson diversity appeared to increase in the oil-exposed community compared to the control, there were no statistical differences to support these findings.

Thus, the water communities were more diverse than fish gut-associated communities.

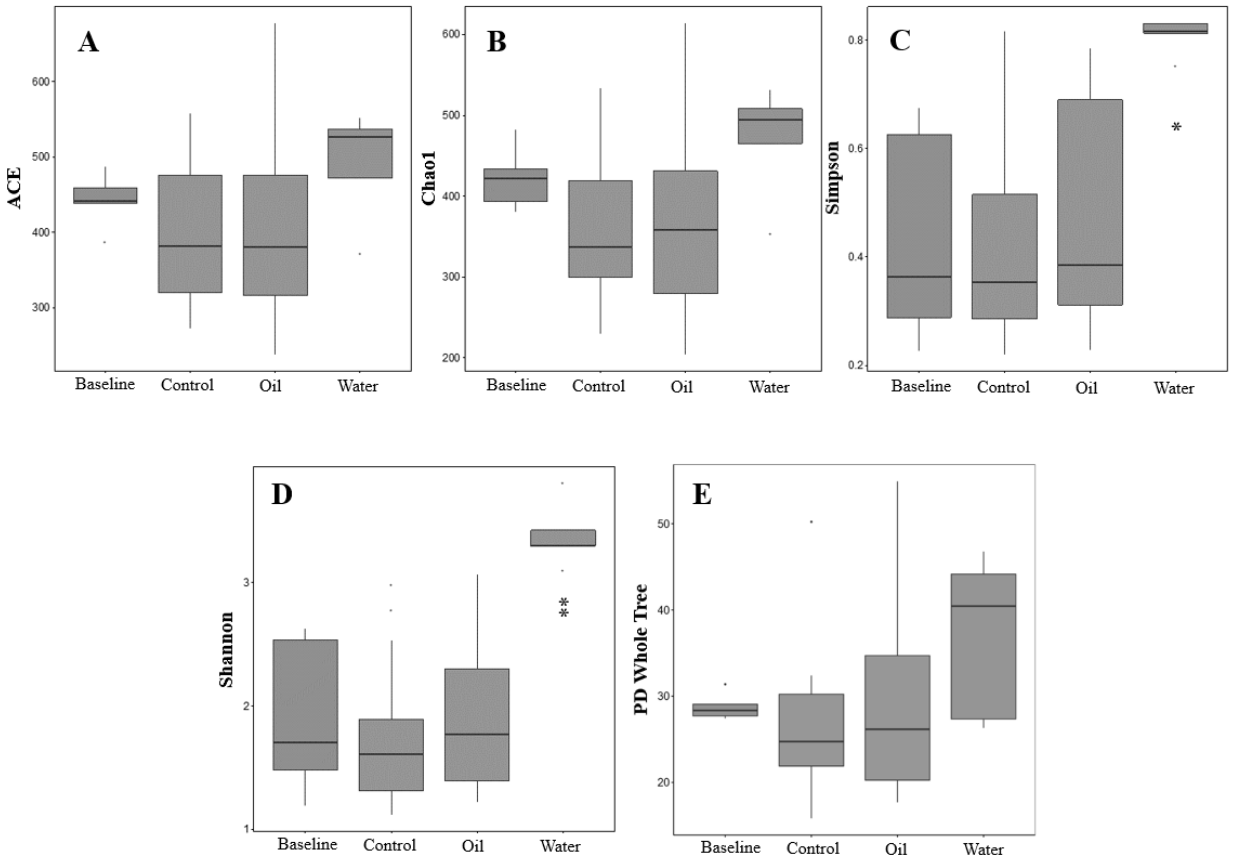


Figure 2.6 ACE (Abundance-based Coverage Estimator; $p > 0.05$ for all groups) (A), Chao1 (B), Simpson diversity (C), Shannon diversity ($p < 0.05$ for water) (D), PD Whole Tree (E) indices of alpha diversity within treatment groups (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

2.4.2.2 Beta Diversity

Beta diversity shows water is significantly different from gut-associated communities, while control and oil-exposed fish are similar in composition

To assess the beta diversity between groups, we used a Non-Metric Multidimensional Scaling (NMDS) ordination of Bray-Curtis dissimilarity at the genus level and calculated statistical differences between groups using PERMANOVA. NMDS is an ordination method that plots the rank order of pairwise dissimilarities in a community, and the Bray-Curtis is the distance metric used to calculate this dissimilarity which is a ratio that calculates the shared species per total number of species at each site or sample (Bray & Curtis, 1957; Paliy & Shankar, 2016).

The NMDS plot including all groups showed that only water-associated taxa were significant drivers of clustering (PERMANOVA, $p < 0.01$) and all host-associated communities clustered closer together (**Fig. 2.11-A**). A separate plot of only the control and oil-exposed groups did not indicate that treatment or experimental run were drivers of clustering (PERMANOVA, $p > 0.05$) (**Fig. 2.11-B**). Thus, the water microbiome was significantly different from that of fish gut microbiomes.

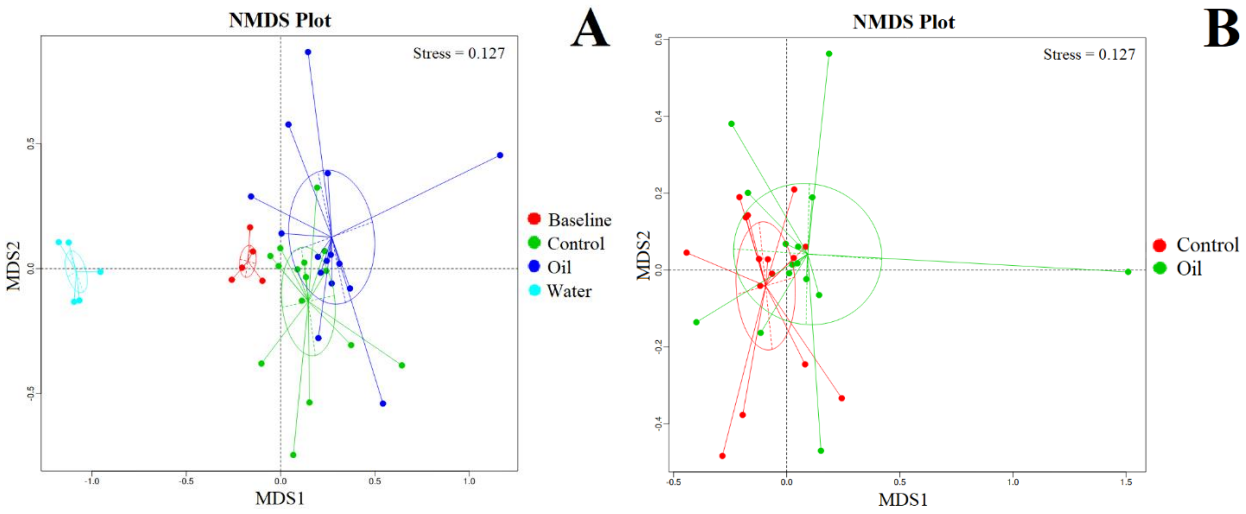


Figure 2.7 Non-Metric Multidimensional Scaling (NMDS) ordination of Bray-Curtis dissimilarity calculated at the genus level across all treatment groups (A) and between control and oil treatments (B). Water was the only significant driver of clustering across groups ($p < 0.01$). Neither experiment number nor treatment were found to be drivers of clustering between control and oil treatments ($p > 0.05$).

We further visualized the beta diversity between groups by creating Principal Coordinates Analyses (PCoA) based on Yue-Clayton theta distance matrices (**Fig. 2.12**). Both PCoA and NMDS first start by calculating a given distance matrix between samples, but instead of plotting rank order based on all variance in the data, PCoA plots object distances on only the first two largest eigenvector-based gradients of variability (Paliy & Shankar, 2016). Yue-Clayton theta distance matrices are another way to analyze the similarity of communities, which incorporates the proportions of both shared and unique species (Yue & Clayton, 2005). This index was more useful to determine if there were any differences between control and oil exposed fish, considering that the oil treatment showed a much higher abundance of unique OTUs compared to the control. However, none of the treatment groups were significantly different from one another based upon

a Tukey's HSD analysis ($p > 0.05$; **Table S.6**), even though it appeared that water-associated communities were different from gut-associated communities. Separating the control and oil groups from the other treatments also did not yield any significant differences between these two treatments ($p > 0.05$; **Table S.6**). Given these results, we cannot conclude that water or oil-exposure were drivers of clustering.

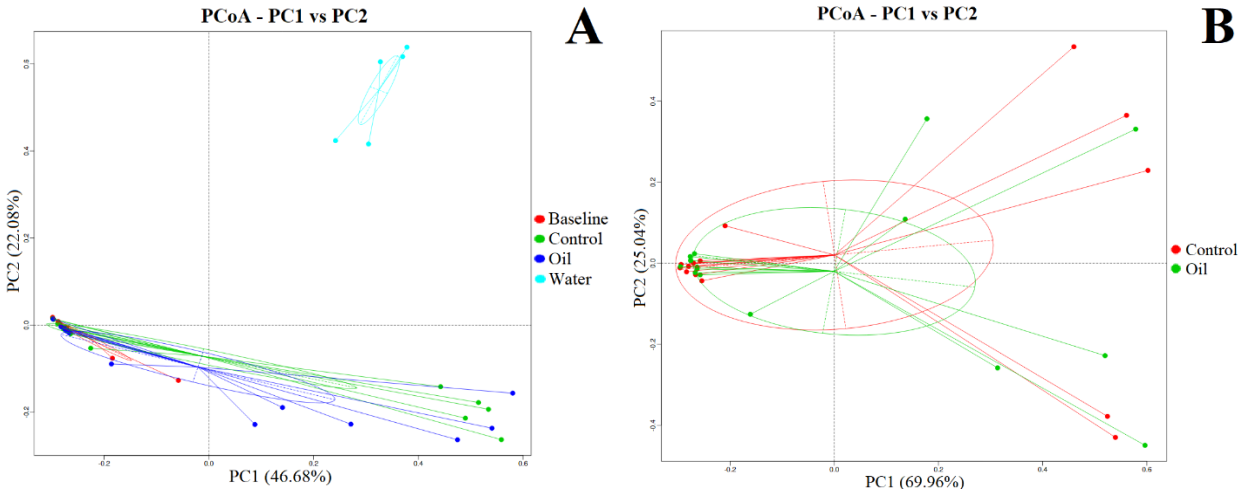


Figure 2.8 Principal Coordinate Analysis ordination based on Yue-Clayton theta distance matrices calculated at the genus level across all treatment groups (A) and between control and oil treatments (B).

2.4.2.3 Taxonomic Composition

Composition of the water microbial community is distinct from the sheepshead minnow gut microbiome, yet taxonomic makeup in oil-exposed fish remained relatively stable

In general, the microbiome of sheepshead minnow GI tracts as well as that of the surrounding water revealed distinct taxonomic patterns. The number of unique and shared OTUs found within each treatment group is illustrated in a Venn Diagram (**Fig. 2.5**), with the oil group showing the most unique OTUs between all groups (267) as well as only compared to the control group (358). These OTUs are explored in further detail below.

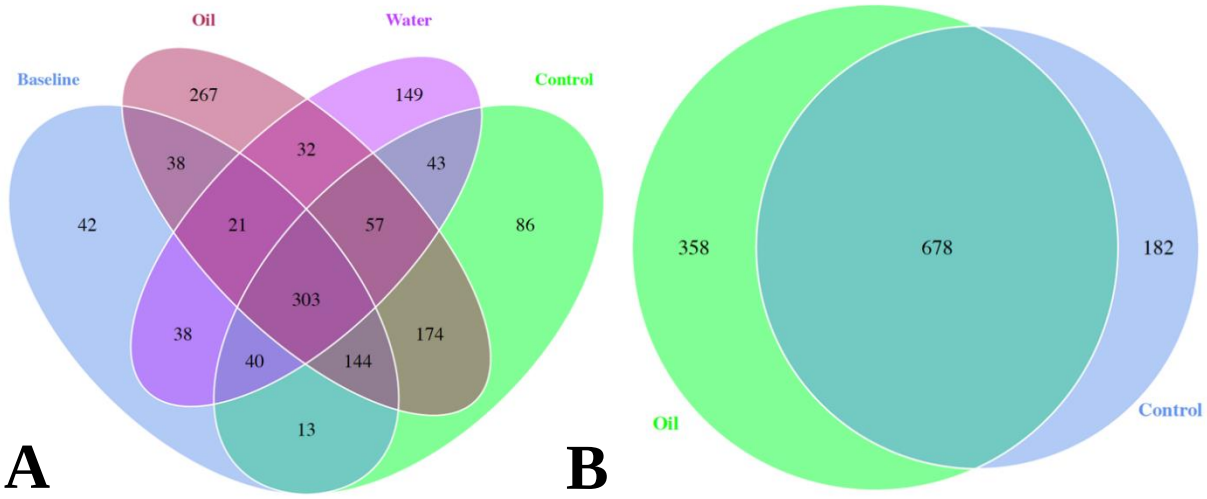
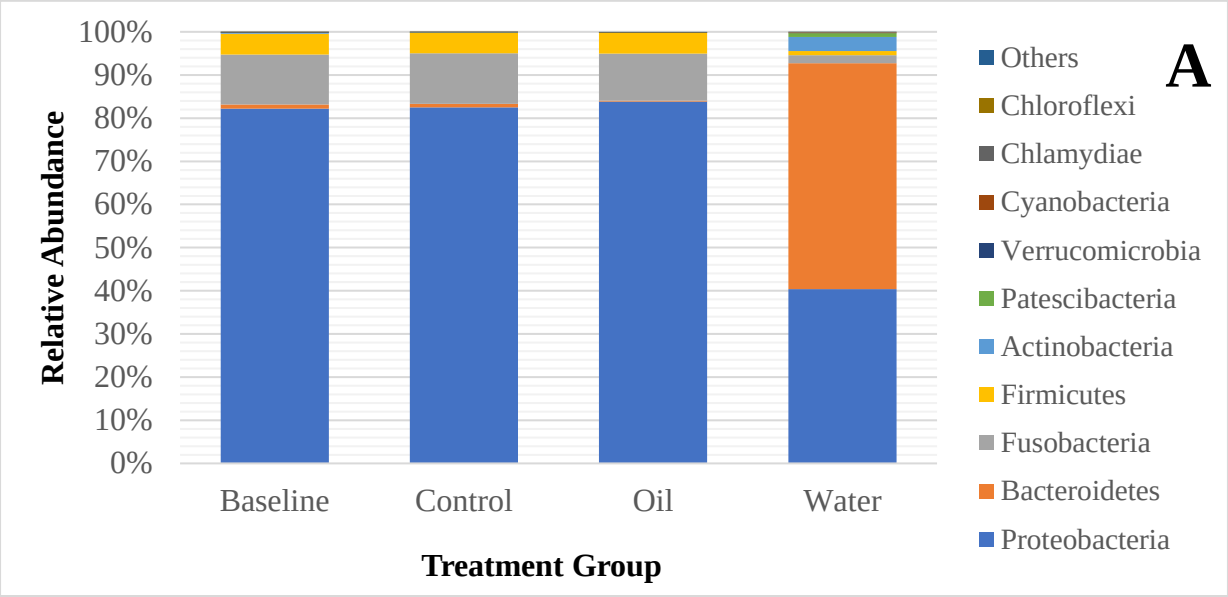


Figure 2.9 Venn diagram of the number of common and unique OTUs from all treatment groups (A) and from only control and oil treatments (B). OTUs were obtained at 97 % similarity for each sample.

