THE PRESENCE OF PARTICULATE MATERIAL AND COMPARATIVE TOXICITY OF CRUDE OIL IN FINFISH FROM THE NORTHERN GULF OF MEXICO

By

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A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Environmental Science

Written under the direction of

Keith R. Cooper

And approved by

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New Brunswick, New Jersey

October 2016

ABSTRACT OF THE DISSERTATION

THE PRESENCE OF PARTICULATE MATERIAL AND COMPARATIVE TOXICITY OF CRUDE OIL IN FINFISH FROM THE NORTHERN GULF OF MEXICO

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The Deepwater Horizon oil spill (DHOS) was an unprecedented event causing the release of 4.9 million barrels of crude oil into the northern Gulf of Mexico on April 20th, 2010. This spill occurred at a depth of 1500 m approximately 66 km offshore. The unique nature of the spill caused potential exposure scenarios to major coastal regions of the Gulf of Mexico including wetland habitats, benthic reefs, the air-sea interface, and pelagic zones between the leaking wellhead and the coast. In the aftermath of large disasters such as the DHOS, the main question that is asked is, "What are the long term impacts to the natural resources and habitats affected?" One such resource, the economically and ecologically important filter-feeding species Gulf menhaden (*Brevoortia patronus*), was the primary species of concern in this dissertation. Menhaden play a key role in the transfer of energy up the food chain and serve as an important forage species for many secondary and tertiary consumers. It was hypothesized that Gulf menhaden exposed to crude oil will show lesions in the gills and heart representative of acute and chronic exposures, based on the time of collection and exposure scenario. Additional studies examined the role of particulates in

the toxicity of crude oil, as well as comparative evaluation of the sensitivity of menhaden and differences in target organ/phenotype of fish species that inhabit similar habitats. We found that there was evidence of crude oil exposure to Gulf menhaden based on gill, stomach, and heart lesions in the years following the spill as well as an increase in whole fish tissue polycyclic aromatic hydrocarbon (PAH) concentrations relative to a reference sample. Particulates with strong PAH signatures were also found in the hearts of the collected fish; however, our additional studies show that penetration of particulate material into the vasculature of selected fish is not likely due solely to particle exposures. Gulf menhaden were also shown to be more sensitive to crude oil exposures than Florida pompano. In our experiments, craniofacial neuromast cells and olfactory lamellae were severely impaired in exposed Gulf menhaden while pompano displayed a much higher prevalence of secondary gill lamellar adhesion as a result of the exposures. This dissertation highlights the importance of evaluating different species for their sensitivity to crude oil, the lesions present in fish exposed to crude oil, and also presents data on the ability of particulates to cross epithelial barriers. These findings may be relevant to both industrial particulate matter and micro plastics within the aquatic environment.

ACKNOWLEDGEMENTS

First and foremost, I must thank my advisor, Dr. Keith Cooper, for his years of support and guidance in all aspects of my graduate work and development as a professional. It is easy to say that I would not be where I am today if it were not for his insight and belief in my abilities as a scientist. He is an unparalleled advisor, and truly cares about the wellbeing of his students in addition to passing along his wisdom as an environmental toxicologist.

I am grateful for the support of collaborator and committee member, Dr. Carolyn Bentivegna, and committee members Dr. John Reinfelder and Dr. Donna Fennell, whose inquisitive questions always guided this work in the right direction.

I would like to thank all of the collaborators for making this work possible, specifically Dr. Ralph Portier, Dr. Greg Olson, Dr. Edward Chesney, Dr. Donald Baltz, Erin Saal, William Childress, and any of the other individuals involved in the field collections and sample preparation. This dissertation includes pieces to a much larger project and without the coauthors of the chapters and work done by members from Louisiana State University, LUMCON, and Seton Hall, it would not have been possible.

To the Cooper/White Lab members, Dr. Lori White, Dr. Carrie Jantzen, Dr. Tiffany Kung and Kate Annunziato, I may be the most indebted to you. Thank you for putting up with the constant whirlwind of stories/requests/favors/distractions, and always keeping me grounded when I got a little too…enthusiastic. I couldn't have done it without you. Also, a big thank you to the remainder of the game night crew, Brian Estrella and Allison Isola, for the Thursday night exercises in logical progression used to meet a common or competitive goal.

A special thanks to everyone from Lipman, especially Jessie Maguire, Peter Anderson, and Dr. Peter Kahn for the countless favors, as well as the undergraduates, Fatima Toor, Ghadeer Abuhamdeh, Kathleen Vincent, Melissa Gronske, Kat Mai, Krista Barone, Gina Moreno, Marcin Ciesla, Victoria Oriole, Keith Moffatt, and Carlos Domingues (and anyone else who was trapped in lab with the fish) for their willingness to learn and patience with my roundabout style of teaching a necropsy.

Lastly, I must thank my friends and family for their continuous and unwavering support. I especially would like to thank my parents, Eileen and Bob; through every bump and crossroads, they were always there to lend sage advice and help me stay the course.

DEDICATION

I would like to dedicate this dissertation to my Grandmother, Carol Ann Millemann and my Grandfather, Robert Nicholas Millemann. It was from spending time with them at a young age that I developed an appreciation for the beauty of nature and the world around me. These two remarkable individuals were instrumental in my earliest scientific experiences and always encouraged me to do my best in every aspect of my life. My grandparents bought me my first microscope, which is what I can attribute my original fascination with the aquatic world. I still remember taking a "sample" of the algae, only to realize when we got home that there were two little bugs swimming in the cup. I will never forget the sense of wonder and curiosity I had for those two bugs (what I now know were ostracods). For every trip to the shore, game of chess, sci-fi movie, crab we caught, sleepover, "supper", and for every ounce of love and support, I say thank you.

Table of Contents

LIST OF TABLES:

LIST OF FIGURES:

LIST OF ABBREVIATIONS:

API	American Petroleum Institute
B[a]P	Benzo [a] pyrene
B[b]F	Benzo [b] fluoranthene
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
CEWAF	Chemically enhanced water accommodated
CI	Confidence Interval
CYP1A	Cytochrome p450 1A
DCM	Dichloromethane
DHOS	Deepwater Horizon oil spill
DO	Dissolved Oxygen
dpf	days post fertilization
GCMS	Gas Chromatography-Mass Spectroscopy
GI	Grand Isle
LC50	Lethal concentration to 50 % of organisms
LUMCON	Louisiana University Marine Consortium
MC252	Mississippi Canyon Block 252 Oil
MMP-9	Mixed Metalloproteinase-9
MS222	Tricaine Methanosulfonate
NOAA	National Oceanic and Atmospheric Administration
PAH	Polycyclic Aromatic Hydrocarbon
PM	Particulate Matter
ppb	parts per billion
ppm	parts per million
psu	practical salinity units
SFS	Scanning Fluorescent Spectroscopy
TBTEX	Total Benzene, Toluene, Ethylbenzene, Xylene

TGF-β	transforming growth factor β
TPAH	Total Polycyclic Aromatic Hydrocarbon
TPH	Total Petroleum Hydrocarbons
VB	Vermilion Bay
VOC	Volatile Organic Compound
WSF	Water Soluble Fraction
YOY	Young-of-the-year

CHAPTER 1: INTRODUCTION

1.1 General Introduction

In the immediate aftermath of large disasters, resources are quickly mobilized to limit the direct impacts of the disaster on the affected area. Large oil spills have been increasingly studied over the past several decades due to their potential for toxic exposures to wildlife and humans. The primary ecological concerns after previously studied oil spills include the oiling of pelagic and benthic organisms, nearby beaches and estuaries, as well as the compromising of the air-sea interface. These impacts vary based on the type of oil, magnitude, location, and the time of year of the spill (See Table 1.1). In reviews of the previous spills such as the Ixtoc I blowout and Amoco Cadiz oil tanker, initial reports focused on the coastal intertidal regions and reported mass mortalities of invertebrates that occurred in the immediate vicinity of the oiled beaches and estuaries (Teal and Howarth 1984; Jernelöv and Lindén 1981). More recent studies on spills, including the West Falmouth, Exxon Valdez and the Arabian Gulf oil spill, have shown chronic impacts such as continued detection of oil in sediments as well as persistent reductions in biological community diversity (Peterson et al. 2003; Jones et al. 2008; Randolph et al. 1998; Reddy et al. 2002). The importance of this shift towards identifying and characterizing the possible chronic impacts of oil spills on local wildlife and natural resources years after a spill is still poorly understood.

This dissertation examines the acute and chronic impacts of the recent Deepwater Horizon oil spill (DHOS) on native fish species, as well as provides methods for testing the accumulation of particulates in fish vasculature based on our findings in collected fish samples. The following introduction provides an in-depth examination of the fate and

transport of oil released from the DHOS, as well as provides previously examined toxicity and impacts of previous oil spills. Then, crude oil toxicity is described to link the effects seen in field and laboratory studies as well as describe the acute and chronic impacts of crude oil exposure. Lastly, a review of the documented impacts of the DHOS on resident biota and laboratory studies of native and non-native species were examined to compare with the results of this dissertation. Overall this dissertation provides insight into novel toxicological responses of a relatively understudied filter feeder, Gulf menhaden, as well as comparative toxicology between Gulf menhaden and Florida pompano. The findings of these field based studies motivated us to also investigate, under laboratory controlled conditions, particulate toxicity using different hydrocarbon and plastic based surrogates for particulate matter (PM).