To visualize the taxonomic makeup of both the fish gut and water-associated microbial communities, we calculated the top 10 most abundant taxa at the phylum and genus levels based upon the maximum relative abundance of an OTU across all samples. This was determined across all treatment groups, as well as for only oil and control. At the phylum level, the environmental microbiome of the tank water was comprised of Bacteroidetes (52.3%), Proteobacteria (40.40 %), Actinobacteria (3.25 %), Fusobacteria (1.92 %), Firmicutes (0.91 %), and Patescibacteria (0.9 %), with all other phyla (Verrucomicrobia, Cyanobacteria, Chlamydiae, and Chloroflexi) contributing ≤ 0.1 % to the total observed taxonomic groups. Across all treatment groups, the phyla in the fish gut microbiome were predominantly composed of Proteobacteria (> 80 %), Fusobacteria (≥ 11 %), Firmicutes (> 4 %), and to a lesser extent, Bacteroidetes (≤ 1 %) (**Fig. 2.6-A**). When comparing only the control and oil groups, the predominant phyla across both treatments remained largely the same, comprising Proteobacteria, Fusobacteria, and Firmicutes as the top three most abundant with all others represented at < 1% (**Fig. 2.6-B**). In sum, the phyla of the water community were markedly different from those of the fish gut communities. These differences become more apparent at the genus level.



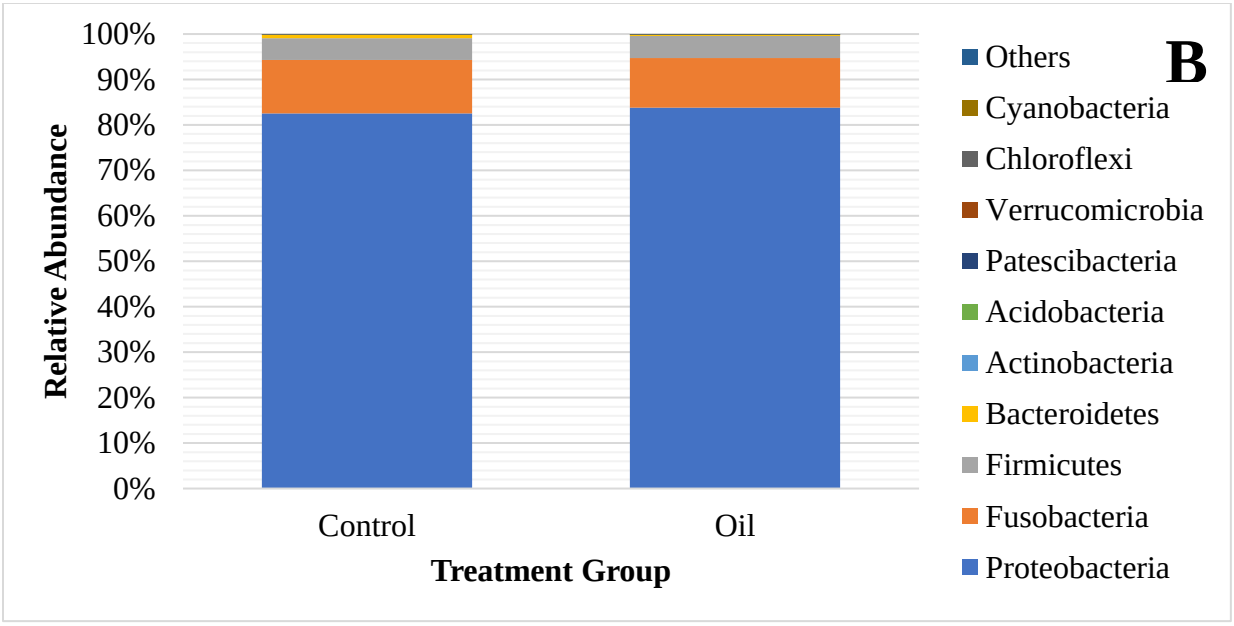


Figure 2.10 Relative abundance of the top ten phyla represented across all treatment groups (A) and between only the control and oil treatments (B). All other taxa are combined into the "Others" category.

Across all treatment groups at the genus level, there are more distinctions between gut-associated microbiota and free-living aquatic microbiota (**Fig. 2.7-A**). Water samples were composed of *Phaeocystidibacter* (17.80 %), *Phaeodactylibacter* (16.16 %), NS3a marine group (15.87 %), *Marivita* (12.63 %), *Stenotrophomonas* (6.85 %), *Pseudohongiella* (4.33 %), and *Cetobacterium* (1.69 %), with all other genera < 1%. The gut of baseline, control, and oil-exposed fish contained *Vibrio* (10.26 % ± 6.25), *Stenotrophomonas* (64.60 % ± 7.07), *Cetobacterium* (10.38 % ± 0.09), *Photobacterium* (3.31 % ± 1.65), and *Propionigenium* (1.24 % ± 0.01). When comparing only the control and oil groups, the top genera were *Stenotrophomonas*, *Vibrio*, *Cetobacterium*, and *Marivita* (**Fig 2.7**). Other genera were detected here that were less abundant in the baseline and water groups, which include *Ruminococcus 2*, *Lactobacillus*, *Blautia*, [*Eubacterium*] *coprostanoligenes* group, *Streptococcus*, and *Dialister*. All other taxa comprised > 5 % of the total. In general, the water communities comprised noticeably different genera than those of the fish gut-associated communities, although some taxa were shared between these two groups. However, it is imperative to discern if there are differences in less abundant taxa between the oil-exposed communities and the control.

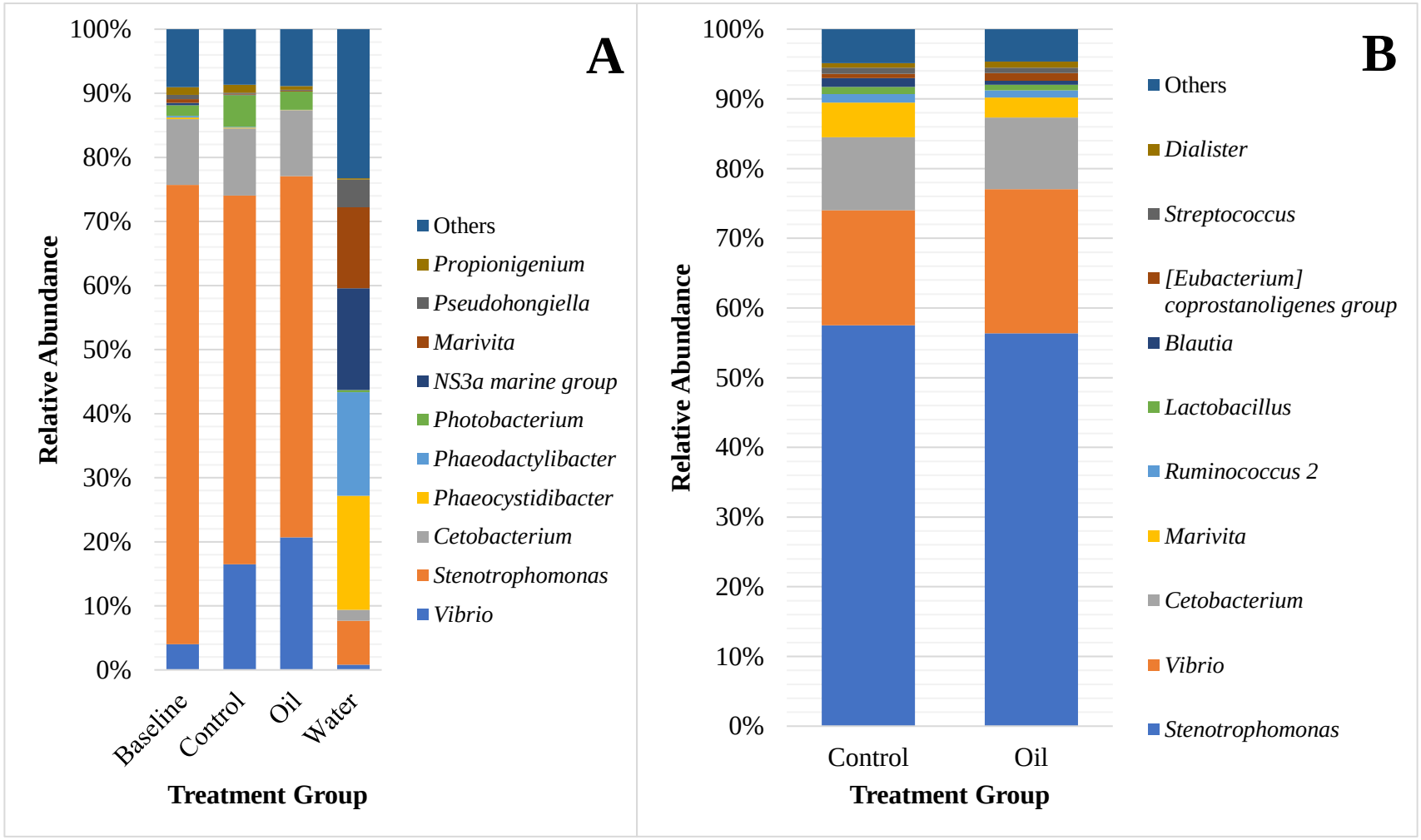


Figure 2.11 Relative abundance of the top ten genera represented across all treatment groups (A) and of only the control and oil treatments (B). All other taxa are combined into the "Others" category.

To visualize broad patterns in relative abundance, we created a heatmap at the genus level to compare individual samples in both the control and oil treatment groups (**Fig. 2.8**). The distribution of taxa (listed in the *y*-axis) did not show any particular patterns between treatment groups, with some taxa observed in higher abundances in only one sample (displayed in dark red). The dendrogram on the *x*-axis also shows that many individual samples did not cluster solely according to the water treatment group. This suggests that a few samples with higher abundances of rare taxa may have been driving these differences between samples.

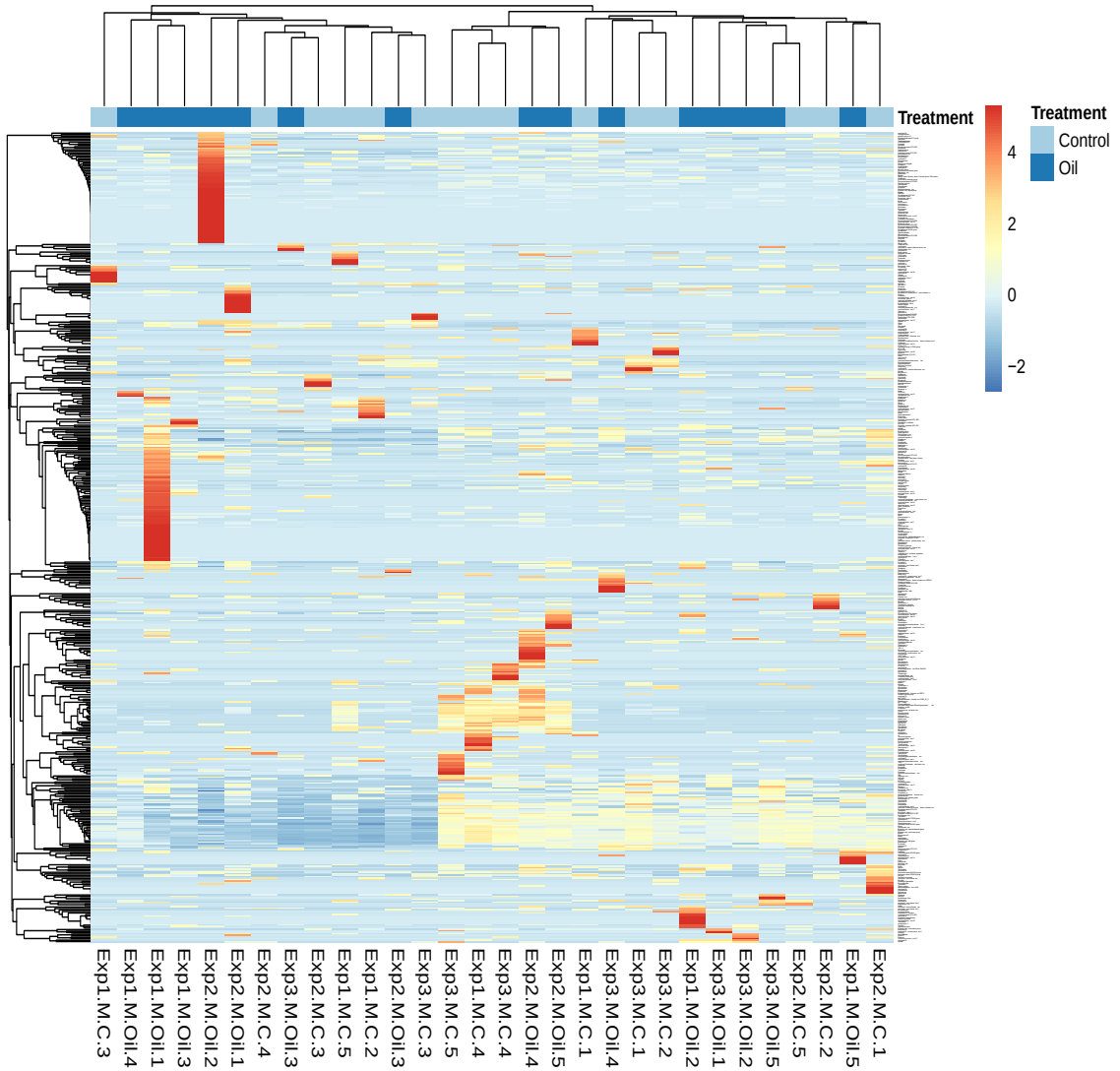


Figure 2.12 Heat map of abundances of all genera observed between control and oil treatments. Unit variance scaling was applied to rows, and both rows (n = 538) and columns (n = 30) are clustered using correlation distance and average linkage. The color scale indicates relative abundance from the mean.

2.4.3 Taxonomic Differential Abundance Comparisons

Significant shifts in taxa reflect host- and water-associated compositions

When comparing the water and fish gut-associated microbiomes, several statistical differences were found at both the phylum and genus levels. These results were calculated from METASTATs analyses to compare pairwise differences between hundreds of different taxa in the various treatment groups, where *q*-values ($q < 0.05$) were used as they take into account the false discovery rate and are a more stringent indicator of statistical significance. It should be noted that only the top ten taxa are displayed in **Figure 2.13** to show differences in the most abundant taxa between treatment groups. All other taxa with significant differences were much less abundant and caveats regarding this are discussed later in **Ch. 2.5.2**. Notable differences between water-associated and gut-associated microbiomes included a much higher prevalence of Proteobacteria in the fish gut compared to the water (42.4 % ± 0.5) in contrast to Bacteroidetes which showed the opposite trend (51.6 % ± 0.2) (**Fig. 2.13 D-F**). Firmicutes was also lower in the water samples compared to the gut (3.9 % ± 0.02) (**Fig. 2.13 D-F**). Other taxa were more common in water samples compared to the fish gut (Chlamydiae and Hydrogenedentes), while Acidobacteria, Spirochaetes, and Thermotogae had small but significant decreases from the fish gut compared to water samples (**Figs. 2.13 D-F**). Several genera also showed significant changes between the fish gut and water environments. *Stenotrophomonas* had a much higher abundance in the gut to compared to the water samples across all treatment groups (-55 % ± 4.9), while the NS3a marine group was less abundant in the control and oil treatments compared to the water samples (-15.7 % ± 0.1) (**Fig. 2.13 J-L**). *Vibrio* also increased in oil-exposed communities compared to the water environment (-19.87 %) (**Fig 2.13-L**). Overall, these relationships reflect the unique differences between fish gut and water communities. However, changes between gut-associated treatment groups are less discernable.

Although none of the topmost abundant phyla were significantly different in the baseline community compared to the control, a few genera were found to be significantly different. These included the NS3a marine group (0.004 baseline, 0.001 control), *Marivita* (0.006 baseline, 0.001 control), and *Pseudohongiella* (0.007 baseline, 0.002 control), which all slightly decreased from the baseline community to the control (**Fig. 2.13-G**). Comparing the shifts from the baseline to oil-exposed communities, *Phaeocystidibacter* (-0.21 %), *Phaeodactylibacter* (-0.21 %), NS3a marine group (-0.37 %), *Marivita* (-0.43 %), and *Pseudohongiella* (-0.54 %) decreased to a small degree

(**Fig 2.13-H**). However, some caution should be used in interpreting comparisons between baseline and control or oil-exposed fish, as sample sizes were 5 and 15, respectively. In general, these differences between baseline and control/oil-exposed communities may be a factor of the different timepoints in which they were sampled. The comparisons between control and oil treatment groups are even more negligible.

Interestingly, no significant differences were found in relative abundances at either the phylum or genus level in control communities compared to the oil-exposed group (**Fig. 2.13 C & I**). The only taxa that showed any significance ($q < 0.05$) were the Chloroflexia and Dehalococcoidia classes, and the genera UBA1819 and *Ethanoligenens* (both members of the Ruminococcaceae family). These taxa were all prevalent at very low abundances, along with the majority of taxa that had p-values < 0.01. There were many other significant p-values at other taxonomic levels, yet none of the other levels had significant q-values which may indicate a high number of false positives (**Table 2.2**). In sum, none of the topmost abundant taxa when comparing control and oil-exposed communities showed any significant differences, yet a few rare taxa were significant between these two treatment groups. To elucidate further differences between these treatments, we sought to factor in biomarkers of differences using LEfSe.

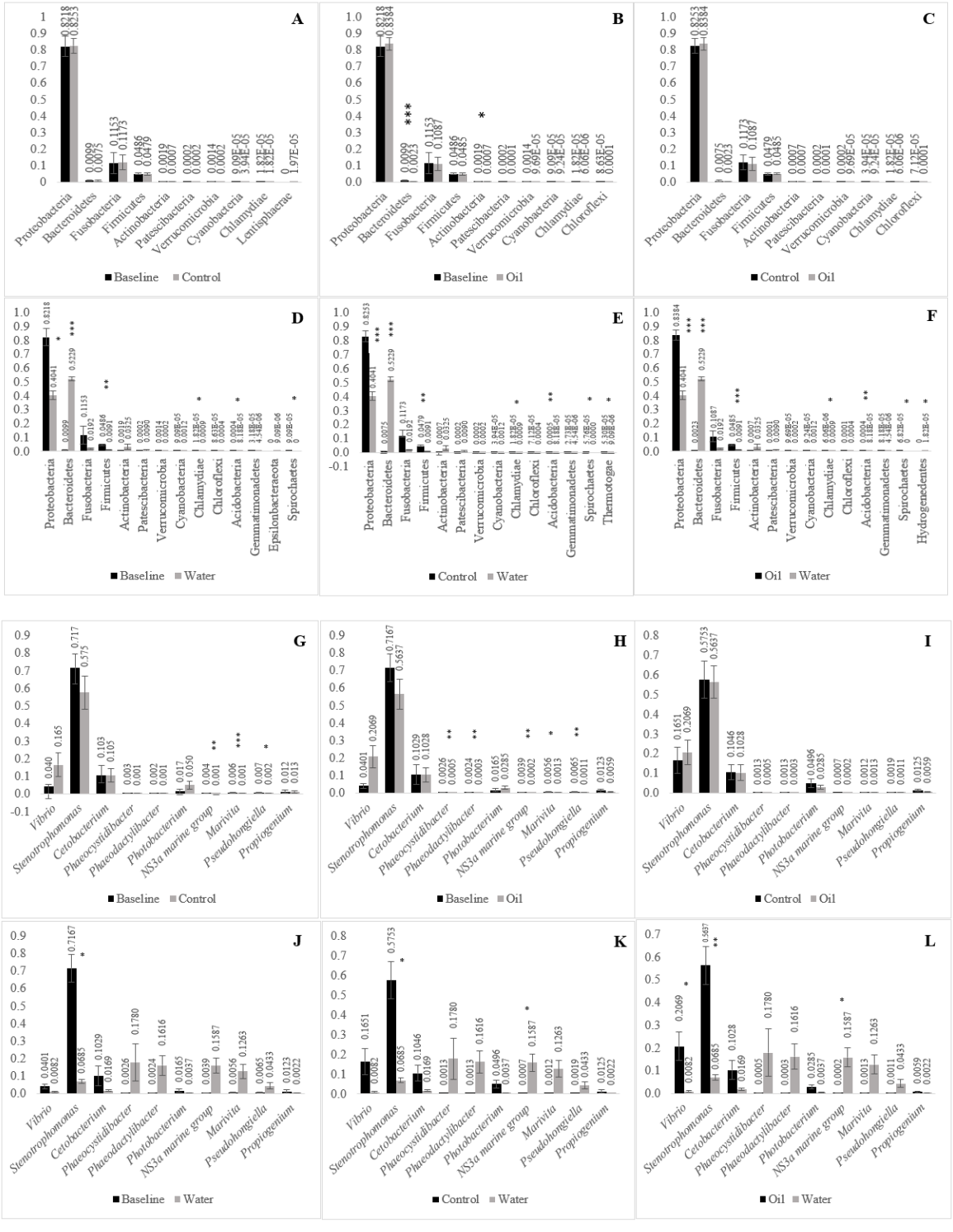


Figure 2.13 Relative abundances of the top ten phyla (A-F) and genera (G-L) across all treatment groups. Significant METASTAT results are indicated by the stars above the corresponding taxa (* = $q < 0.05$, ** = $q < 0.01$, *** = $q < 0.001$).

Table 2.2 METASTATs results of taxa at the class and genus levels, showing means, variance, and standard error (SEM) between control and oil-exposed fish microbial communities.

	Control			Oil				
Taxa	**Mean**	**Variance**	**SEM**	**Mean**	**Variance**	**SEM**	***p*-value**	***q*-value**
Chloroflexia	0	0	0	3.33 E-05	1.37 E-08	3.02 E-05	0.001	0.038
Dehalococcoidia	0	0	0	2.27 E-05	4.94 E-09	1.81 E-05	0.001	0.038
UBA1819	0	0	0	2.12 E-05	6.75 E-09	2.12 E-05	0.0001	0.039
Ethanoligenens	0	0	0	2.12 E-05	6.75 E-09	2.12 E-05	0.0001	0.039
Ercella	0	0	0	1.82 E-05	4.14 E-09	1.66 E-05	0.001	0.09
Uncultured Bacterium (Cellvibrionaceae family)	1.67 E-05	4.03 E-10	5.18 E-06	0	0	0	0.001	0.09
Prevotella 9	0	0	0	3.79 E-05	2.15 E-08	3.79 E-05	0.001	0.09
Alloprevotella	0	0	0	3.33 E-05	1.67 E-08	3.33 E-05	0.001	0.09
Arcobacter	0	0	0	2.73 E-05	1.12 E-08	2.73 E-05	0.001	0.09
Ruminococcaceae *UCG-008*	0	0	0	1.51 E-05	3.44 E-09	1.51 E-05	0.002	0.14
MND1 (Nitrosomonadac-eae)	1.51 E-06	3.44 E-11	1.51 E-06	3.94 E-05	9.62 E-09	2.53 E-05	0.002	0.14
Azohydromonas	1.36 E-05	5.75 E-10	6.19 E-06	5.15 E-05	3.57 E-09	1.54 E-05	0.003	0.18
Uncultured Bacterium (Betaproteobacter-iales; TRA3-20)	1.51 E-06	3.44 E-11	1.51E -06	1.82E -05	1.27 E-09	9.20E -06	0.003	0.18
Sphaerochaeta	0	0	0	1.36 E-05	2.79 E-09	1.36 E-05	0.004	0.18
Prevotellaceae *UCG-001*	0	0	0	1.36 E-05	2.20 E-09	1.21 E-05	0.004	0.18

Table 2.2 continued

Uncultured soil bacterium (Dehalococcoidia; SAR202 clade)	0	0	0	1.36 E-05	1.31 E-09	9.36 E-06	0.004	0.18
Micromonospora	1.51 E-06	3.44 E-11	1.51 E-06	3.64 E-05	6.55 E-09	2.09 E-05	0.005	0.21
Haliea	2.27 E-05	5.90 E-10	6.27 E-06	4.54 E-06	1.62 E-10	3.29 E-06	0.006	0.22
Oscillibacter	1.51 E-06	3.44 E-11	1.51 E-06	1.67 E-05	2.25 E-09	1.22 E-05	0.006	0.22
Caproiciproducens	1.51 E-06	3.44 E-11	1.51 E-06	1.67 E-05	1.36 E-09	9.53 E-06	0.006	0.22
Hoeflea	2.88 E-05	1.14 E-09	8.72 E-06	4.54 E-06	8.85 E-11	2.43 E-06	0.007	0.22
Thauera	1.06 E-05	2.85 E-10	4.36 E-06	5.15 E-05	5.57 E-09	1.93 E-05	0.007	0.22
Prevotella 7	1.51 E-06	3.44E-11	1.51 E-06	3.33 E-05	5.74E-09	1.96 E-05	0.009	0.27

To determine which taxa were significant discriminators of the various treatment groups, we performed a LEfSe analysis which only displayed groups that had an LDA score > 2 (**Fig. 2.14**). Taxa labeled as "uncultured" in this analysis have not been previously cultured and are only known because they have been sequenced before. Several taxa were discriminant of the water samples, including Bacteroidetes, Bacteroidia, Flavobacteriales, Cryomorphaceae, *Phaeocystidibacter*, Chitinophagales, Flavobacteriaceae, Saprospiraceae, *Phaeodactylibacter*, NS3a marine group, Stappiaceae, Hyphomicrobiaceae, *Thalassospira*, Actinomarinales, Cellvibrionaceae, and Thalassospiraceae. The Paenibacillaceae family, *Pseudomonas pachastrellae*, and the MND1 genus were discriminant of oil-exposed gut communities, while two *Bacteroides* strains were discriminant of the control group. Several other taxa had differential characteristics within each of the treatment groups and are displayed in **Fig. S-1**. Between only the control and oil, the LEfSe analysis indicated that only the order Pseudomonadales was discriminant of the oil treatment (**Fig. 2.15**). This order also increased in abundance in the oil treatment (**Fig. S-2**). The control group showed a higher number of discriminant taxa, including

NS3a marine group, Flavobacteriaceae, *Phaeocystidibacter*, Cryomorphaceae, Saprospiraceae, *Phaeodactylibacter*, Chitinophagales, Flavobacteriales, Bacteroidetes, and Bacteroidia. The uncultured bacterium refers to a taxa that has only been previously sequenced and not cultured.