1.2 DHOS magnitude

The Deepwater Horizon oil well blew out on April 20th, 2010, causing the release of an estimated 779 million liters (l) (4.9 million barrels) of crude oil into the Gulf of Mexico (McNutt et al. 2012), more than doubling the annual amount of oil typically released in the entire Gulf of Mexico via natural seeps and small spills (Kvenvolden and Cooper 2003). Over the next three months, the well discharged an average of 60 thousand barrels of Mississippi Canyon Block 252 (MC252; American Petroleum Institute (API) gravity 35.2) oil per day, amounting to the largest marine oil spill in United States history. In addition to the oil, nearly 8 million l of dispersant were used to alleviate the aggregation of oil at both the surface and the wellhead (Kujawinski et al. 2011). The leak occurred nearly 80 km offshore at a depth of approximately 1500 m and the oil was composed of various types of hydrocarbons including short chain alkanes, long chain alkanes, aromatics,

and also polar hydrocarbons (Camilli et al. 2010; Reddy et al. 2012). The fate of these components has been evaluated with regards to their accumulation in surface and subsurface waters, as well as deposition on the sea floor. A unique feature of the DHOS was the formation of a large subsurface plume around 1100 m that was dominated by lower molecular weight hydrocarbons, primarily benzene, toluene, ethylbenzene, and xylenes (BTEX) (See table 1.2.) (Camilli et al. 2010; Reddy et al. 2012; Joye et al. 2011). Within the plume, a large increase in microbial activity was observed which correlated with reduced oxygen levels (Hazen et al. 2010; Valentine et al. 2010; Kessler et al. 2011). Heavier molecular weight constituents of oil either rose to the surface and formed a surface slick or were deposited on the sea floor (Reddy et al. 2012; Montagna et al. 2013). The surface slick, enriched in higher molecular weight polycyclic aromatic hydrocarbons (PAHs) such as phenanthrenes and fluoranthenes (Diercks et al. 2010), created an emulsion as it drifted towards coastal shorelines or bound to plankton (Federal Interagency Solutions 2010). The formation of marine snow was also associated with the surface slick before it sank to the bottom of the Gulf, depositing organic matter and high molecular weight PAHs to the seafloor (Passow et al. 2012). Each of these environmental fates of the Deepwater Horizon oil components has its own potential for impacting wildlife and likely resulting in both acute and chronic toxicological exposures.

The massive release of oil became an immediate national concern because of the potential economic and ecological impact on Gulf of Mexico resources. Commercial fisheries landings in the Gulf averaged approximately $662 million in revenue over the five years prior to the DHOS (National Marine Fisheries 2012). Based on the size of the spill, just over a third of that amount ($247 million) was estimated to be lost due to closures of

Gulf of Mexico fisheries in the months following the oil spill (McCrea-Strub et al. 2011). Numerous studies have attempted to characterize the effects of shore oiling on estuarine and pelagic biota after the DHOS, including the continued detection of PAH exposure in killifish and the loss of sensitive marsh habitats to physical oiling (Whitehead et al. 2012; Silliman et al. 2012; Mascarelli 2010). Other studies examined pelagic and benthic species for larval recruitment, signs of oil exposure, and survival in affected areas (Murawski et al. 2014; Coglianese 2010; Muhling et al. 2012; Rooker et al. 2013). Compared to other spills, the magnitude of the DHOS and the time of year created a great deal of uncertainty for people who earn their livelihood along the coast because of the potential impacts on both the ecological and economic resources in the region.

Clean-up efforts were rapidly mobilized in response to the spill and utilized a variety of techniques, some that have never been used before at such a large scale. These included the use of booms to capture oil, burning of oil that reached the surface, as well as the use of an unprecedented amount of dispersants to increase the bioavailability of oil to microbes at both the leaking wellhead and on the surface. At the time of the capping of the well it was estimated that 17% of the total oil released was recovered directly from the well head, 3% was skimmed from the surface, 25% evaporated or dissolved, 16% was naturally dispersed, 5% was burned at the surface, 8% was chemically dispersed, and 26% remained unaccounted for in the environment (Federal Interagency Solutions 2010). The variety of remediation techniques used during the DHOS had the intention to alleviate disastrous effects in coastal habitats and reduce the potential for toxic exposure to offshore marine organisms (Peterson et al. 2012).

1.2.1 Subsurface plume

Oil from the Deepwater Horizon wellhead was immediately impacted by a number of biotic and abiotic factors, collectively defined as weathering, which influenced the physical and chemical breakdown of the oil. Physical weathering began after release from the wellhead due to the high pressure (153 bar) and dispersants used nearly a mile beneath the ocean surface (Joye et al. 2011; Kujawinski et al. 2011). Lighter components of crude oil dissolved as they were released and led to the formation of a deep plume between 1100 and 1300 m. The combined pressure and dispersant use at the leaking wellhead induced the formation of droplets which rapidly dissipated into the water column increasing the dissolution of smaller hydrocarbons (Camilli et al. 2010; Hazen et al. 2010; Atlas and Hazen 2011). Although crude oil is considered hydrophobic, many hydrocarbons are slightly soluble based on the number of carbons in the molecule as well as their arrangement and aromaticity (See Table 1.3). Gaseous alkanes (C1-C5) were quickly dissolved at the wellhead or formed hydrates that rose to the surface and dissolved slowly as the temperature increased and pressure decreased higher in the water column (Reddy et al. 2012). Gaseous hydrates that had formed have not been well studied but likely dissolved slowly as they rose to the surface, distributing hydrocarbons throughout the water column (Joye et al. 2011; Kessler et al. 2011). Significant releases of methane at the wellhead also played a role in the transport of heavier oil constituents throughout the water column. Dense PAH's likely adhered to methane bubbles and hydrates, leading to increased distribution in the water column. Volatile monoaromatic hydrocarbons, including BTEX, were effectively dispersed at the wellhead and found to be the primary constituents of the deep plume at concentrations exceeding 50 µg/l in some samples (Camilli et al. 2010) (See

Table 1.2). Subsurface fluorescence of dissolved organic matter was found to correlate with elevated levels of naphthalenes at sites near the well head, indicating some dissolution of smaller PAHs (Diercks et al. 2010). Samples taken in mid-May 2010 had total PAH (TPAH) concentrations of up to 189 µg/l (97.1% of which were naphthalene and its congeners) at a depth of 1320 m not far from the wellhead (Diercks et al. 2010). The combination of dissolved and dispersed short alkanes and monoaromatic hydrocarbons made up the majority of the deep plume which spread in the west-southwest direction from the wellhead source (Camilli et al., 2010). Trace elevations of hydrocarbons were detected as far as 35 km from the wellhead approximately 2 months after the initial blowout (Camilli et al., 2010).

One significant benefit of the dispersion of the crude oil was the increase in surface area of oil and the resultant biodegradation of hydrocarbons by microorganisms (Atlas and Hazen, 2011; Prince and Butler, 2013; Prince et al., 2013). This biotic form of weathering breaks down crude oil into basic hydrocarbon components and ultimately CO_2. Numerous species of fungi and bacteria have developed mechanisms metabolize hydrocarbons for energy and growth (Das and Chandran 2010; Atlas and Bartha 1992). The Gulf of Mexico has a robust microbial community that has evolved to take advantage of crude oil for metabolism (Atlas and Hazen, 2011; Joye et al., 2011). In the months following the DHOS, evidence of biodegradation was seen in a number of studies based on increased microbe densities, shifts in dissolved oxygen (DO) concentrations, and decreased contaminant concentrations. Within the deep plume, overall cell density increased from 2.73×10^4 cells/ml to 5.51×10^4 cells/ml and was dominated by *Oceanospiralles*, an order of γ-Proteobacteria known to breakdown and degrade crude oil components (Hazen et al.,

2010). Oxygen levels associated with this increase in cell density averaged 59% of saturation, compared to 67% outside the plume, indicating slow rates of biodegradation not limited by DO (Hazen et al., 2010). The ratio of 26-carbon alkanes to 15-carbon alkanes increased substantially with distance from the wellhead, suggesting that there was preferential biodegradation of linear hydrocarbons (Hazen et al., 2010). Another study also reported evidence of biodegradation in the deep plume based on a fairly consistent decrease from DO levels within the plume (5.98 mg/l) compared to the mean values for the region under those conditions (6.11 mg/l) (Camilli et al., 2010). Albeit a seemingly minor difference, the oxygen drawdown noted in these studies indicates the slow degradation of hydrocarbons by microbes, which is consistent with the 2-fold increase in bacterial cell density in the plume.