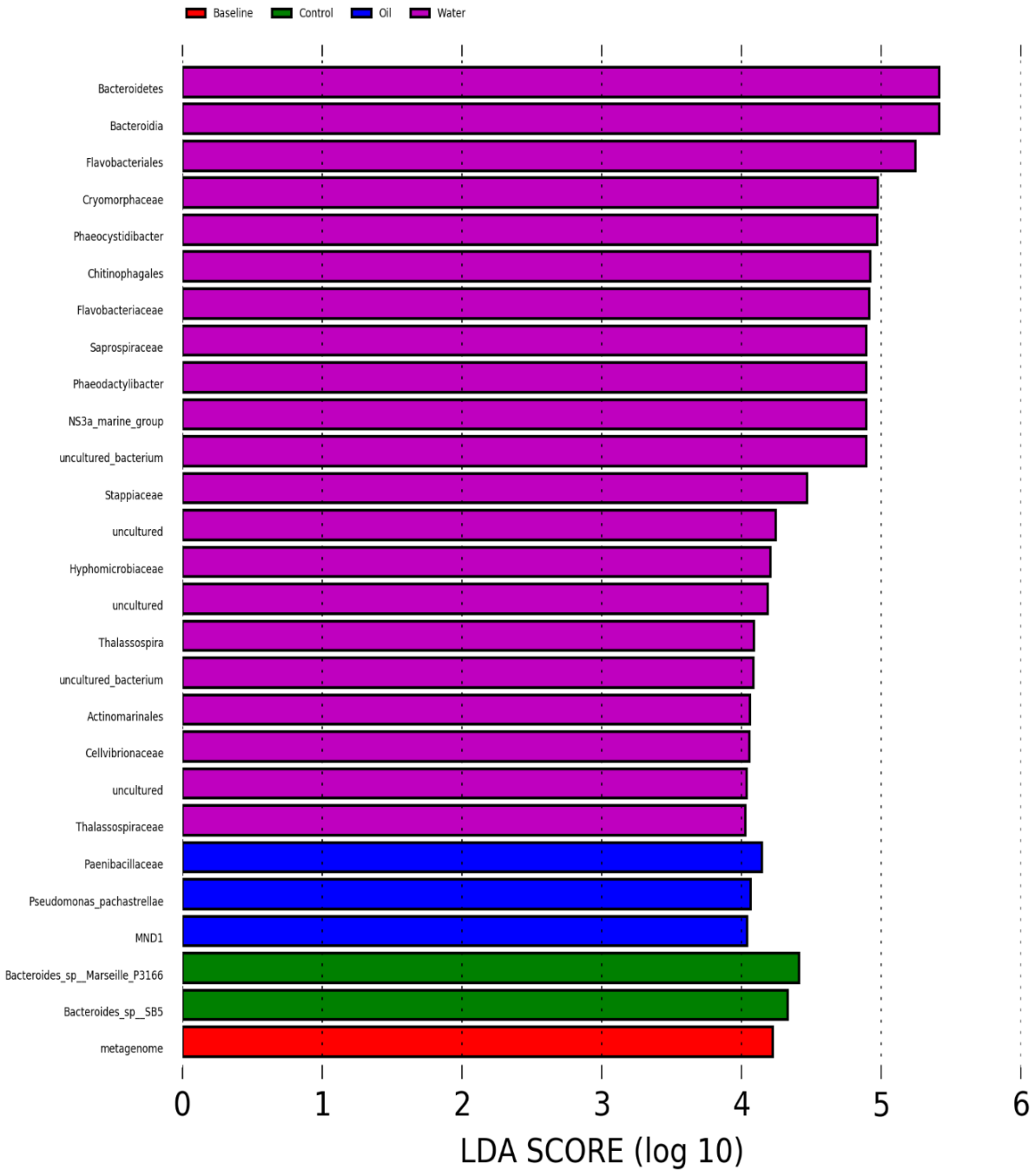


Figure 2.14 Linear Discriminant Analysis (LDA) score distribution histogram, indicating differentially taxa in each of the treatment groups. The length of a bar represents the effect level of the taxa, with LDA scores > 4 displayed here. Uncultured refers to taxa that have been sequenced yet not cultured.

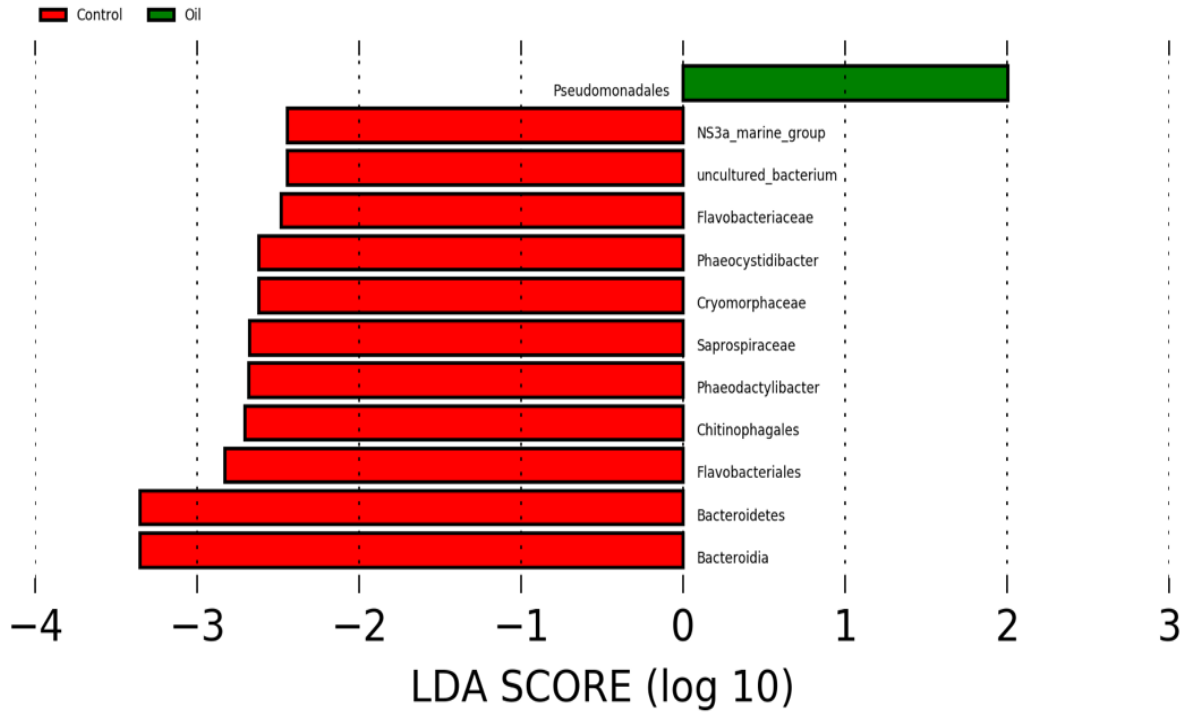


Figure 2.15 Linear Discriminant Analysis (LDA) score distribution histogram, indicating enriched taxa in each of the treatment groups. The length of a bar represents the effect level of the taxa, with LDA scores > 2 displayed here. Uncultured refers to taxa that have been sequenced yet not cultured.

2.4.4 Foraging Behavior Trials

It should initially be noted that there was an accidental deviation in protocol on day seven of the first experimental run, which was significant enough to cut the first experimental run from the analysis. The normality check using the Shapiro-Wilk Test resulted in a significant p-value ($p < 0.0001$), indicating that the data did not assume a normal distribution. The non-parametric Fligner-Killeen Test for checking the homogeneity of variances was used and indicated that the variances were homogenous ($p = 0.57$). The results of the binomial GLM indicated that there were no significant differences in prey capture rate between day one and day seven in both the control and oil groups ($p > 0.05$; **Fig. 2.16, 2.17**).

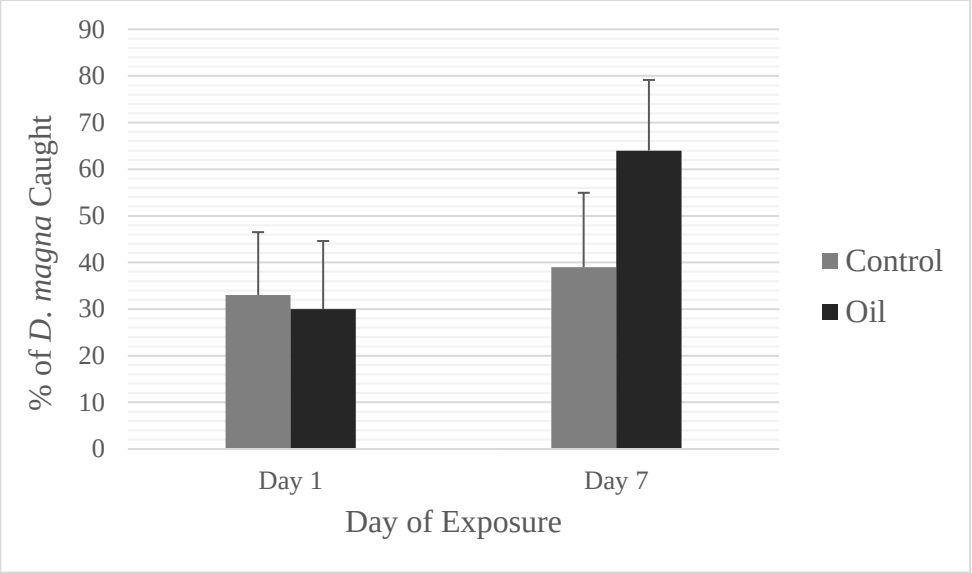


Figure 2.16 Mean ± SEM percent of prey capture of control and oil-exposed fish on days one (before exposure) and seven (after exposure).

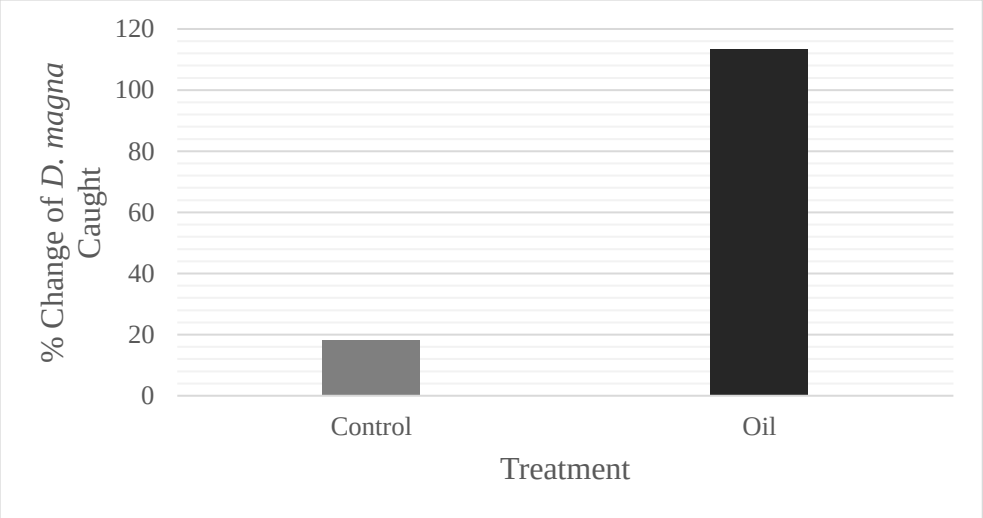


Figure 2.17 Percent change from averages (illustrated in Fig. 2.16) in prey capture rate of control and oil-exposed fish between days one (before exposure) and seven (after exposure).

2.5 Discussion

This study investigated the impacts of PAHs in weathered oil from the DWH spill on the gut microbiome and foraging behavior of *C. variegatus* adult females. Microbial taxonomic characterizations (shifts in diversity, relative abundance, and discriminant taxa) of the gut-microbiome and control water as well as prey capture rate were measured from control and simulated oil spill conditions. The nominal 5% HEWAF concentration did not induce any mortality in either experiment and no significant effects were seen in the foraging behavior of fish. However, the dose was enough to show modest shifts in some gut-associated microbial taxa although overall alpha and beta diversity were not affected by oil exposure. The microbiome of the water samples was much more diverse than the gut-associated environments.

2.5.1 Fluorescence and tPAHs

The chemical composition of our HEWAF stocks is quite similar to that of the publicly available database of >10,000 samples of water chemistry data obtained from the DWH spill (BP Gulf Science Data, 2016). Our composition and concentrations of total PAHs in HEWAF stock solutions can be compared to one study that identified differences in WAF and HEWAF preparations of various types of oil collected from the DWH spill, including slick A oil. The PAH composition found in slick A in that study was similar to our data, as it is composed mostly of fluorenes, phenanthrenes, and dibenzothiophenes (Sandoval et al., 2017). Although their salinity was slightly higher at 33 ppt, they found that HEWAF stocks of slick A oil yielded an average of 1387 ± 207 µg/L with the HEWAF method producing larger amounts of oil in the particulate phase (Sandoval et al., 2017). However, the tPAH concentrations of all prepared HEWAFs in this study were all significantly higher than what has been reported in the public database (BP Gulf Science Data, 2016; Sandoval et al., 2017). In another study examining transcriptional effects on sheepshead minnows exposed to slick A oil, HEWAF was prepared with 15 ppt artificial saltwater and yielded stock solution tPAH50 concentrations of 2467.39 ± 199.91 µg/L (Jones et al., 2017). Our concentrations for all stock HEWAFs were closer to those found by Jones et al. (2017). It should be noted that our tPAH concentrations included compounds that are not considered true PAHs and are instead heterocyclic compounds, such as dibenzothiophene, carbazole, and dibenzofuran, or others such as C30-Hopane. However, some aquatic organism exposure studies

still include these as PAHs in their tPAH counts (E. G. Xu et al., 2016). Another caveat to note is that the fluorometer used in this study to estimate tPAHs operates in a excitation wavelength range of 340-500 nm and an emission wavelength range of 410-600 nm, which is within the range of where most PAHs and other polycyclic aromatic compounds fluoresce, though it may miss some compounds that have wavelengths below 340 (Driskill et al., 2018; Ferretto et al., 2014; Fetzer & Tucker, 1991; Kumke et al., 1995). Thus, our tPAH concentrations are likely an overestimate compared to other studies using the same oil and methods.

The significant decrease in PAH concentrations from 0 to 24 hours in our exposures is similar to what Sandoval et al. (2017) found, where HEWAF concentrations of slick A oil decreased by 86.6% in a day. For the microbiome study, tPAHs decreased by 91%, while in the foraging study they decreased by 77.5%. PAHs are known to undergo biodegradation from environmentally occurring bacteria, photodegradation, which could have been due to the 16:8 light cycle within the incubator where exposures were conducted, and to a lesser extent, chemical oxidation (Abdel-Shafy & Mansour, 2016b; John et al., 2016). Overall, these results show that PAHs degrade quickly within 24 hours.

2.5.2 16S rRNA Sequencing

Higher alpha diversity in the water microbial community yet no differences in beta diversity across treatment groups

With both alpha and beta diversity measures utilized in this study, only the water samples were found to have significantly higher alpha diversity and were drivers of community structure between treatment groups. While ACE (Abundance-based Coverage Estimator) and Chao1 simply estimate species richness, both Simpson and Shannon indices take both species richness and evenness into account, though the Shannon index has a higher bias towards richness and Simpson towards evenness (Kim et al., 2017). On the other hand, PD Whole Tree, or Faith's Index, is calculated by identifying the minimum sum of the branch lengths on a phylogenetic tree for a group of taxa (Faith, 1992). Our results did not indicate any significant differences between groups even though the oil treatment had a higher number of unique OTUs which would have contributed to a higher score. Microbial populations in aquatic environments are typically of a similar magnitude to that of external fish microbiomes, which are still smaller than gut-associated populations (Merrifield & Rodiles, 2015a). Although they have been documented to be smaller in

terms of population size, our results indicated a higher species richness but lower evenness within the water samples. Interestingly, only our NMDS ordination based on Bray-Curtis dissimilarity index found that water was significantly different from other treatment groups. The PCoA based on Yue-Clayton theta distance matrices appeared to show that water was grouped much further away from the fish gut-associated communities, yet this was found to not be significant. This may be due to inherent differences in ordination techniques and the indices used to calculate taxonomic similarities but could also indicate a large number of lower abundant shared taxa between the water and gut communities.

Of the four studies that we found to document the effects of oil and PAHs on fish gut microbiomes, only one described both alpha and beta diversity indices. This study investigated the impacts of three concentrations of crude oil (low = 10 μg/L, medium = 50 μg/L, and high = 100 μg/L) in water on adult Atlantic cod gut microbiomes exposed for 28 days (Bagi et al., 2018b). The authors did not find any significant differences in the observed number of OTUs, Shannon diversity, or Inverse Simpson diversity between treatments, although the number of OTUs seemed to decrease with higher oil concentrations (Bagi et al., 2018b). However, they did find significantly different Shannon diversity when the two lower treatments (control and low) and two upper treatments were coupled together, with decreased diversity in the upper treatments (Bagi et al., 2018b). Their PERMANOVA results also indicated significant differences in beta diversity with an overall decreased diversity from low to high exposure groups. Our results are thus in contrast to what these authors described. Our measures of both species richness and Shannon diversity did not yield any significant differences, similar to findings in the Atlantic cod study where alpha diversity was not significant from control to low oil treatments. With our beta diversity analysis, there were no significant differences in clustering between control and oil. Both treatment groups showed strong patterns of overlap, even when different ordination methods and distance matrices were used to separate shared and unique taxa. Two other studies that exposed juvenile southern flounder to much lower oil concentrations (approximately 0.05 μg/L) in sediment for 7 and 32 days, respectively, did not account for alpha diversity measures but found that oil-exposed microbial communities were significantly different from control in terms of beta diversity (Bayha et al., 2017c; Nancy J. Brown-Peterson et al., 2015a). These differences could be due to several factors, including exposure duration, route of exposure (sediment vs. water suspension) oil

concentration, and perhaps the study species. Thus, the lack of clear patterns in alpha or beta diversity as a result of oil exposure in our study should be investigated further.

Water microbial communities are distinct from fish gut-associated communities, yet oil exposure did not show many changes in taxa composition

The water ecosystem supports a diverse array of species (Campbell et al., 2015; Zhang et al., 2014), and the results from the water samples collected in this study support this observation. Several taxa identified here have some associations with marine or estuarine environments, including the phyla Bacteroidetes (Campbell et al., 2015), Verrucomicrobia (Freitas et al., 2012), Hydrogenedentes (Momper et al., 2018), Thermotogae (Nesbø et al., 2015), Gemmatimonadetes (Zeng et al., 2016), Cyanobacteria (Murrel, 2004), and Chlamydiae (Pizzetti et al., 2012), as well as genera NS3a marine group (Bacteroidetes) (Lindh et al., 2016) and *Pseudohongiella* (L. Xu et al., 2016). Only Patescibacteria was not found to be associated with marine environments in existing literature, but with groundwater, which may have originated from the RO water in our lab (Herrmann et al., 2019). In particular, the dominance of both Bacteroidetes and Proteobacteria was consistent with what has been found in previous studies (Campbell et al., 2015). Some taxa had been previously isolated from algal hosts (specifically *Phaeocystidibacter*, *Phaeodactylibacter*, and *Marivita*) (Z. Chen et al., 2014; Hwang et al., 2009; Zhou et al., 2013), which may explain the presence of some of these microbes in the gut-associated microbiomes of the fish in our study, as the fish readily consumed the various types of algae growing on the walls of our tank system that the fish were housed in. In sum, the results of overall microbial taxonomic characterization of our water samples are consistent with previously identified environmental microbes. However, the community composition here was markedly different from that of fish gut-associated communities.

The taxonomic makeup of the *C. variegatus* gut-microbiome is characteristic of what is commonly found in fish gut-associated microbiomes. To our knowledge, this is the first study to characterize the gut microbiome of *C. variegatus*, and our results may show some taxa that comprise the core microbiome of this species. The large abundance of Proteobacteria, mainly comprising members of the *Vibrio* and *Photobacterium* genera, as well as Fusobacteria comprising the *Cetobacterium* genus, are common in marine fish (Pérez et al., 2010). However, the striking abundance of the genus *Stenotrophomonas* found in our study has not been commonly described as a core member of marine fish gut-associated microbiomes. In the past, *Stenotrophomonas* was classified as a member of the *Pseudomonas* genus (another common member of fish gut

microbiomes) until it was later moved to this different genus (Palleroni & Bradbury, 1993). The genus consists of eight known species that are commonly found to colonize plants, soil, and marine habitats, and strains of *Stenotrophomonas maltophilia* in particular have highly versatile abilities that include beneficial effects for plants, degradation of anthropogenic pollutants, and pathogenic tendencies (Ryan et al., 2009). It was recently described in zebrafish exposed to polystyrene microplastics as well as another study exposing them to a fungicide, where it increased in abundance in response to each toxicant (C. Jin et al., 2017; Y. Jin et al., 2018). *S. maltophilia* has also been recognized as a fish pathogen, notably in channel catfish (*Ictalurus punctatus*) (X. Wang et al., 2016). Additionally, *Propionigenium* is a genus of anaerobic bacteria that were found to a lesser extent (1.24 % ± 0.01) in the gut microbiome but have been found in estuarine sediment and are known for their ability to grow by decarboxylating succinate to propionate, which is a common SCFA (Cani & Knauf, 2016; Janssen & Liesack, 1995). This genus was abundant in the gut microbiome of middle-age African turquoise killifish (*Nothobranchius furzeri*) which were experimentally recolonized by bacteria that are common to younger fish, thereby extending the lifespan of the host (Smith et al., 2017). Overall, the relatively large abundance of *Stenotrophomonas* in the *C. variegatus* gut microbiome is intriguing and should be explored further to elucidate what role it plays in this environment. These taxa described here are all fairly typical of what is found in fish gut-microbiomes, yet oil-exposed communities were found to have a higher number of unique OTUs compared to the control. Exploring relative abundances of genera between these groups revealed some caveats of this finding.

The dispersion of relative abundances of genera across both treatment groups as seen in our heat map showed that both treatment groups were fairly similar as there was a high degree of overlap between individual samples in the dendrogram. Many of the more abundant genera were also concentrated in individual samples (indicated by the bright red areas on the heat map), and as a result were represented at very low average abundances across treatment groups. While there were several unique OTUs in the oil treatment, the majority of unique taxa seemed to be found in only one or two individual fish. We further explored some of these trends to determine genera that were enriched in one treatment group compared to another.

Fish gut microbiomes may have selected some taxa from the water; time shows some impact on baseline vs experimental fish, yet weathered oil had little effect on the gut microbiome

The shifts in microbiota between the fish gut and the water samples are reflected in the taxa that are associated with each system as described above. Our METASTATs results show that the fish gut microbiome may have selected some environmental microbes from the water. Significant differences between the water and baseline, control, and oil samples indicated that certain taxa with known free-living aquatic species were present in the gut. This includes the phyla Bacteroidetes, Thermotogae, and Hydrogenedentes, along with the NS3a marine group genus. In the fish guts alone, the differences between baseline compared to control or oil-exposed fish showed small yet significant increases of some water-associated taxa in the baseline fish, including Bacteroidetes, NS3a marine group, *Phaeocystidibacter*, *Phaeodactylibacter*, and *Marivita*. This supports the possibility that the baseline fish guts may have been influenced more by environmental microbes, including those associated with algae growth. In sum, the free-living aquatic microbial community may have played a role in the gut microbiome that should be elucidated further. Because the baseline fish appeared to have more water-associated taxa, these differences could also bring light to the potential that these samples differed over time compared to the control fish.

The baseline fish were sampled on day 7 of the first experimental run, concurrent with the first set of control fish. However, the majority of the control fish were sampled at a later time. Hence, some of the significant differences in taxa between the baseline and control could have been a factor of the time in which they were sampled. These differences included decreases in NS3a marine group, *Marivita*, and *Pseudohongiella* from the baseline to control communities. The SEM of baseline fish also appeared to be tighter, which is likely due to less variability from being sampled all at the same time. Additionally, the fact that the baseline fish had an n = 5 compared to control fish with n = 15 should be considered and these results should be interpreted with some caution. Thus, the timing of sampling could have played a factor in some of the differences seen in the baseline compared to control communities. However, differences between control and oil-exposed communities were mostly among rarer taxa.

We found no significant relationships between taxa in the control and oil treatments among the most abundant phyla or genera. This may suggest that the gut microbiome of sheepshead minnows is relatively robust to xenobiotic stressors. The only significant *q*-values were found in

more rare bacterial taxa: at the class (Chloroflexia and Dehalococcoidia, $q < 0.05$) and genus (*UBA1819* and *Ethanoligenens*, both members of the Ruminococcaceae family, $q < 0.05$) levels. Chloroflexia are a group of filamentous photosynthetic bacteria, while Dehalococcoidia are known for their ability to break down chlorinated compounds (Biderre-Petit et al., 2016; Matturro et al., 2017). While the majority of PAHs are not chlorinated, some such as dibenzofurans can be and were detected in our GC/MS results. On the other hand, the Ruminococcaceae family is a common member of mammalian gut microbiomes and are specialized to degrade plant material (Biddle et al., 2013). Several other taxa had significant *p*-values (< 0.01) yet non-significant *q*-values. However, the low abundances of these four OTUs within either treatment group may suggest that they are relatively transient in the gut microbiome of these fish, as 16S rRNA sequencing inherently only characterizes a snapshot of the microbiome at a specific point in time. When closely observing our heatmap, we also found some of these rare taxa to be highly abundant in only one sample from either treatment group, causing them to have low average abundances across all samples. Another caveat to note is that many of these taxa have high standard errors, oftentimes slightly lesser than or equal to the mean (**Table 2.2**). Because of the high amount of variability in the data, significant yet miniscule changes in rare OTUs from one treatment group to another, and a large amount of singleton OTUs, it is difficult to draw many concrete conclusions from this analysis. In sum, even though the oil treatment had a much higher number of unique OTUs as shown in the Venn diagram, it appears that most of the differences were driven by rare taxa that were found in higher numbers in a single sample.

Known oil-degrading taxa are discriminant of oil-exposed fish gut communities

In control fish, the LEfSe analysis comparing all treatment groups identified two species from the genus *Bacteroides* that were discriminant of this treatment. Although typically found in freshwater fish, this genus is known for its role in producing vitamin B12, as well as SCFAs (Gómez & Balcázar, 2008; Koh et al., 2016; Sugita et al., 1991). This suggests that this genus could be playing a beneficial role in the gut of control fish. More taxa were represented when only comparing the control group against the oil treatment, including many that were previously identified as water associated (e.g., NS3a marine group, *Phaeocystidibacter*, and *Phaeodactylibacter*). All of the taxa listed are members of the Bacteroidetes phylum, and many of the parent families and/or orders of these water associated genera were present in control fish

(Hahnke et al., 2016). This may mean that control fish gut microbiota composition was influenced more by the surrounding water. However, it is interesting to note that no known hydrocarbon-degrading taxa were among those identified in this analysis in the control fish, which supports our hypothesis that these bacteria would be more present in the oil treatment.

Notably, two taxa of gut microbes that had the highest effect sizes (LDA score > 4) in oil exposed fish guts when compared against all treatments, Paenibacillaceae and *Pseudomonas pachastrellae,* are known to be involved in hydrocarbon degradation. Paenibacillaceae is a family consisting of eight genera, including the genus *Paenibacillus*, (Grady et al., 2016) which was the only one detected in our samples. This genus consists of over 200 species that conduct a large diversity of functions and include strains that are capable of bioremediation of PAHs by producing oxygenases, dehydrogenases, and lignolytic enzymes (Grady et al., 2016; Haritash & Kaushik, 2009). It is unknown if the two species of *Paenibacillus* found in our samples (*P. macerans* and *P. borealis*) are involved in oil degradation, and further research is warranted to determine their role in this activity. This genus has been previously detected in fish gut microbiomes and associated with metabolism of cellulose from dietary fiber in herbivorous blunt snout bream (*Megalobrama amblycephala*) and acting antagonistically towards fish pathogens when applied to cultured red tilapia (species not listed) as a probiotic (H. Liu et al., 2016; Yee et al., 2013), but to our knowledge, has not been found in other studies examining the effects of PAHs on fish.