During the DHOS, a large portion of gaseous oil components such as methane, ethane, and propane were released in addition to large alkanes, aromatics, and polar compounds (Joye et al., 2011; Kessler et al., 2011; Valentine et al., 2010). An estimated gas to oil ratio (defined as cubic feet of gas/barrel of oil released at standard temperature and pressure) of 1,600 was calculated by Reddy et al. (2012), which is equivalent to 1.7 x 10^{11} g of C1-C5 hydrocarbons being released. Valentine et al. (2011) determined a gas to oil ratio of 3,000 while Joye et al. (2011) had an estimate nearly four times greater than Kessler et al. (2011), indicating variability in estimates of the release of natural gas during the DHOS. Regardless, the amount of gaseous hydrocarbons released was substantial and dissolved in the water column either immediately at the well head or at shallower depths due to temporary hydrate sequestration (Joye et al., 2011, Kessler et al., 2011).

Degradation of dissolved hydrocarbons by methanotrophs and other bacteria can cause significant decreases in DO concentrations in marine environments (Peterson et al., 2012, Du and Kessler, 2012). The release of these compounds during the DHOS stimulated methanotrophs and other bacteria capable of rapidly depleting oxygen levels as they metabolized the newly abundant carbon source (Joye et al., 2011; Kessler et al., 2011; Valentine et al., 2010). Based on estimates of the amount of natural gas released during the DHOS, the total oxygen demand for its biodegradation was estimated to be between 9.6×10^{11} g and 1.24×10^{12} g (Kessler et al., 2011; Valentine et al., 2010). The disappearance of methane and abundant presence of methanotrophs by mid-August in the deep plume indicated a relatively rapid consumption of gaseous hydrocarbons (Kessler et al., 2011) and potentially dangerous oxygen depletion. DO concentrations below 2.0 mg/l are considered dangerously hypoxic and areas of the Gulf of Mexico are already susceptible to substantial oxygen depletion from nutrient overloads from the Mississippi River and other smaller inputs (Rabalais and Turner 2006). None of the aforementioned reports monitoring DO levels indicated oxygen depletion to the 2.0 mg/l threshold.

Depletion of DO concentrations was initially a concern during the DHOS because of the potential impact on species of ecological or economic importance in the Gulf of Mexico (Mascarelli 2010). The magnitude of the oil spilled and the resulting evidence of biodegradation, especially of gaseous components, indicated a massive demand for oxygen. Monitoring of DO determined that dangerous environmental thresholds were not approached, likely due to sufficient mixing of water at the depths of concern. This does not eliminate the possibility of smaller, unmonitored regions developing hypoxic zones but it seems that initial concern of large-scale drops in DO concentrations associated with the

deep plume were avoided. Deep regions with low DO concentrations were a cause for concern because biotic responses such as fish kills would have been extremely difficult to observe or quantify.

The use of dispersants at the leaking wellhead increased the efficiency of biodegradation in the deep plume (Du and Kessler 2012) but also had the potential to increase marine organisms' exposure to toxic hydrocarbon compounds and oil-dispersant mixtures. Nearly 8 million l of COREXIT 9500A and COREXIT 9527 were used as surfactants during the DHOS to prevent oil from reaching coastal areas (Kujawinski et al. 2011). Toxicity studies found COREXIT 9500A to be mildly toxic to mysid shrimp, with 42 µl/l being the calculated lethal concentration to 50% of organisms (LC50). Inland silverside minnows had a calculated LC50 of 130 µl/l (Hemmer, Barron, and Greene 2011). The overall toxicity of COREXIT 9500A was comparable to alternative dispersants. COREXIT 9500A was also found to be toxic to coral larvae, causing mortality of 87% of animals exposed to 50 µl/l of the dispersant (Goodbody-Gringley et al. 2013). Dispersants enhance the dissolution of hydrocarbons and thus increase the bioavailability for all types of organisms in the Gulf, not just microbes.

The actual effects of the subsurface plume on pelagic organisms during the DHOS have not been reported, if there were any major effects at all. Biodegradation and dispersion were shown to have reduced the concentrations of low molecular weight hydrocarbons in the deep plume and based on measurements of DO concentrations the DO would not appear to have been a major concern in the water column. Previous measurements of low molecular weight hydrocarbons at concentrations above known toxic effect levels could have impacted larval and other life stages of fish and invertebrate species inhabiting the

deeper pelagic zones. Other fates of DHOS oil, including areas surface as well as the benthos, will have toxicological questions and organisms affected by the potential exposure.

1.2.2 Surface plume

It was reported that approximately 50% of the crude oil released during the DHOS reached the surface and formed a large surface oil slick, ultimately affecting a total of just over 176,000 square km of ocean surface between April 25th and July 16th 2010 (Federal Interagency Solutions 2010; Ryerson et al. 2012; Norse and Amos 2010). The surface slick varied from thin oil sheens (~0.1 µm) to thick mats of oil (>100 µm) and also included emulsions that reached deeper into the subsurface (Federal Interagency Solutions 2010; Leifer et al. 2012). Elevated concentrations of hydrocarbons originating from surface slicks were seen at depths up to 25 m below the surface (See Table 1.2). As the spill progressed, the combination of weathering and remediation efforts affected the composition of surface oil by removing lighter hydrocarbons and oxidizing heavier ones (Aeppli et al. 2012). Volatile organic compounds (VOCs; including BTEX and C1-C5 alkanes) were mostly dissolved in the water column but the small fraction that reached the surface likely evaporated within the first two-three hours (Ryerson et al. 2012). Atmospheric samples from research flights on June 8th and 10th 2010 showed increases in VOC as well as secondary organic aerosols immediately downwind of the surface plume (De Gouw et al. 2011; Ryerson et al. 2012). Oil samples taken from the shoreline and near shore areas had no detectable levels of BTEX present also indicating they volatilized or degraded before they ever reached shore (Atlas and Hazen 2011).

Medium sized PAHs, such as napthalenes, were found in atmospheric samples (Atlas and Hazen 2011) as well as subsurface (Diercks et al. 2010) and early surface samples from the Gulf of Mexico (Aeppli et al. 2012), which is not surprising due to napthalene's higher solubility and vapor pressure compared to other PAHs. Surface samples of oil taken during the spill had a higher presence of PAHs such fluorenes, phenanthrene, and anthracene (Spier et al. 2013; Aeppli et al. 2012), indicating a markedly different composition of oil constituents in the surface slick compared to the deep plume. The significant weathering and photo oxidation affected the composition of the surface slick by increasing the relative concentrations of heavier hydrocarbons and oxidized carbons (Aeppli et al. 2012). Surface slicks had relatively constant ratios of n-C18/phytane (2.5-3) throughout the study indicating very low loss of larger hydrocarbons. Surface samples from shorelines showed larger relative concentrations of chrysene and its congeners, while naphthalene congeners were completely removed by the time the oil reached shore (Aeppli et al. 2012). The enrichment of heavier crude oil components such as chrysene at the surface promoted the formation and stabilization of emulsions in the open ocean and decreased the efficiency of dispersants (Bejarano, Levine, and Mearns 2013). Emulsions of oil on the surface of the gulf were observed by researchers in the field and could penetrate deeper below the surface, likely impacting species of birds, fish, and mammals' dependent on the upper water column for feeding or reproductive purposes.

1.2.3 Remedial efforts and possible increased toxicity

Remedial efforts including the burning and skimming of surface oil were used to reduce the amount of oil potentially moving towards shore. According to the Federal Interagency Services Group (2010), 5% of the total oil released evaporated from the surface

and an additional 3% was skimmed and 5% burned by cleanup crews. Recovery of surface oil was accomplished using skimmers, but to date no data has been released defining the amount of oil or oil/water mixture that was collected. A total of 410 controlled burns took place after the spill resulting in the combustion of an estimated 18-23 million l of surface oil (Schaum et al. 2010). The combustion of crude oil is known to release toxic compounds into the atmosphere, including carcinogenic PAHs such as B[a]P (Peterson et al. 2012; Schaum et al. 2010). These compounds, as well as PM like black carbon, can be deposited back to the seawater surface of the Gulf by atmospheric deposition. Estimates determined that between 10% and 20% of the burned oil remained as burn residue in the environment, increasing risk for workers and populations downwind of the burn (Peterson et al. 2012; Schaum et al. 2010). Atmospheric samples taken on monitoring flights over the deliberate surface burns during the DHOS showed elevated levels of black carbon aerosol, carbon monoxide, and carbon dioxide (Perring et al. 2011). The black carbon reported in Perring et al. (2011) was "remarkably larger" than black carbon aerosols from previous burning studies, likely contributing to its reduced atmospheric effects but also to an increased deposition rate back in the Gulf. This data was based on a relatively small sample size, thus a more thorough analysis of the magnitude, composition, and fate of the burned oil residues would benefit the understanding of marine organisms' potential exposure to pyrogenic PAHs formed following large combustion events.