The other species discriminant of the oil-exposed group, *Pseudomonas pachastrellae*, was first isolated from a deep-sea sponge (Romanenko et al., 2005). Whole-genome sequencing has been completed for this particular species, where genes encoding pathways for PAH degradation were identified (Gomila et al., 2017). Furthermore, the *Pseudomonas* genus is widely recognized as having strains that are active in the biodegradation of PAHs, and a *P. pachastrellae* in particular has been isolated as a known oil-degrading bacteria from contaminated beach sands after the DWH oil spill (Ghosal et al., 2016; Kostka et al., 2011; Lamendella et al., 2014). To our knowledge, neither this species nor the only other taxa with an LDA score > 4 (MND1) have been previously documented in fish gut microbiomes. MND1 is a genus belonging to the Nitrosomonadaceae family, which is known for their ability to oxidize ammonia to nitrites (Prosser et al., 2014).

Several other taxa that are known for the ability to degrade PAHs had smaller effect sizes with LDA scores > 2 (**Fig. S-1**). These include Aeromonadales, Micrococcacaeae, *Serratia marcescens, Ralstonia pickettii, Acinetobacter,* Moraxellaceae, Burkholderiaceae, Bacillales,

Citrobacter freundii, Sphingomonadaceae, *Thauera*, and *Lysobacter* (Alegbeleye et al., 2017a; Bayoumi, 2009; Flanagan et al., 2014; Ghosal et al., 2016; Korenblum et al., 2012; Nzila et al., 2018; Pandey et al., 2012; Toledo et al., 2006; Yetti et al., 2018). Besides using enzymes to degrade PAHs, many of these bacteria can exclusively use specific PAHs such as phenanthrene, naphthalene, or anthracene as their carbon or energy source (Nzila et al., 2018; J. S. Seo et al., 2009). However, when the LEfSe analysis was conducted only between the control and oil groups, the number of differential taxa in the oil treatment dropped to just one, the Pseudomonadales order. This order increased in abundance in the oil treatment compared to the control and comprises a few of the above taxa that are known for oil degradation, including *Pseudomonas pachastrellae*, *Acinetobacter*, and Moraxellaceae. Because Paenibacillaceae and *Pseudomonas pachastrellae* were missing from the LEfSe between control and oil, it may indicate that they have more biological similarities to taxa in the baseline or water samples. Overall, these findings suggest that some taxa in the oil treatment group may be actively degrading PAHs in the gut when fish are exposed to oil.

2.5.3 Foraging Behavior Trials

Although a reduction in feeding rate is a common ecological assessment of stress in fish, especially as a result of contaminant exposure, the results gathered from this study are inconclusive as foraging was not significantly affected by oil exposure. The previous studies that were used to develop the methods for our foraging behavioral trials had assessed prey capture rate in larval sheepshead minnow and both larval and adult mummichogs (*Fundulus heteroclitus*), and to our knowledge, this is the first study to examine this endpoint of PAH toxicity in adult sheepshead minnows. Similar to our results, PAHs did not impact larval and adult mummichog feeding rates at contaminated field sites, where other contaminants played a larger role in this (J. S. Weis et al., 2001; Judith S Weis et al., 2003). However, in other studies that investigated this endpoint with PAHs as the sole contaminant, prey-capture rates were significantly reduced. In larval sheepshead minnows (10 dph) exposed to 1.7 and 17 µg/L tPAHs in HEWAF created from DWH oil, prey-capture rates were significantly reduced at the 17 µg/L concentration after 2 minutes (Jasperse et al., 2019b). Darter goby (*Gobionellus boleosoma*) exposed to diesel-contaminated sediment above 0.2 µg/kg tPAH experienced a 50-100% reduction in feeding rates, with no feeding occurring at 0.687 µg/kg (Gregg et al., 1997). Similarly, juvenile spot (*Leiostomus xanthurus*), a species that

feeds by manipulating sediment in its buccal cavity to capture prey, significantly reduced the amount of strikes towards sediment contaminated with 122,000 ppb tPAH and had a 70% discontinuation of feeding before the end of the trials, likely due to the narcotic effect of PAHs in the re-suspended sediment (Hinkle-Conn et al., 1998). Both darter gobies and spot are bottom feeders similar to sheepshead minnows, who dwell near the muddy bottoms of shallow, turbid water and primarily eat detritus, algae, and other microinvertebrates (Froese & Torres, 2011). Thus, adult sheepshead minnows are likely to experience PAH exposure and a possible reduction in foraging behavior due to the resuspension of sediment when feeding. In sum, this work highlights the need for more studies that investigate prey capture ability of adult sheepshead minnows in response to oil exposure, as significant effects were seen in their larvae. More information is needed on this behavioral endpoint as well as the physiological mechanisms behind it (such as gut microbiome dysbiosis), as feeding rates directly impact growth which can cause population declines and drops in reproduction from less energy.

It is known that the gut-microbiome can impact host behavior through the production of microbial metabolites, and there is a need for future studies to directly investigate the connections between gut microbiome dysbiosis and feeding behavior impairment as a result of contaminant exposure. Research focusing on the link between gut microbiome dynamics and behavioral endpoints is an emerging field, yet some key questions remain unclear regarding connections between specific microbes and host responses as well as the mechanisms and extent to which microbes play a role in nervous system function (Vuong et al., 2017). Several studies have documented stress-induced microbial dysbiosis linked to behavioral changes, yet few studies have investigated the link between stress-induced behaviors or neurological dysfunctions as a result of xenobiotic contaminant exposure and gut dysbiosis (Vuong et al., 2017). For example, one study found that glyphosate-based herbicide exposure in mice resulted in microbial dysbiosis linked to anxiety and depression behaviors (Aitbali et al., 2018). In another, rats exposed to silver nanoparticles (Ag-NPs) exhibited behavioral changes in a maze which was correlated with reductions in gut microbiome diversity (Javurek et al., 2017). Finally, gut microbiome dysbiosis was correlated with anxiety and depression behaviors in mice chronically exposed to alcohol (Z. Xu et al., 2019). However, much of this research has been conducted with mice or rat models, with less focus on fish or wild species.

In the present study, we hypothesized that gut dysbiosis as a result of oil exposure could play a role in altered feeding behavior via the gut-brain axis through a possible change in microbial metabolite production. Our 16S rRNA sequencing results did not show any significant compositional changes and diversity remained relatively stable between control and oil treatments, and the results of our foraging study did not show any significant changes in behavior. To our knowledge, this is the first study to explore the possible connection between altered behavior and gut microbiome dysbiosis in fish due to oil exposure. Future studies that investigate altered foraging behavior in fish should investigate the role of microbially-produced SCFAs as they are most often associated with appetite regulating behaviors. Correlation analyses between changes in microbe abundance and host phenotypic traits as a result of contaminant exposure are necessary. In general, gut dysbiosis may be a contributing factor towards changes in host behavior that should be explored further.

2.5.4 Limitations

There are some limitations within this study that should be acknowledged, starting with the ones related to biological endpoints measured. Although we were highly careful to not introduce any bacterial contamination when dissecting fish for DNA extraction, fish were only cleaned dry and not dipped in an ethanol solution to remove any external bacteria as other studies have done (Weiss et al., 2014). This may also explain the presence of known aquatic microbiota in the gut samples. In addition, the timing of the dissections may have introduced some variability in the microbiome data, as microbial communities change over time as well as with diet (e.g., the baseline fish consuming more algae in holding tanks prior to dissection than those of control fish). The accidental deviation in protocol for the foraging study lead us to having a smaller sample size, which could have significantly impacted our results. In sum, a few of these limitations were accidental in nature and others were more inherent to the study design.

Additional caveats should be noted for the oil exposures. Due to logistics and timing of experiments, a few samples in the reports of tPAHs at the 24-hour mark include some that were taken closer to 18 or 20 hours. However, these values were still very similar to those reported at 24 hours. There was also a potential decrease in bioavailability of the oil during the experiments, which may have contributed to the lack of microbial dysbiosis seen in our results. Oil could have had low bioavailability due to a number of factors. It may have adsorbed onto uneaten food flakes

at the bottom of the tanks, which were noticed at the 24-hour mark. Our thought was that one of the ways fish gut microbiota may be disturbed was through the fish consuming food flakes at the surface of the tanks immediately after dosing. However, flakes were consumed quickly after administration, and there may not have been enough time for the oil to adsorb onto the food. The necessary aeration of the tanks may have significantly disturbed the oil to a point where the oil droplets in the HEWAF congealed into larger droplets, thereby decreasing bioavailability. When conducting water changes after 24 hours, oil slicks were adhered to the plastic tubing on the air stones as well as the aluminum foil covers. The visibility in the tanks was also much clearer at this time compared to the initial dose time. During troubleshooting before the experiment, we also noticed that stirring multiple HEWAFs together caused the fluorescence measurements to be much lower than a HEWAF which was not disturbed as such. Therefore, the disturbance via aeration or adsorption of oil onto uneaten food could have decreased the bioavailability of oil in the experimental tanks.

Along with this, the fish could have had low bioaccumulation in the GI tract compared to other tissues such as the gills, possibly due to increased elimination rates or via enterohepatic circulation. There have been several studies that have investigated bioconcentration factors (BCFs) of PAHs in fish. For example, sheepshead minnows exposed to select PAHs (naphthalene, phenanthrene, and pyrene, with associated alkylated homologues) at total concentrations of 7.57 and 72.31 ppb in the water column over 36 days showed rapid uptake in tissues at day 4 of the high exposure group (Jonsson et al., 2004). The authors found BCFs for phenanthrene and pyrene parent and homologues to be lower than those of naphthalene, with BCFs showing an increasing trend with alkylation of parent PAHs. The high concentration in this study is closer to our estimated total PAH value of 80.99 ± 12.5 µg/L, and BCFs measured from our exposure concentration could be similar to what was found by Jonsson et. al. (2004). Thus, a combination of these factors could have caused a limited uptake of PAHs from the oil in the fish GI tracts.

One of the ways that researchers can identify whether oil toxicity was induced in a fish is through the induction of the cyp1a gene. Our concentrations of oil were similar to those of other studies with sheepshead minnows or other small estuarine fish (such as *Fundulus grandis*) that noted an induction of the cyp1a gene after exposure (Rodgers et al., 2018; Serafin et al., 2019; Simning et al., 2019). Although future studies should conduct qPCR to determine if oil had an

impact on the host, we could reasonably infer that our exposures had an effect on the fish as a whole that could have also induced microbial dysbiosis.

2.6 Supplemental Information

Table S.1 Average ± SEM of female fish body mass (g), total body length (mm), total DNA yield (ng/µL), and DNA purity measured as absorbance (nm) per experimental run for oil, control, baseline, and water groups (n = 5 for each group) for the microbiome study.

Collection Date	Experimental Run	Exposure Group	Body Mass (g)	Total Body Length (mm)	Total DNA Yield (ng/µL)	260/280 (nm)
8/12/2019	1	Oil	2.94 ± 0.31	50.44 ± 1.82	437.59 ± 38.49	2.08 ± 0.01
8/19/2019	2	Oil	2.54 ± 0.15	50.44 ± 1.15	275.69 ± 20.51	2.06 ± 0.01
8/26/2019	3	Oil	3.06 ± 0.38	52.40 ± 1.91	265.34 ± 43.04	2.07 ± 0.01
8/12/2019	1	Control	2.43 ± 0.09	48.40 ± 1.18	297.44 ± 69.56	2.08 ± 0.02
8/19/2019	2	Control	2.43 ± 0.12	48.58 ± 1.11	167.57 ± 49.93	2.03 ± 0.02
8/26/2019	3	Control	2.88 ± 0.18	51.00 ± 0.76	112.45 ± 27.54	2.05 ± 0.01
8/12/2019	N/A	Baseline	2.69 ± 0.13	49.42 ± 0.89	241.37 ± 86.32	2.06 ± 0.01
8/19/2019	N/A	Water	N/A	N/A	14.33 ± 0.51	1.94 ± 0.01

Table S.2 Water Quality data across both experiments, with means ± SE. NA = data not available due to malfunctions with the DO meter.

Experiment	Date	Temperature (°C)	Salinity (ppt)	pH	DO (mg/L)	Ammonia (total)	Nitrates	Nitrites
Microbiome	8/06/2019	25.4	25	8	9.28	0	0	0
	8/07/2019	25.2	25	8	9.34	0.25	0	0
	8/08/2019	25.5	25	8	9.105	0	0	0
	8/09/2019	25.3	25	8	9.33	0.25	0	0
	8/10/2019	25.5	25	8	9.37	0	0	0
	8/11/2019	25.6	25	8	9.38	0	0	0
	8/13/2019	25	25	8	9.53	0.25	0	0
	8/14/2019	25.6	26	8	9.29	0	0	0
	8/15/2019	25.7	26	8	9.33	0	0	0
	8/16/2019	25.7	25	8	9.32	0	0	0
	8/17/2019	25	25	8	NA	0	0	0
	8/18/2019	25	25	8	NA	0.25	0	0
	8/20/2019	25	25	8	NA	0.25	0	0
	8/21/2019	25	25	8	NA	0	0	0
	8/22/2019	25	25	8	NA	0.25	0	0
	8/23/2019	26	25	8	NA	0	0	0
	8/24/2019	25	25	8	NA	0	0	0
	8/25/2019	25	25	8	NA	0	0	0
Foraging	9/20/2019	26	25	8	NA	0	0	0
	9/21/2019	26	25	8	NA	0	0	0
	9/22/2019	26	25	8	NA	0	0	0
	9/23/2019	26	25	8	NA	0	0	0
	9/24/2019	25.35	23	7.7	8.5	0	0	0
	9/25/2019	25.5	24	7.9	8.61	0	0	0
	9/27/2019	25.25	25	7.9	8.51	0	0	0
	9/28/2019	25.5	24	8	7.56	0	0	0
	9/29/2019	25.45	25	8	7.63	0	2.5	0.125
	9/30/2019	25.7	24	8	7.4	0	0	0.125
	10/01/2019	25.65	24	7.9	7.52	0.125	2.5	0.125
	10/02/2019	25.65	24	8	7.72	0.125	2.5	0.125

Table S.2 continued

10/04/2019	25.6	26	8	7.91	0	0	0
10/05/2019	25.25	26	8	7.96	0	0	0
10/06/2019	25.55	25	8	7.91	0	0	0
10/07/2019	25.35	25	8	7.96	0	0	0
10/08/2019	25.45	24	8	7.93	0	0	0
10/09/2019	25.25	23.5	8	8.00	0	0	0
Microbiome Mean ± SE	25.31 ± 0.08	25.11 ± 0.08	8.00 ± 0.00	9.33 ± 0.03	0.08 ± 0.03	0 ± 0	0 ± 0
Foraging Mean ± SE	25.58 ± 0.06	24.58 ± 0.19	7.97 ± 0.02	7.94 ± 0.1	0.01 ± 0.01	0.42 ± 0.23	0.03 ± 0.01
Overall Mean ± SE	25.44 ± 0.05	24.85 ± 0.11	7.98 ± 0.01	8.52 ± 0.15	0.05 ± 0.02	0.21 ± 0.12	0.01 ± 0.01

Table S.3 Summary statistical results of PAH data from both microbiome and foraging experiments. NS = non-significant, $p > 0.05$.