The chemical dispersion of offshore oil spills has been an effective remediation technique because of its effectiveness in increasing biodegradation and natural weathering of crude oil (Prince and Butler 2013; Bejarano, Levine, and Mearns 2013). The dispersants COREXIT 9500A and COREXIT 9527 were both applied to the surface slicks in the Gulf

of Mexico, resulting in increased penetration of hydrocarbons into the water column (BenKinney et al. 2011; Bejarano, Levine, and Mearns 2013) in addition to decreased surface visibility of oil. More oil is effectively dispersed when there is more wave energy to facilitate vesicle/micelle formation. Crude oil constituents were detected nearly 10 m below the surface. The dense emulsions observed during recovery efforts were fairly resistant to dispersant applications because of the small droplet size and the heavy nature of the remaining oil. A number of studies have examined dispersant use and they generally demonstrated an increase in toxicity of dispersed oil to organisms such as mesozooplankton (Almeda et al. 2013), fish (Milinkovitch et al. 2013; Hemmer, Barron, and Greene 2011), and corals (Goodbody-Gringley et al. 2013) when compared to oil or dispersant alone. This result is not unexpected since the dispersed oils release higher concentrations of oil derived hydrocarbons into a larger volume within the water column.

Remedial efforts used during the DHOS have the short term goal to eliminate visible portions of oil, but these methods may increase toxic exposures. Pyrogenic PAHs, such as B[*a*]P, are released upon the burning of crude oil and were likely deposited back into the Gulf. Dispersants significantly increase biodegradation potential (Prince et al. 2013) but also significantly increase the volume of water that becomes contaminated (BenKinney et al. 2011). Risk assessments evaluating the potential increases in exposure to species dependent on the upper pelagic zones (including crustaceans, finfish, mollusks, and squid) will be beneficial to continue the development of our understanding on the ecological impacts of dispersant use.

1.2.4 Marine snow

Marine snow is defined as an aggregation of small PM greater than 0.5 mm diameter (Alldredge and Silver 1988). A large marine snow accumulation occurred during the DHOS and was believed to be responsible for the fate of a portion of the surface oil slick (Federal Interagency Solutions 2010). The marine snow formation was hypothesized to be initiated by bacterial degradation of surface oil creating mucous webs that eventually collapsed on themselves and settled to the sea bottom (Passow et al. 2012). Field samples of the marine snow from the DHOS were found to have a settling velocity between 68 and 553 m/d, slightly faster on average than marine snow observed elsewhere (Passow et al. 2012). As these particles settle, they would scavenge plankton and other suspended organic matter (Passow et al. 2012). The sinking marine snow is a biologically active aggregation of living organisms as well as detrital matter (Alldredge and Silver 1988), and is thus a potential food source for a variety of organisms. Many organisms in the Gulf rely on planktonic matter as food, including zooplankton and menhaden. The associations of marine snow with crude oil likely contributed to the observed bioaccumulation of hydrocarbons in the food chain and likely contributed to exposures in pelagic species of fish (Graham et al. 2010). The particulate nature of marine snow would also have increased the likelihood of direct contact with organisms using gill respiration, causing another type of exposure scenario that could be further investigated.

Marine snow formations had disappeared by June and most had settled to the benthic regions of the Gulf as the surface slick was transported towards shore. During the six months after the spill, sediment accumulation was four times greater in areas associated with marine snow and surface slicks (Daly et al. 2016). One of the less understood aspects

of the DHOS is the impacts of this accumulation of oil and marine snow at the seafloor. Organisms living at the bottom of the Gulf of Mexico are responsible for a number of ecologically important functions including organic matter decomposition, organic matter transfer to higher trophic levels, and the recycling of nutrients (Danovaro et al. 2008). The productivity and efficiency of these functions increases substantially with higher levels of biodiversity in the benthic community (Danovaro et al. 2008). Studies examining benthic communities and sediments affected by the DHOS have found increases in nematode presence and decreases in micro and macrofaunal diversity which may be associated with increased rates of sedimentation or toxic exposure (Montagna et al. 2013). The composition of deep sea benthic communities indicates very slow recruitment (Grassle 1977) and thus a potentially slow recovery from any significant diversity loss, such as was seen in the DHOS. This loss of benthic diversity could deplete the valuable nutrient cycling abilities of benthic communities.

Other similar benthic habitats have been significantly affected by the increased deposition of marine snow and hydrocarbons to the sea floor. Coral colonies approximately 11 km southwest of the spill (in the vicinity of the deep plume) showed significant signs of stress compared to a previous survey and was associated with elevated, naphthalene, phenanthrene, dibenzothiophene, benzo[*a*]anthracene, and chrysene concentrations in brown flocculent material on the surface of affected corals (White et al. 2012). Other coral sites that were not affected showed no differences from the previous survey. Closer to shore, many important fish species such as tilefish, grouper, and snapper are associated with deep (100-400 m) lime scale burrows and reef systems that can be severely disrupted by excess sedimentation and accumulation of heavy PAHs that may fall out of the surface

slick. Marine snow and heavy or weathered oil components that reached the sea floor have disrupted sensitive habitats and have not shown evidence of being biodegradable (Reddy et al. 2012).

Previous studies on oil degradation have determined a half-life of lightly weathered Alaska North Slope crude oil to be 13.8 days at 8 ºC in aerated seawater (Prince et al. 2013) which is comparable to the conditions at the bottom of the Gulf (~5 ºC). The half-life of light alkanes was estimated to be between 1.2 and 6.1 days in the deep plume of the DHOS (Hazen et al. 2010) and light alkanes were essentially not present in the oil/marine snow deposits at the bottom of the Gulf due to rapid degradation. The oil and marine snow that reached the bottom of the Gulf consists of heavily weathered denser components of crude oil and is likely buried under multiple layers of detrital material. The anoxic sediments created will not degrade crude oil components as efficiently as the aerated experiments and other field observations. Studies conducted after the Exxon Valdez oil spill in Prince William Sound indicated the persistence of crude oil components for decades in anoxic sediments along the coast (Peterson et al. 2003) and the same is likely true for benthic deposits of crude oil components. Some higher molecular weight PAHs and crude oil constituents may not degrade at all in the dark, cold, anoxic sediments at the bottom of the Gulf. The persistence of these recalcitrant crude oil components can cause chronic exposures in benthic and demersal species, shifting community compositions and altering valuable ecosystem roles such as bioturbation and oxygenation of benthic sediments. Additionally, the increased sediment deposition on substrates ideal for settlement of bivalves and other benthic organisms such as corals could result in substantial depletion of habitat recolonization on the seafloor.

1.3 Crude oil toxicity

The toxicity of crude oil is based on a variety of factors such as the duration of exposure and type of constituents present in the oil. Crude oils are complex mixtures of a variety of hydrocarbons, short chain alkanes, alkenes, BTEX, and low and higher molecular weight aromatic and nonaromatic hydrocarbons. A number of constituents in crude oil included benzene, PAHs, and remediation by products are known or probable human carcinogens (See Table 1.3); benzene, is a known human carcinogen. Benzo[*a*] pyrene (B[*a*]P) is a classically studied human carcinogenic PAH that has been well characterized for its association with lung and epithelial tumor formation in rats and mice after laboratory exposures (NTP 2011). The toxicity of environmental exposures to crude oil will vary depending on the relative concentrations and bioavailability of the more acute or chronic toxic constituents, like B[*a*]P. For example, some organisms (such as mummichogs and bivalves) are more closely associated with sediments (whether for feeding or shelter), where accumulation of heavier crude oil constituents tends to be greater relative to the water column (Whitehead et al. 2012). Different species also have different sensitivities to crude oil constituents and may be affected in different ways. When assessing toxicological data, the concentrations of specific toxic oil constituents and the duration of exposure are most important to determine the potential acute and chronic toxic responses.

In the aftermath of an oil spill, one of the first major concerns is the large accumulation of oil at the sea surface or in sediments which can cause acute toxicity and mortality to biota. Many different organisms will perish as a result of the coating of skin and feathers or accidental ingestion of significant volumes of crude oil. Acute exposure to volatile components (BTEX) of crude oil can cause disorientation, making affected

organisms more susceptible to predation or drowning in marine environments (Peterson et al. 2003). In coastal areas, salt marshes can become coated in oil causing the die-off of *Spartina* spp. leading to significant erosion and loss of sensitive marsh habitat (Silliman et al. 2012). Not all marine organisms, however, experience the significant physical oiling seen immediately following an oil spill but are exposed to lower concentrations of oil constituents over extended periods of time.

Exposure to crude oil and crude oil constituents, most notably PAHs, results in a variety of toxicological endpoints (See Table 1.4). Biochemically, induction of cytochrome P450 expression (specifically CYP1A) is an established biological marker to a number of xenobiotics and can be used to characterize PAH exposure in both laboratory and field studies (Spies et al. 1996). As was previously mentioned, B[*a*]P is a well-studied carcinogenic PAH. Metabolism of B[*a*]P begins with its conversion to one of two epoxides, B[*a*]P 4,5-oxide or B[*a*]P 7,8-oxide by CYP1A (Klaassen and Watkins III 2015). Epoxide hydrolase converts these epoxides to their respective dihydrodiols, at which point these compounds are eliminated. B[*a*]P 7,8-dihydrodiol, however, can go through another round of CYP1A metabolism and create B[*a*]P 7,8-dihydrodiol-9,10-epoxide. This final form of B[*a*]P is resistant to hydroxylation, known to cause DNA adducts, and has been associated with liver lesions and neoplasms (Szeliga and Dipple 1998). Other PAHs and their metabolites have a variety of potential mechanisms and are continuously being studied for their roles in lesion development and carcinogenesis.

A number of PAHs are known carcinogens in fish, but most laboratory exposure studies have not been carried out long enough to detect neoplasms. Field studies from sites that are chronically polluted with PAHs, however, have shown substantial increases in

hepatic tumor prevalence. Over 90% of mummichogs collected from a site in the Elizabeth River in Virginia, a river with consistently elevated creosote levels, had gross liver neoplasms (Vogelbein et al. 1990). One third of these lesions were determined to be hepatocellular carcinomas. Rose et al. (2000) found a significant prevalence of PAH derived DNA adducts in mummichogs collected from the same area, likely resulting in the observed increase in hepatic neoplasms (Rose et al. 2000). In wild brown bullhead catfish (age 3-4) from the Black River in Ohio, hepatic tumors were present in 40% of the population which decreased to 10% after 5 years (Baumann and Harshbarger 1995). This decrease in hepatic tumors was correlated to a 99% decrease in sediment PAH concentrations over the same time frame due the closing of a nearby coking plant. These results suggest a strong causality between chronic, multi-year PAH exposures and hepatic tumor formation.

Laboratory studies on fish exposed to crude oil have not typically been carried out long enough to detect neoplasms, but they show a wide range of developmental and physiological responses (See Table 1.4). Adult fish show typical toxicity to crude oil exposures when concentrations are above 50 µg/l (Khan and Kiceniuk 1984). Atlantic cod showed delayed gonadal development after exposure to the water accommodated fractions of crude oil between 10 and 50 µg/l, which may affect overall fecundity (Khan 2013). Histological observations in fish exposed to crude oil include gill hyperplasia, chronic inflammatory responses, and elevated levels of macrophage centers in liver and kidney sections ((Agamy 2013, 2012a, 2012b; Khan 1995; Khan and Kiceniuk 1984). Liver lesions such as sinusoid dilation, nuclear degeneration, and necrosis significantly increased in experimental rabbitfish exposed to Arabian light crude oil when compared to controls

(Agamy, 2012a,b). Fibrosis in heart tissue was also present in a few adult Atlantic cod exposed to crude oil (Khan and Kiceniuk, 1984). The prevalence of these lesions in adult fish could impact fitness and likely decrease reproductive success because of shifts in the allocation of metabolic resources. More energy will be directed towards inflammatory responses and repair mechanisms instead of reproduction.

Significantly lower concentrations of crude oil have shown to influence an array of developmental lesions in the embryonic stages of fish. Exposures of weathered crude oil to herring and pink salmon embryos resulted in decreased hatch rates, smaller size, and poor survivability in the wild compared to controls (Short et al., 2003). Ingvarsdottir et al. (2012) reported significantly increased mortality and craniofacial asymmetry in larval Atlantic herring exposed to 15 µg/l of crude oil that may affect feeding. Exposure to PAH concentrations as low as 1 µg/l have induced lesions such as yolk sac edema and premature hatching in rainbow trout and pacific herring embryos (Carls et al., 1999; Heintz et al., 1999). In de Soysa et al. (2012) it was hypothesized that the developmental lesions seen in larval fish, specifically craniofacial and heart malformations, could be a result of a defect in cranial neural crest development as a result of crude oil exposure. Neural crest cells differentiate during development into a variety of cells, including portions of head cartilage and heart which are spatially associated and may be a target for developmental PAH exposure.

Heart abnormalities during development were common in zebrafish larvae exposed to crude oil PAHs and have recently been studied as a major endpoint of crude oil exposure in fish species because of the discovery of developmental sensitivity to low levels (1 µg/l) of PAHs compared to adult fish (Incardona et al., 2010). Developmental lesions including

pericardial edema, looping defects, and elongated or rounded ventricles have been observed in embryonic fish exposed to crude oil, suggesting that the heart is heavily influenced by PAH exposure during development (Incardona et al., 2004; Zhang et al., 2012). Exposure of developing zebrafish to phenanthrene induced significant looping defects in the heart and increased interstitial fibrosis that coincided with upregulation of matrix metalloproteinase-9 (MMP-9) and transforming growth factor β (TGF-β) (Zhang et al., 2013). Exposures of embryonic zebrafish to low levels of weathered PAHs have shown decreases in heart efficiency and slower swimming ability after reaching adulthood (Hicken et al., 2011), reducing the fitness of these fish if they were in the wild. Deformities and decreased physiological efficiency of the heart can reduce the fitness of individuals and will likely lead to increased predation and mortality of affected individuals in a natural environment.

In summary, a variety of histological lesions in the gills, gonads, liver, and heart have been observed following exposure to crude oil and its constituents. Damage to gill filaments in fish would decrease osmotic regulation in addition to the loss of respiratory capacity. Reductions in respiratory efficiency will result in altered circulating DO and CO_2 concentrations, which will likely result in altered systemic pH. The loss of osmoregulation could affect membrane potentials resulting in irregular nerve cell conductance and heart contractions. Developmental lesions to the heart have shown to decrease cardiac output (Milinkovitch et al., 2012; Hicken et al., 2010) likely resulting in decreased oxygenation of other tissues. The increase in CYP1A induction would indicate that PAHs are reaching the liver to result in increased presence of PAH metabolites, and thus more prominent damage to the liver and DNA adduct formation. Increased lesions in fish will ultimately

result in the diversion of metabolic resources from feeding and spawning to the maintenance and repair of affected tissues.

1.4 Documented Ecological Effects of the DHOS

The effects of exposure to crude oil from the Deepwater Horizon have been studied by multiple groups in the Gulf of Mexico ecosystem. Field and laboratory studies, reports, and formal observations make up much of what is known about the impacts of this unprecedented release of crude oil in the Gulf. One of the earliest quantifiable effects of the DHOS was the significant loss of valuable wetland habitat. Oil residues that reached the shoreline were divided into three basic types: submerged oil mats in the subtidal zones, small surface residual balls left behind after mechanical removal of oil from beach sands, and supratidal buried oil which was buried beneath sand or organic matter (OSAT Final Report, 2011). As was seen in studies of the surface oil, much of the oil that made it to shore had been significantly weathered, leaving only heavy oil constituents that are persistent and not easily degraded. Despite remedial efforts, a total of nearly 1,773 kilometers of shoreline were oiled in the months following the oil spill (Michel et al., 2013). At least 212 linear kilometers of Gulf coastline were significantly oiled which has led to rapid die offs of marsh grasses which are key to maintaining shoreline structure and preventing erosion (Mascarelli 2010). More than 75 of those kilometers were in Louisiana and caused the erosion of Louisiana marshes to increase by 125% compared to reference locations (Silliman et al., 2012). Other areas, specifically islands that were oiled as a result of the spill, saw up to a 275% increase in erosion rates, most likely due to the loss of marsh grasses and vegetation (Turner, McClenachan, and Tweel 2016). These rates have since dropped oil from both the Deepwater Horizon and other unknown sources persists in marsh

sediments, with the potential to cause long term exposures to resident species during storms or via bioturbation processes (Kırman et al. 2016).

Early analysis of field observations immediately after the spill included the presence of visible oil in crab larvae connective tissue, increased cetacean carcass recovery, and elevated activation of the AhR pathway in resident marsh fish (Mascarelli, 2010; Williams et al., 2011; Whitehead et al., 2011; Restoring a Degraded Gulf of Mexico, 2013). Oil based hydrocarbons linked to the DHOS were shown to accumulate in the food chain via plankton (Graham et al., 2010), and likely made it into higher trophic level consumers based on reports of fish with increased PAHs in both estuarine and pelagic habitats. Gulf killifish were one of the first fish species to be studied from oiled estuaries in the Gulf (Whitehead et al. 2012; Dubansky et al. 2013) and were shown to have increased activity of the AhR signaling pathway compared to fish collected from unoiled sites, indicating exposure to PAHs from the spill. Studies of the remaining weathered oil in sediments and dissolved in the water showed significant elevation in levels of PAHs when compared to reference sites (Whitehead et al., 2011; Silliman et al., 2012). Continuous release of heavy PAHs from subsurface oil residue may lead to a chronic, low dose exposure to species dependent on estuaries for spawning and nursery habitats, however the current data suggest that these coastal communities have either adapted to oil spills or are able to recover quickly (Roth and Baltz 2009).