Effect	Statistical Test	df	t Stat	t Critical two-tail	F	*p*-value
PAH Data: Microbiome Study						
0 hr. (Initial) between experimental runs	ANOVA: Single Factor	2, 15	-	-	3.68	0.05
0 hr. – Experiment 1 vs. 2	t-Test: Two-Sample Assuming Unequal Variances	10	-1.971	2.228	-	0.077
0 hr. – Experiment 1 vs. 3	t-Test: Two-Sample Assuming Unequal Variances	10	0.564	2.228	-	NS
0 hr. – Experiment 2 vs. 3	t-Test: Two-Sample Assuming Unequal Variances	10	2.525	2.228	-	0.03
24 hr. (Final) between experimental runs	ANOVA: Single Factor	2, 15	-	-	1.99	NS
Experiment 1 – 0 vs. 24 hr.	t-Test: Two-Sample Assuming Unequal Variances	6	4.505	2.447	-	0.004
Experiment 2 – 0 vs. 24 hr.	t-Test: Two-Sample Assuming Unequal Variances	10	7.652	2.228	-	1.73E-05
Experiment 3 – 0 vs. 24 hr.	t-Test: Two-Sample Assuming Unequal Variances	9	6.579	2.262	-	0.0001
PAH Data: Foraging Study						
0 hr. (Initial) between experimental runs	ANOVA: Single Factor	2, 15	-	-	1.27	NS
24 hr. (Final) between experimental runs	ANOVA: Single Factor	2, 15	-	-	2.57	NS
Experiment 1 – 0 vs. 24 hr.	t-Test: Two-Sample Assuming Unequal Variances	7	7.33	2.365	-	0.0002
Experiment 2 – 0 vs. 24 hr.	t-Test: Two-Sample Assuming Unequal Variances	9	6.61	2.447	-	6.45E-05
Experiment 3 – 0 vs. 24 hr.	t-Test: Two-Sample Assuming Unequal Variances	6	6.98	2.262	-	0.0006

Table S.4 Summary statistical results of alpha diversity metrics from the microbiome study with Tukey's HSD. NS = non-significant, $p > 0.05$.

Diversity Metric	Effect	Diff	Upper	Lower	*p*-value
ACE	Control-Baseline	-48.279	-187.590	91.032	NS
	Oil-Baseline	-39.938	-179.250	99.373	NS
	Water-Baseline	49.010	-121.611	219.630	NS
	Oil-Control	8.340	-90.168	106.848	NS
	Water-Control	97.288	-42.023	236.599	NS
	Water-Oil	88.948	-50.363	228.259	NS
Chao1	Control-Baseline	-64.165	-195.657	67.326	NS
	Oil-Baseline	-50.048	-181.539	81.444	NS
	Water-Baseline	48.286	-112.757	209.329	NS
	Oil-Control	14.118	-78.860	107.096	NS
	Water-Control	112.452	-19.040	243.943	NS
	Water-Oil	98.334	-33.157	229.825	NS
Simpson	Control-Baseline	-0.005	-0.269	0.259	NS
	Oil-Baseline	0.048	-0.216	0.312	NS
	Water-Baseline	0.373	0.050	0.696	0.018
	Oil-Control	0.053	-0.133	0.240	NS
	Water-Control	0.378	0.115	0.642	0.002
	Water-Oil	0.325	0.061	0.589	0.011
Shannon	Control-Baseline	-0.158	-0.960	0.643	NS
	Oil-Baseline	0.001	-0.800	0.803	NS
	Water-Baseline	1.476	0.494	2.457	0.001
	Oil-Control	0.160	-0.407	0.726	NS
	Water-Control	1.634	0.833	2.435	1.92E-05
	Water-Oil	1.474	0.673	2.276	9.84E-05
PD Whole Tree	Control-Baseline	-2.565	-15.400	10.270	NS
	Oil-Baseline	0.501	-12.334	13.336	NS
	Water-Baseline	8.215	-7.505	23.934	NS
	Oil-Control	3.066	-6.009	12.142	NS
	Water-Control	10.780	-2.055	23.615	NS
	Water-Oil	7.714	-5.121	20.549	NS

Table S.5 Results of observed OTUs, alpha diversity and species richness metric, and Good's Coverage per sample.

Sample ID	Observed OTUs	Mean ± SEM	Shannon Index	Mean ± SEM	Simpson Index	Mean ± SEM	Chao1	Mean ± SEM	ACE	Mean ± SEM	PD Whole Tree	Mean ± SEM	Good's Coverage
Baseline.1	296	301.2 ± 8.83	1.48	1.91 ± 0.287	0.288	0.436 ± 0.091	380.444	422.2± 17.8	386.67	442.45 ± 16.42	27.701	28.79 ± 0.72	0.998
Baseline.2	275		1.193		0.226		421.872		438.33		28.342		0.997
Baseline.3	330		2.628		0.626		433.25		458.721		31.43		0.997
Baseline.4	300		1.707		0.363		393.059		441.397		29.067		0.997
Baseline.5	305		2.537		0.676		482.396		487.139		27.432		0.997
Exp1.M.Control.1	263	234.73 ± 15.24	1.71	1.75 ± 0.15	0.379	0.431 ± 0.051	408.2	358.04 ± 23.33	477.003	394.17 ± 23.37	30.646	26.23 ± 2.21	0.997
Exp1.M.Control.2	177		2.98		0.817		300		321.369		23.647		0.998
Exp1.M.Control.3	230		1.887		0.49		341.766		393.889		22.968		0.998
Exp1.M.C.4	297		1.291		0.253		414.018		441.092		27.404		0.997
Exp1.M.C.5	205		2.529		0.695		270.022		306.348		26.843		0.998
Exp2.M.C.1	333		1.499		0.341		534.333		557.643		50.261		0.997
Exp2.M.C.2	225		1.12		0.239		337.222		381.013		31.106		0.998
Exp2.M.C.3	170		1.61		0.433		307.308		343.48		16.272		0.998
Exp2.M.C.4	156		1.897		0.541		229.484		271.932		15.844		0.998
Exp2.M.C.5	192		1.3		0.295		299.129		318.364		20.822		0.998
Exp3.M.C.1	239		1.518		0.331		314.783		338.618		24.169		0.998
Exp3.M.C.2	247		1.321		0.276		424.073		474.63		24.735		0.997
Exp3.M.C.3	163		2.777		0.795		250.6		289.695		16.502		0.998
Exp3.M.C.4	294		1.127		0.22		455.25		491.683		32.397		0.997
Exp3.M.C.5	330		1.694		0.353		484.393		505.832		29.819		0.997
Exp1.M.Oil.1	362	247.93 ± 18.12	3.066	1.91 ± 0.159	0.786	0.484 ± 0.052	408.683	372.16 ± 29.41	427.108	402.51 ± 32.32	54.917	29.3 ± 2.81	0.998

Table S.5 continued

Exp1.M.Oil.2	306		1.616	0.365	453.424	525.084	45.438	0.997			
Exp1.M.Oil.3	196		2.611	0.757	269.488	298.068	25.618	0.998			
Exp1.M.Oil.4	189		1.43	0.335	274.8	308.142	20.336	0.998			
Exp1.M.Oil.5	207		1.368	0.301	284.341	323.402	26.16	0.998			
Exp2.M.Oil.1	304		2.966	0.77	516.038	563.576	38.364	0.997			
Exp2.M.Oil.2	231		2.028	0.52	259.286	263.555	34.907	0.999			
Exp2.M.Oil.3	191		2.316	0.695	338.231	338.42	17.695	0.998			
Exp2.M.Oil.4	371		1.222	0.228	614.375	677.799	34.505	0.996			
Exp2.M.Oil.5	334		1.77	0.385	525.625	551.807	34.116	0.997			
Exp3.M.Oil.1	223		1.896	0.517	346.75	385.434	20.191	0.998			
Exp3.M.Oil.2	205		1.33	0.302	363.444	354.27	19.445	0.998			
Exp3.M.Oil.3	136		2.29	0.685	204.44	237.829	19.93	0.999			
Exp3.M.Oil.4	231		1.426	0.321	357.923	380.273	26.825	0.998			
Exp3.M.Oil.5	207		1.368	0.301	284.341	323.402	20.985	0.998			
Water.M.8.19.19.1	304	351 ± 28.4	2.966	3.38 ± 0.118	0.77	0.809 ± 0.015	516.038	470.49 ± 31.28	563.576	491.46 ± 32.99	46.76	37.01 ± 4.27	0.997
Water.M.8.19.19.2	231		2.028		0.52		259.286		263.555		27.35		0.999
Water.M.8.19.19.3	191		2.316		0.695		338.231		338.42		26.327		0.998
Water.M.8.24.19.1	371		1.222		0.228		614.375		677.799		44.161		0.996
Water.M.8.24.19.2	334		1.77		0.385		525.625		551.807		40.448		0.997

Table S.6 Summary statistical results of beta diversity at the genus level from the microbiome study using PERMANOVA and Tukey's HSD. NS = non-significant, $p > 0.05$.

Groups	Effect	Df	Sum of Squares	Mean Squares	F Model	R^2	Pr(>F)
NMDS with Bray-Curtis (PERMANOVA)							
Oil vs. Control	Treatment	1	0.0364	0.03636	0.2208	0.00816	NS
	Experiment	1	0.0586	0.058594	0.35583	0.01315	NS
	Treatment : Experiment	1	0.0796	0.079645	0.48366	0.01787	NS
All Treatments	Water-Control	1(18)	1.8274 (3.1269)	1.82744 (0.17372)	10.52	0.36885 (0.63115)	0.001
	Water-Baseline	1(8)	1.41928 (0.81593)	1.41928 (0.10199)	13.916	0.63497 (0.36503)	0.006
	Water-Oil	1(18)	1.9792 (2.6780)	1.97922 (0.14878)	13.303	0.42497 (0.57503)	0.001
	Control-Baseline	1(18)	0.13333 (2.61978)	0.13334 (0.14554)	0.91612	0.04843 (0.95157)	NS
	Control-Oil	1(28)	0.0378 (4.4819)	0.037776 (0.160068)	0.236	0.00836 (0.99164)	NS
	Baseline-Oil	1(18)	0.18748 (2.17088)	0.18748 (0.12060)	1.5545	0.0795 (0.9205)	NS
PCoA with Yue-Clayton (Tukey's HSD)							
Groups	**Diff**	**Lwr**	**Upr**	**P adj**	-	-	-
Oil vs. Control	-0.037	-0.146	0.071	NS	-	-	-
Control-Baseline	0.328	-0.01	0.665	NS	-	-	-
Oil-Baseline	0.291	-0.047	0.629	NS	-	-	-
Water-Baseline	0.39	-0.067	0.846	NS	-	-	-
Oil-Control	-0.037	-0.178	0.104	NS	-	-	-
Water-Control	0.062	-0.276	0.34	NS	-	-	-
Water-Oil	0.099	-0.239	0.437	NS	-	-	-

Table S.7 Q-values from METASTAT analyses in the microbiome study. NS = non-significant, $q > 0.05$. B = Baseline, C = Control, O = Oil, W = Water.

Taxa Classification	Taxa Name	B vs. C	B vs. O	C vs. O	B vs. W	C vs. W	O vs. W
Phylum	Proteobacteria	NS	NS	NS	0.017	9.00E-04	0
	Bacteroidetes	NS	0	NS	0	0	0
	Fusobacteria	NS	NS	NS	NS	NS	NS
	Firmicutes	NS	NS	NS	0.009	0.0024	0.0006
	Actinobacteria	NS	0.041	NS	NS	NS	NS
	Patescibacteria	NS	NS	NS	NS	NS	NS
	Verrucomicrobia	NS	NS	NS	NS	NS	NS
	Cyanobacteria	NS	NS	NS	NS	NS	NS
	Chlamydiae	NS	NS	NS	0.027	0.048	0.021
	Lentisphaerae	NS	-	-	-	-	-
	Chloroflexi	-	NS	NS	NS	NS	NS
	Acidobacteria	-	-	-	0.017	0.004	0.007
	Gemmatimonadetes	-	-	-	NS	NS	NS
	Epsilonbacteraeota	-	-	-	NS	-	-
	Spirochaetes	-	-	-	0.048	0.033	0.013
	Thermotogae	-	-	-	-	0.0306	-
	Hydrogenedentes	-	-	-	-	-	0.02
Genus	Vibrio	NS	NS	NS	NS	NS	0.049
	Stenotrophomonas	NS	NS	NS	0.027	0.013	0.007
	Cetobacterium	NS	NS	NS	NS	NS	NS
	Phaeocystidibacter	NS	0.007	NS	NS	NS	NS
	Phaeodactylibacter	NS	0.004	NS	NS	NS	NS
	Photobacterium	NS	NS	NS	NS	NS	NS
	NS3a marine group	0.002	0.003	NS	NS	0.037	0.033
	Marivita	0	0.016	NS	NS	NS	NS
	Pseudohongiella	0.018	0.006	NS	NS	NS	NS
	Propiogenium	NS	NS	NS	NS	NS	NS

Table S.8 Summary statistical results of binomial general linear models for prey capture rates in oil and control groups for the foraging study. NS = non-significant, $p > 0.05$.