In addition to these initial observations, over 10% of the area of the Gulf of Mexico was closed to fishing due to the oil spill, causing an estimated loss of $247 million in the Gulf fishing industry with Louisiana being hit the hardest (McCrea-Strub et al., 2011). A number of commercially important fish found in the Gulf of Mexico depend on epipelagic

zones for feeding and larval transport. In many of these species, overlaps in surface water affected by oil and areas associated with pelagic spawning of fish could have impacted larval recruitment and survivorship. Blackfin tuna, blue marlin, mahi-mahi, and sailfish larval densities decreased in the Gulf after the DHOS in comparison to the previous three years (Rooker et al. 2013). Bluefin tuna larvae may also have been affected based on samples taken during the DHOS showing overlap between favorable spawning areas in the Gulf and oil affected waters (Muhling et al. 2012). Southwest shifts of blue marlin population densities in the Gulf of Mexico during the summer of 2010 relative to 2009 indicated a preference for non-oiled coastal waters (Rooker et al. 2013). Despite these data, it was found that abundance of many fish species returned to normal in the years following the spill and the predicted community effects on fish assemblages did not occur (Schaefer, Frazier, and Barr 2016). Other species of fish were found to likely be impacted by the spill, specifically red snapper collected in 2011 and 2012, which were determined to have higher concentrations of PAHs in bile the first year of sampling (Murawski et al. 2014). In many cases, increased PAH concentrations were noted, but these concentrations were significantly below seafood safety concerns (Ylitalo et al. 2012).

Laboratory exposures aimed at assessing the impacts of the DHOS on non-traditionally studied commercial fish have determined a suite of impacts, most notably in the hearts of developing embryos and the swim performance of exposed individuals (Incardona et al. 2014; Stieglitz et al. 2016; Mager et al. 2014). Adult and juvenile mahi-mahi exposed to Deepwater Horizon oil had impaired swim performance, and were expected to have a more difficult time capturing prey and escaping predators as a result of the exposure (Stieglitz et al. 2016; Mager et al. 2014). The results of these studies will add

important information to the specific impacts that DHOS oil and its constituents have on a wider range of relevant organisms.

Concentrations of benzene above the aquatic toxicity reference value (defined as >1 µg/l) were found both within and outside of the deep plumes over the course of the spill (Spier et al. 2013) indicating that chemical exposures from the deep plume could have impacted species over a wider range of the water column than initially reported. Commercial fish species such as yellowfin tuna (*Thunnus albacares*) have been tracked to a depth of 432m in the Gulf of Mexico (Weng et al. 2009) but in other parts of the world yellowfin dives have been recorded as deep as 1173m (Schaefer, Fuller, and Block 2007). An abundance of species resides at great depths in the Gulf, many of which may have been affected by this deep plume. These effects were not quantified but any long term impacts of this type of exposure remains to be seen, if they did occur. Other species, such as Gulf menhaden, tend to remain closer to shore and do not have the vast migratory capability of the aforementioned larger pelagic species. Gulf menhaden are known to spawn approximately 80 km offshore between October and March and then return to protected bays and estuaries to feed during summer months (Lewis and Roithmayr 1980). Planktonic larvae drift inshore while they develop and depend exclusively on estuaries for protective habitat, food, and growth during their juvenile stage (Ahrenholz 1991). Estuaries along the coast of Louisiana are particularly productive menhaden habitats (McCrea-Strub et al. 2011) and were severely oiled during the DHOS creating a specific need to study this species and other species dependent on these sensitive habitats. Environmental risk assessments need to examine the species present throughout the water column and their potential exposures based on migration and known concentrations of xenobiotics. Risk

assessments for different times of the year also need to be incorporated to include the variations in life-cycles of different species.

There is overlap between the area affected by the DHOS and numerous other possible sources of xenobiotics that could distort the full picture of toxicity from this single large scale event such as the DHOS. Nearly 200 million l of crude oil are released into the Gulf of Mexico annually via natural seeps, atmospheric deposition, and small scale losses from commercial or recreational activities (Kvenvolden and Cooper 2003). Black carbon from atmospheric or riverine deposition may also distort data pertaining to the DHOS (Mitra et al. 2002). As time goes on, decreases in the large acute effects from the DHOS may start to blend with the elevated background lesions likely found in marine species inhabiting the northern Gulf of Mexico. The Gulf of Mexico is host to a variety of possible contaminants that may all influence the interpretation of results from the area affected by the DHOS.

1.5 Chronic Implications

Perhaps the most understudied and complicated aspect of oil spill ecology is the potential for dynamic shifts in ecosystem functionality. Impacts on keystone species or vulnerable habitats may drastically change the productivity and diversity found in their respective environments. For example, sea otters in Alaska play a vital role in maintaining trophic interactions between sea urchins and kelp and thus ecosystem stability (Peterson et al. 2003). Sea otters suffered mass mortality as a result of the Exxon Valdez oil spill due to direct oiling, and were further stressed by predation from killer whales. The decrease in sea otter numbers could have caused a significant increase in sea urchin population, causing a catastrophic decrease in kelp productivity and thus habitat. In this ecosystem, there is a

delicate balance between sea otters, sea urchins and kelp forests that maintain a thriving productive ecosystem in Prince William Sound.

1.6 Rationale and Hypothesis of the Dissertation

In the Gulf of Mexico, one could argue that Gulf menhaden may play a similar role in ecosystem function. Menhaden feed on plankton and are a key prey species for tuna, dolphins, and other higher trophic level consumers. Without substantial populations of menhaden, much of that trophic transfer of energy could be lost. Any shifts in populations greatly affect the fishery ecology and economics of the area. These population shifts, should they occur, can be explained by organismal responses to contamination.

This dissertation examines the possible impacts of DHOS at an organismal level and provides insight into species comparisons of the acute and chronic toxicity of crude oil. The overall goal of this dissertation was to evaluate the response of Gulf menhaden exposed to crude oil by determining the specific lesion types present and relative sensitivity to crude oil. It was hypothesized that fish species exposed to constituents of crude oil spills will show lesions representative of acute and chronic oil exposures, based on the time of collection and exposure scenario. Chapter 2 describes testing of this hypothesis using menhaden collected from the northern Gulf of Mexico from sites oiled after the DHOS as well as from reference sites in the region. Menhaden collected from Louisiana waters in 2011 and 2012, one and two years following the DHOS, showed varying severities of gill lesions as well as an unusual accumulation of black particulates visible at necropsy in the heart and stomach vasculature. Laboratory exposures described in Chapter 3 are based on a study conducted in coordination with the Louisiana University Marine Consortium (LUMCON) in which juvenile Gulf menhaden and Florida pompano were exposed to a

crude oil dispersant mixture to determine species specific responses. Gulf menhaden were determined to be much more sensitive, and displayed hemorrhage and necrosis of the olfactory lamellae and lateral line neuromast. Pompano had a higher prevalence of secondary lamellar adhesion and gill epithelial hyperplasia, lesions more typically associated with acute exposures in fish species. Chapter 4 describes an investigation of the accumulation of particles in zebrafish and mummichog motivated by our observation of particulate material in the gills, stomach, and heart vasculature of field collected fish. Particulate studies in these fish have further application in the impacts of urban PM, diesel soot, and microplastic pollution and their potential accumulation in fish tissue.

Hypothesis of the dissertation: Fish species exposed to constituents of crude oil spills will show lesions representative of acute and chronic oil exposures, based on the time of collection and exposure scenario.

Chapter 2 Aim: Evaluate the histopathological condition of Gulf menhaden from the northern Gulf of Mexico in the years following the DHOS. Hypothesis: Histological evaluation of wild Gulf menhaden collected from waters overlapping with areas affected by the DHOS would reveal a spectrum of reversible to permanent lesions in the years following the spill.

Chapter 3 Aim: To determine the acute sensitivity of Gulf menhaden and Florida pompano, two economically and ecologically relevant Gulf of Mexico species, to dispersed crude oil. Hypothesis: Gulf menhaden will be more sensitive than Florida pompano to dispersed crude oil exposure. Sensory tissue (neuromast and olfactory epithelium) of Gulf menhaden will be more sensitive than gill tissue to a dispersed crude oil exposure.

Chapter 4 Aim: Determine if particulate material of different size and composition can cross epithelial barriers of zebrafish and mummichog. Hypothesis: Acetylene black and fluorescent microspheres will cross gastrointestinal and gill epithelium and enter the vascular system of zebrafish and mummichog.

Table 1.1. Selected major oil spill characteristics ranked by volume of oil discharged into the environment (in millions of l).