Coefficients	**Estimate**	**Standard Error**	**Z value**	**Pr(> |z|)**
(Intercept)	-0.7082	0.6725	-1.053	0.292
GroupOil	-0.1391	0.9636	-0.144	0.885
Day 7	0.2609	0.9341	0.279	0.78
GroupOil : Day 7	1.1618	1.3352	0.87	0.384
Fixed Effects	**Estimate**	**Standard Error**	**Z value**	**Pr(> |z|)**
(Intercept)	-0.8162	1.1793	-0.692	0.489
GroupOil	-0.7138	1.6263	-0.439	0.661
Day 7	0.0002	1.2231	0	1.000
GroupOil : Day 7	2.3272	1.9774	1.177	0.239

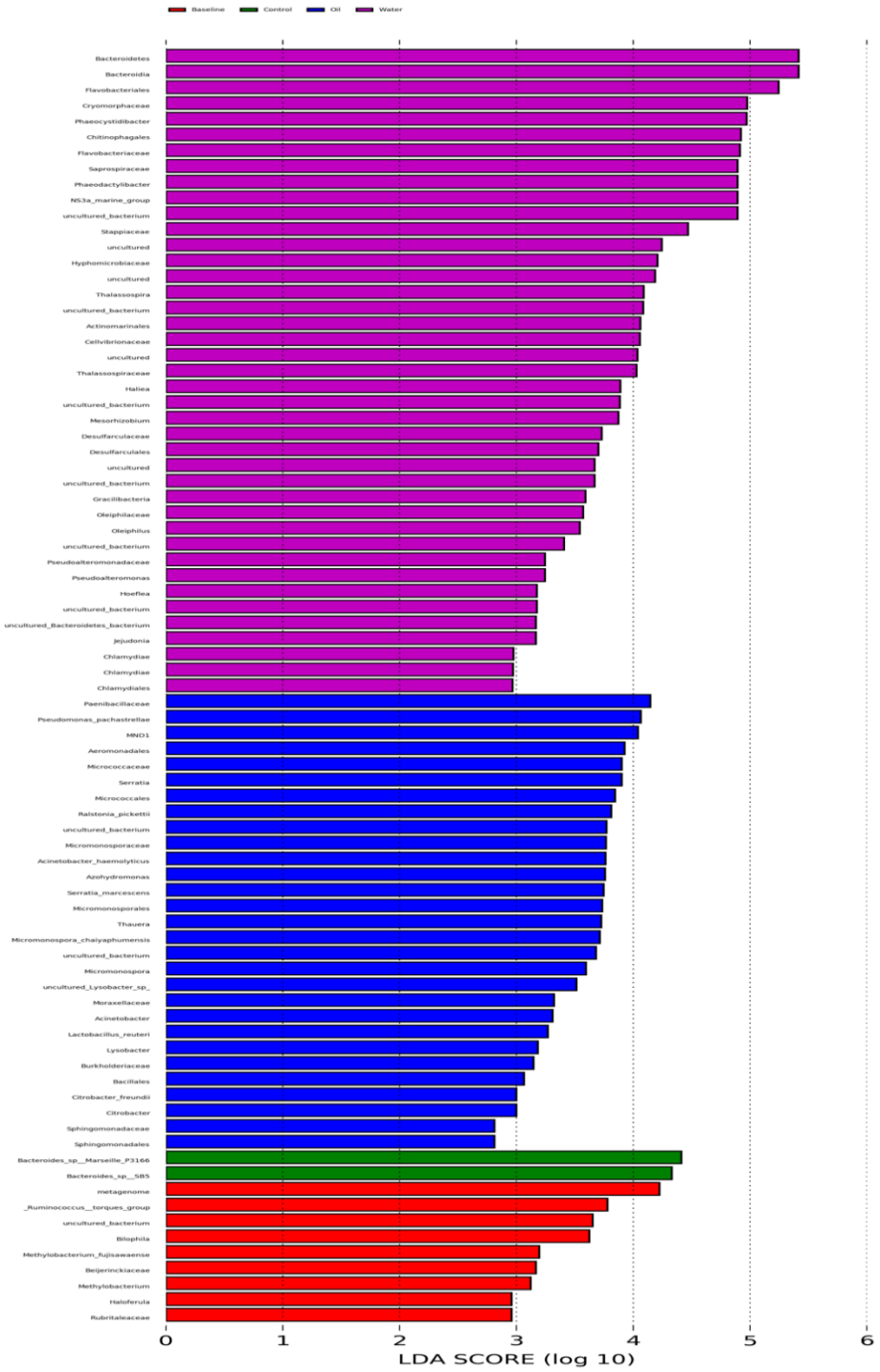


Figure S.1 Linear Discriminant Analysis (LDA) score distribution histogram, indicating enriched taxa in each of the treatment groups. The length of a bar represents the effect level of the taxa, with LDA scores > 2 displayed here. Uncultured refers to taxa that have not been identified.

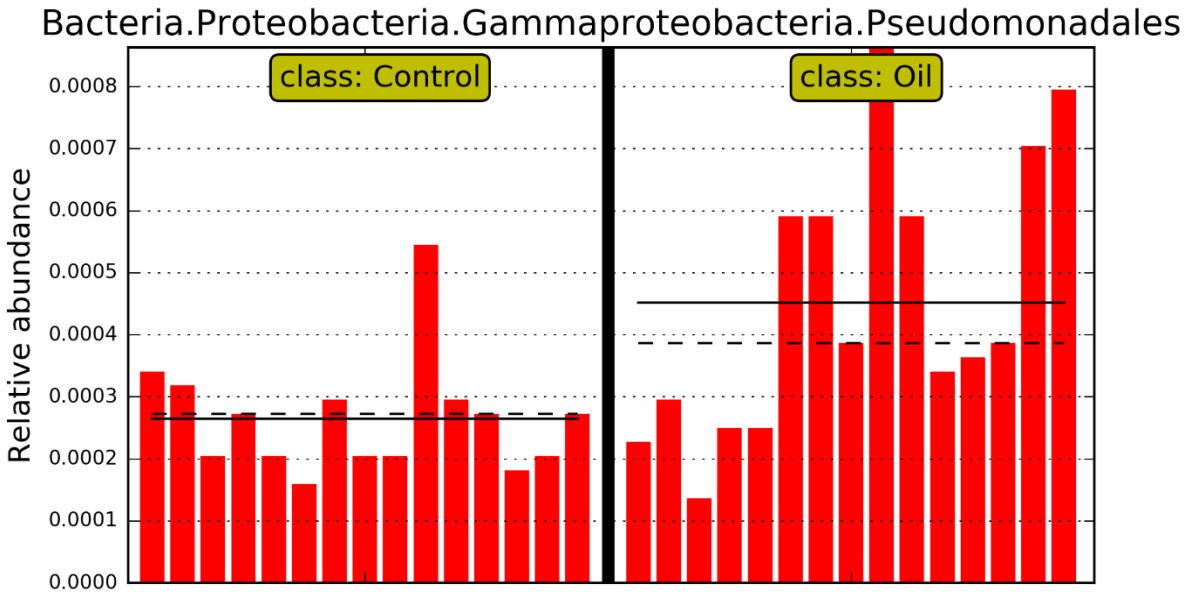


Figure S.2 Histogram of the relative abundance of the Pseudomonadales order from our LEfSe analysis of only the control and oil treatments. Bars represent individual samples in both treatments, with the mean and median represented by the solid and dashed lines, respectively.

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CHAPTER 3. CONCLUSIONS AND FUTURE DIRECTIONS

3.1 Chapter 1

Despite the knowledge gained regarding fish microbiome structure, several challenges and knowledge gaps still remain in this field. Among studies, there is wide variation in the species and associated tissues studied, and even within some of the established dominant phyla, there are still considerable inter- and intraspecific differences to be considered. This can pose some difficulties for establishing a core microbiome as a means of baseline comparison for healthy fish in the wild and within aquaculture. Within ecotoxicology, most studies have generally only reported taxonomic characterizations along with previous knowledge on OTU function. While this is necessary as many taxa are still unclassified, it could be considered reductionist to only address previously described functional roles of OTUs with shifts in taxonomic data. As such, more investigation into correlations between host trait values and microbial community compositions as well as functional metagenomic analyses are needed. Research focusing on host-associated microbiomes is a rapidly developing field, particularly within aquatic ecotoxicology as it can establish a broader scope of the impacts chemical contaminants have on an organism. Next-Generation Sequencing (NGS) tools are becoming more widely used in microbiome studies, as culture-dependent methods can only capture a small fraction of the microbiome. On a broader scale, it is imperative that the methods and techniques used in microbiome studies are homogenized for reproducibility. It can be difficult to compare studies that use a variety of methods to characterize and quantify microbial communities, especially when culture-based methods are becoming outdated in favor of NGS. However, this process of homogenization may be difficult for the time being as the field is still relatively young and constantly evolving. There exist many other reviews that highlight the best methods and practices for conducting microbiome studies, which are particularly helpful for those starting out in the field. Overall, this is an exciting field with many practical applications in a variety of areas, notably in ecotoxicology.

3.2 Chapter 2

Evidence has been presented in previous studies that crude and weathered oil can disrupt the gut-microbial communities of fish. Specifically, bacterial taxa that are known for hydrocarbon

degradation were present in fish guts in response to oil exposure. The DWH oil spill has had lasting effects on the fish inhabiting the Gulf of Mexico and examining the impact to their microbiomes is an important physiological endpoint to quantify as it regulates several aspects of host homeostasis. The aim of this chapter was to determine the effects of a 7-day sublethal concentration of weathered oil on the sheepshead minnow gut microbiome as well as their foraging behavior. To our knowledge, this is the first study to examine the microbiome of an estuarine fish in response to oil. Our data suggest 1. the fish gut may be selecting microbes from the environment, resulting in a less diverse community than the water environment, 2. The fish gut microbiome may change over time as the baseline fish were sampled at an earlier timepoint than the majority of control and oil fish, and 3. that our concentration of oil (80.99 ± 12.5 µg/L) was not enough to induce overall microbial dysbiosis, yet it did identify known oil-degrading bacterial taxa that were discriminant of the oil exposure treatment through a LEfSe analysis. Specifically, the order Pseudomonadales, Paenibacillaceae family, and *Pseudomonas pachastrellae* were found, and to our knowledge, have not been previously described in fish exposed to oil. Functional metagenomics analyses are needed to verify these findings and determine if pathways related to oil degradation increased as a result of exposure. Although we predicted that oil exposure would decrease the feeding rates of *C. variegatus*, this concentration did not show any significant effects towards inducing behavioral change, and future studies are needed to elucidate any potential effects on the feeding behavior of adult sheepshead minnows exposed to weathered oil as well as their connection to the microbiome.

3.3 Future Directions and Implications

3.3.1 Future Directions

Due to the lack of apparent microbial dysbiosis or alterations in foraging behavior in the present study, there are several other research questions that could be explored further with this work as the foundational study. First, there are alternative directions that could be taken in regard to the oil exposures. Future studies could test multiple oil concentrations and use an oil with a known chemical constituency as a positive control. This could also be done over a longer time span, as previous studies that found oil-induced microbial dysbiosis exposed fish chronically over several weeks. A chronic exposure may also be more realistic to environmental conditions post-

oil spill. Because of the potential decreased bioavailability of the oil due to disturbance from air stones and/or adsorption to uneaten food, it will be imperative to examine the concentration of water-soluble components within the oil, and to directly test water samples from the exposure tanks to determine which chemicals remain after 24 hours. It may also be prudent to conduct oil exposures within sediment instead of the water column, as this may be a more environmentally relevant exposure route for these fish as they predominantly occupy the sandy or muddy bottoms of shallow estuarine water. Furthermore, other tissues such as the skin or gills could be examined for how their local microbial communities react to oil exposure as they are in more direct contact with oil in the water column. This can also be coupled with PAH body burden analyses to determine the amount of bioaccumulation in the tissues.

Although the gut microbiome of *C. variegatus* remained relatively stable even after oil exposure, our LEfSe analysis revealed that some taxa related to oil degradation were present in exposed fish and not in control. These results will be followed up with analysis of shotgun metagenomic sequencing that was performed on the same samples. In these analyses, we will explore the functional capabilities of microbes in each treatment to determine if any metabolic pathways or genes related to oil degradation increased in abundance. Beyond metagenomics, it may be interesting to explore how hypoxia, salinity, and temperature along with oil exposure affects the gut microbiome, as oil toxicity is known to be exacerbated with multiple stressors.

There are also many other avenues that can be explored with assessing behavioral shifts in fish exposed to oil. We did not see any effects of oil on the foraging behavior of fish in the present study, but future work could address this with a higher sample size, more oil concentrations, and a longer exposure duration. This study was quite exploratory in nature with making the connection between behavioral shifts and gut microbiome dysbiosis, but future studies can work to try to directly test the two. This could be in the form of using shotgun metagenomics to assess both the microbial community composition and functional capabilities of the microbiome of oil-exposed fish that also underwent foraging trials, where researchers could look for pathways and/or bacteria known for SCFA production, which is known to potentially play a substantial role in appetite regulation. SCFAs could also be directly measured in fish before and after trials. Gnotobiotic, or germ-free, fish could also potentially be used as a control.

3.3.2 Implications

Microbial ecology is a rapidly expanding field within ecotoxicology and could benefit efforts for environmental risk assessment. Our data suggest that some microbes involved in oil degradation may be present in fish exposed to oil. This could allow for the development of environmental monitoring of microbes that are bioindicators of pollution in fish. Increased knowledge regarding this physiological aspect of host health is imperative, and could have implications in the conservation, management, and culturing of certain fish species, especially when larger stressors such as overfishing, habitat loss, and climate change are factored into the stresses caused by pollutants.