Major Oil Spill	Date	Volume (millions of l)	Type of Oil (API*)	Spill characteristics	Effects Observed	Concerns	Citation
Arabian Gulf/Kuwait	January 23, 1991	900-1270	Kuwait crude oil (30)	Terrestrial and marine spill from multiple sources; intertidal species and near shore benthic communities in the western Arabian Gulf were most affected.	Dense mats of oil covered biota and accumulated in coastal sediments. Loss of species diversity was persistent for decades.	Contamination is still present in the most recent studies evaluating coastal habitat. Low flushing rates increase retention of pollutants in sediment and tidal zones.	Al-Awadhi et al., 2012; Jones et al., 2008; Randolph et al., 1998
Deepwater Horizon	April 22, 2010	779	Light Louisiana, Macondo 252 (35.2)	Drill rig explosion caused deep sea (1500m) release of oil creating subsurface and surface plumes. Significant shore oiling occurred in coastal regions of the northern Gulf of Mexico.	Acute mortality of exposed organisms and loss of significant marsh habitat after shore oiling. Fishing was closed in most of the Gulf for months. Unique partitioning of oil into the subsurface and marine snow.	Persistence of oil in benthos may be significant due to cold temperatures and anoxic sediments. Pelagic fish and mammals may have been more affected than initially reported.	Atlas and Hazen, 2011; Peterson et al., 2012
Ixtoc 1	June 3, 1979	560	Light Louisiana (32)	Drill rig explosion caused shallow sea (50m) release of oil into southern Gulf of Mexico. Affected much of Mexico and Southern Texas.	Fishing limitations were put into effect; benthic communities were disrupted by oil and detrital matter. Coastal bird species and marshes were impaired.	First spill of its kind making preparation and remediation efforts difficult to implement. The need for baseline monitoring became apparent.	Jernelov and Linden, 1981; Teal and Howarth, 1984
Amoco Cadiz	March 16, 1978	260	Light Arabian and Iranian crude oil (35)	Tanker grounded near Brittany, France. Wave action from storms naturally dispersed oil in the coastal area. Concentrations of crude oil up to 500 µg/l were detected.	Significant deposition of oil into sensitive habitats with low flushing rates. Oyster mariculture was most heavily affected. Mechanical cleanup and biodegradation were played key remedial roles.	Abundant low energy environments were affected and rely on biodegradation for remediation. Anoxic sediments and heavier crude oil constituents decrease biodegradation.	Gundlach et al., 1983
Exxon Valdez	March 24, 1989	41.6	Alaska North Slope Heavy Crude (29)	Tanker spill on Bligh Reef in Prince William Sound, Alaska. Nearly 2000 km of pristine coastal habitat was oiled.	Significant declines in bird, fish, invertebrate populations. Oil persisted in sediments and evidence of exposure lasted for decades.	Pristine habitat and abundant wild life severely contaminated and affected by spill. Development of a shift from acute toxic exposures to possible chronic effects as well as increased appreciation for seasonal variation during exposure assessments.	Peterson et al., 2003; Atlas and Hazen, 2011

API: American petroleum institute gravity.

Location	Approximate Depth (m)	Date Collected	Compound/s*	Sample Concentrations (µg/l)	Source
Sites near wellhead	1	May 2010	TPAH	84.8	Dierks et al., 2010
Areas with surface oil	1	June 2010	TPH Pre/Post dispersant	190/990	BenKinney et al., 2011
Areas with surface oil	10	June 2010	TPH Pre/Post dispersant	110/40	BenKinney et al., 2011
Areas with surface slicks	1-10	May-July 2010	TPAH	<0.1-77.3	Bejarano et al., 2013
Grand Terre, coastal Louisiana	Sub-surface	May 2010	TPAH	213	Whitehead et al., 2012
Sites near wellhead	1160	May 2010	TPAH	29.4	Dierks et al., 2010
Sites near wellhead	1320	May 2010	TPAH	189	Dierks et al., 2010
2.3 km Southwest of wellhead	1085	June 2010	TBTEX	43.5	Reddy et al., 2012
6.1 km Southwest of wellhead	1155	June 2010	TBTEX	77.2	Reddy et al., 2012
16 km Southwest of wellhead	1125	June 2010	TBTEX	53.6	Camilli et al., 2010
16.5 km Southwest of wellhead	1125	June 2010	TBTEX	52.9	Reddy et al., 2012
27 km Southwest of wellhead	1124	June 2010	TBTEX	33.4	Reddy et al., 2012

Table 1.2. Measured concentrations of crude oil constituents in the Gulf of Mexico after the DHOS.
*DHOS: Deepwater Horizon oil spill; TPAH: Total polycyclic aromatic hydrocarbons; TPH: Total petroleum hydrocarbons; TBTEX: Total benzene, toluene, ethylbenzene, xylenes.

Table 1.3. Selected aromatic hydrocarbons associated with crude oil and their chemical characteristics (NTP, 2011; ATSDR, 2004; ATSDR, 1995). Modified from Bojes and Pope, (2007).

Aromatic hydrocarbons*	# of rings	Molecular weight (g/mole)	Solubility (mg/l)
Benzene	1	78.1	1790
Toluene	1	92.1	470
Ethylbenzene	1	106.2	150
Xylenes	1	106.2	106
Naphthalene	2	128.2	31
Acenaphthene	3	154.2	3.8
Acenaphthylene	3	152.2	16.1
Anthracene	3	178.2	0.045
Phenanthrene	3	178.2	1.1
Fluorene	3	166.2	1.9
Fluoranthene	4	202.3	0.26
Benz(a)anthracene	4	228.3	0.011
Chrysene	4	228.3	0.0015
5-Methylchrysene	4	242.3	0.062
Pyrene	4	202.3	0.132
Benzo(a)pyrene	5	252.3	0.0038
Benzo(b)fluoranthene	5	252.3	0.0015
Benzo(k)fluoranthene	5	252.3	0.0008
Dibenz(a,h)anthracene	6	278.4	0.0005
Benzo(g,h,i)perylene	6	276.3	0.00026
Indeno[1,2,3-cd]pyrene	6	276.3	0.062

*Bold compounds are at least “reasonably anticipated to be human carcinogens”; Benzene is a known human carcinogen (NTP 2011).

Table 1.4. Previous histological effects seen in fish after exposure to crude oil and crude oil constituents.

Species	Chemical	Concentration and Compound Measured in Experiments*	Exposure Time	Effects	Reference
Atlantic Cod	Venezuelan Crude Oil	50-100µg/l	12 Weeks	Moderate to extensive hyperplasia, clubbing, and filament fusion	Khan and Kiceniuk, 1984
Atlantic Cod	Venezuelan Crude Oil	150-300 µg/l	12 Weeks	Extensive hyperplasia, clubbing, and filament fusion	Khan and Kiceniuk, 1984
Atlantic Cod	Hibernia Crude Oil	50-100µg/l	13 Weeks	Extensive hyperplasia, clubbing, and filament fusion	Khan and Kiceniuk, 1984
Atlantic Herring (larval)	Arctic Crude Oil	15 µg/l-750 µg/l; multiple points	12 Days	Significant increase in mortality, Craniofacial asymmetry	Ingvarsdottir et al., 2012
Capelin (Embryo)	Kobbe Crude Oil	<40.4 µg/l TPAH; decreasing over time	32 Days	Significantly decreased hatch rates; no observed abnormalities in hatched embryos	Franzten et al., 2012
Capelin (Embryo)	Pyrene	50 µg/l	32 Days	Significantly decreased hatch rates; no observed abnormalities in hatched embryos	Franzten et al., 2012
Neotropical Sucker	Commercial Diesel Oil	50% (WSF)	6 hours	Gill epithelial lifting, hyperplasia, aneurysm	Simonoto et al., 2007
Pacific Herring	Alaska North Slope	(Field Collection)	3 Weeks Post Exxon Valdez	Significant increases in multifocal hepatic necrosis as well as naphthalene concentrations in tissues	Marty et al., 1999
Pacific Herring (embryo)	Alaska North Slope Crude Oil (less Weathered)	34 µg/l; decreasing over time	16 Days	Yolk sac edema, premature hatching, increased larval mortality	Carls et al., 1999
Pacific Herring (embryo)	Alaska North Slope Crude Oil (more Weathered)	0.4 µg/l; decreasing over time	16 Days	Yolk sac edema, premature hatching, increased larval mortality	Carls et al., 1999
Pink Salmon (embryo)	Alaska North Slope Crude Oil (weathered)	18 µg/l TPAH; decreasing over time	7-9 months	Significantly increased mortality in embryos	Heintz et al., 1999
Pink Salmon (embryo)	Alaska North Slope Crude Oil (weathered)	2.1-66.5 µg/l TPAH; decreasing over time	7-9 months	Significantly increased mortality at higher doses, pericardial edema associated with ascites in exposed fish. Skin-cell apoptosis increased with dose.	Marty et al., 1997
Pink Salmon (Reddy et al.)	Alaska North Slope Crude Oil (weathered)	25-34 µg/l (WSF)	10 Days	Increased prevalence of liver necrosis and pyknotic nuclei. Some increase in epithelial lifting and hyperplasia in gills.	Brand et al., 2001

*TPAH: Total polycyclic aromatic hydrocarbons; WSF: Water soluble fraction.

Table 1.4 Continued.

Plaice	Ixtoc oil	(Field Collection)	Post Ixtoc I	Fin necrosis, telangiectasis, hyperplasia, lamellar fusion	Haensley et al., 1982
Rockfish (adult, field study)	Alaska North Slope	(Field Collection)	Post Exxon Valdez	Fibrosis and glycogen depletion in liver, elevated pigmented macrophage centers in liver, kidney and spleen	Marty et al., 2003
Turbot	North Sea Heavy Fuel Oil	321 ng/L (WSF)	4 Days	Significant increase in CYP1A expression; Decreased mucocytes and chloride cells in gills	Goanvec et al., 2010
Winter Flounder	Grand Banks Crude Oil	100-300 ug/g (sediment)	8 Weeks	Slight hyperplasia of branchial epithelium	Khan, 1995
Winter Flounder	Grand Banks Crude Oil	600 ug/g (sediment)	8 Weeks	Moderate hyperplasia and hemosiderosis of the spleen	Khan, 1995
Winter Flounder	Grand Banks Crude Oil	1mg/g (sediment)	8 Weeks	Extensive hyperplasia with fusion of ~50% of primary lamellae	Khan, 1995
Zebrafish (embryo)	Pyrene	1 µg/l	72h	Pericardial edema, looping defects, elongated ventricle	Zhang et al., 2012
Zebrafish (embryo)	Phenanthrene	0.89 µg/l	72h	Pericardial edema, looping defects, enlarged ventricle	Zhang et al., 2012b
Zebrafish (embryo)	Alaska North Slope (unweathered)	60 µg/l (TPAH)	48h	100% mortality due to pericardial edema	Hicken et al., 2011
Zebrafish (embryo)	Alaska North Slope (weathered)	24-36 µg/l (TPAH)	48h	After 10 months recovery: Increased mortality, reduced cardiac output, reduced swimming speed, "rounder" hearts based on width/length ratio	Hicken et al., 2011

*TPAH: Total polycyclic aromatic hydrocarbons; WSF: Water soluble fraction.

CHAPTER 2: PARTICULATE ACCUMULATIONS IN THE VITAL ORGANS OF WILD BREVOORTIA PATRONUS FROM THE NORTHERN GULF OF MEXICO COLLECTED AFTER THE DEEPWATER HORIZON OIL SPILL

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*This chapter appears as a manuscript in Ecotoxicology, November 2015, Volume 24, Issue 9, pp 1831–1847 and is reprinted with permission from the publisher (License Number: 3925510801940).

Abstract

Histopathologic lesions were observed in the commercially important filter-feeding fish, *Brevoortia patronus* (Gulf menhaden), along the Louisiana Gulf Coast. Menhaden collected from Louisiana waters in 2011 and 2012, one and two years following the DHOS, showed varying severities of gill lesions as well as an unusual accumulation of black particulates visible at necropsy in the heart and stomach vasculature. The PAH derived particulates were typically 2-5 µm in diameter, but larger aggregates were observed in the coronary vessels on the ventricle surfaces and their location and size was confirmed by light microscopy. Composited particulate composition was consistent with weathered petrogenic PAH mixtures based on GCMS analysis. Particulates were present in 63% and 80% of fish hearts and 70% and 89% of stomach muscularis collected in 2011 and 2012,

respectively. Tissue embedded particulates can lead to localized cellular damage from bioavailable compounds, as well as chronic effects from occlusion of sensitive tissues' blood flow. The PAH derived particulates appeared to act as emboli in small capillaries, and could associated with localized inflammation, focal necrosis and inappropriate collagen and fibroblast tissue repair. We believe large volume filter feeding teleosts, such as menhaden (up to 3 million gallons per year/fish) with high lipid content, have a higher exposure risk and greater potential for toxicity from toxic particulates than other higher trophic level finfish. Suspended PAH derived particulates following an oil spill therefore, should be considered when assessing long-term ecological impacts and not be limited to physical contact (coating) or water soluble fraction (WSF) for assessing toxicity (gill and neurologic).

2.1 INTRODUCTION

Each year, approximately 200 million l of crude oil are released into the Gulf of Mexico as a result of natural seeps from underground reserves as well as from small commercial oil drilling and shipping spills (Kvenvolden and Cooper 2003). The natural presence of crude oil in the environment can cause toxic exposures to wildlife, but this yearly release of oil is relatively widespread and can be metabolized by an enhanced microbial population in addition to physical weathering processes. The Deepwater Horizon oil spill (DHOS), however, introduced millions of barrels of oil from a single source into a localized region in the Gulf of Mexico likely causing substantial toxic exposures to native wildlife. This unprecedented release of oil into the environment, and the ensuing remedial efforts, can significantly affect populations of a variety of organisms over a large area of the Gulf.

Approximately 715 million l (4.9 million barrels) of Louisiana light crude oil (from the Macondo Prospect) were released into the northern Gulf of Mexico following the April 20th, 2010 DHOS blowout, creating the largest marine oil spill in United States history (McNutt et al. 2012). The leaking wellhead was eventually capped on July 15th, 2010 and was declared dead on September 19th, 2010. Oil was primarily released at a depth of approximately 1500m which led to the formation of a large deep plume at 1100m characterized by light hydrocarbons, microbial activity, and depleted oxygen levels (Camilli et al. 2010; Hazen et al. 2010; Joye et al. 2011; Kessler et al. 2011; Valentine et al. 2010). The surface slick was enriched in higher molecular weight, PAHs including phenanthrene, fluoranthene, and their congeners (Diercks et al. 2010; Incardona et al. 2013). Aggregates of organic and inorganic PM typically less than 0.5 mm in diameter

were also associated with the surface slick and were termed “marine snow” (Passow et al. 2012). This fine particulate formation is likely responsible for introducing oil constituents into the food chain (Almeda et al. 2013; Graham et al. 2010) before settling to the bottom of the Gulf and impacting benthic organisms such as deep sea corals (White et al. 2012).

First responders utilized oil spill remediation techniques such as mechanical recovery, in-situ burning, and dispersant applications to alleviate the visible volume of oil. It was estimated that 20% of the oil was mechanically recovered, 5% was burned, 49% was dispersed, evaporated or dissolved, and 26% was unaccounted for, either sinking to the bottom or accumulating on shorelines (Federal Interagency Solutions 2010). A total of 410 controlled burns took place after the spill leading to the combustion of between 18-23 million l of surface oil (Schaum et al. 2010). Analysis from the spill shows that between 10%-20% of the burned crude oil persisted as ash, smoke, or burn residue in the environment (Federal Interagency Solutions 2010). This incomplete combustion of crude oil is known to create toxic PAHs such as B[a]P (a known carcinogen) in addition to PM that can negatively impact marine organisms (Peterson et al. 2012).

Previous studies have shown that the use of dispersants significantly increases biodegradation of crude oil (Prince and Butler 2013) and nearly 8 million l of dispersant were used during the DHOS emergency response (Kujawinski et al. 2011). Surface samples taken in May and June of 2010 showed toxicologically relevant concentrations of aromatic hydrocarbons in surface waters (Diercks et al. 2010; Spier et al. 2013; BenKinney et al. 2011) which coincided with a number of marine fish species spawning seasons including Bluefin tuna and Gulf menhaden (Muhling et al. 2012; Rooker et al. 2013). Dispersant use increased penetration of the PAH enriched surface slick into the epipelagic

zone (BenKinney et al. 2011), which increased the bioavailability of these toxic components of crude oil (Allan, Smith, and Anderson 2012). The dispersed and dissolved surface oil was partially degraded by microbes (Passow et al. 2012), but significant portions of weathered crude oil products remained in the environment for over 18 months after the spill (Aeppli et al. 2012). Dispersant-oil mixtures have also repeatedly shown increases in toxicity to fish, coral, and other wildlife when compared to crude oil alone (Agamy 2013; Goodbody-Gringley et al. 2013; Milinkovitch et al. 2013; Rico-Martínez, Snell, and Shearer 2013). In these studies increased mortality as well as exacerbation of lesions caused by crude oil was commonly associated with chemically dispersed oil. Oil spill remediation techniques such as surface burning and dispersant use can create environmental circumstances that increase the potential toxicity of PAHs from the DHOS to commercially important fish species in the northern Gulf of Mexico (Muhling et al. 2012; Adams, Hernandez, and Cato 2004).

A number of commercially important fish species inhabiting the Gulf of Mexico were estimated to be affected by the DHOS including various species of snapper, grouper, tuna, and menhaden (National Marine Fisheries 2012). Specifically, Gulf menhaden (*Brevoortia patronus*) were of particular concern because their winter spawning season had just ended and juveniles were moving inshore towards estuaries throughout the duration of the spill. Menhaden distribution is most dense off the coast of Louisiana (Shaw et al. 1985), coinciding with the area affected by the Deepwater Horizon surface slick. These fish constitute the second largest fishery (by weight) in the United States and contributed to an average of 163 million dollars in revenue between 2000 and 2005 (McCrea-Strub et al. 2011; Vaughan, Shertzer, and Smith 2007). As pelagic filter feeders menhaden have high

metabolic demands and are sensitive to hypoxia; as a result they constantly filter ocean water for food and oxygen (Lassuy 1983). A single adult menhaden fish can filter in the magnitude of 10 million l (3 million gallons) of water per year (Durbin and Durbin 1975), and schools can consist of thousands of individuals. This behavior, in addition to the seasonal accumulation and storage of lipid (Deegan 1986; Dubrow, Hale, and Bimbo 1976), make them particularly susceptible to exposure to oil associated with plankton or tiny droplets of oil floating in the water column. The areas closed to fishing as a result of the DHOS overlap with a large portion of menhaden foraging and breeding grounds indicating that menhaden may be specifically at risk for increased exposures to toxic crude oil constituents such as PAHs from the spill (McCrea-Strub et al. 2011; Ahrenholz 1991).

A number of impacts on biota while the DHOS spill was ongoing have been reported, but the effects of continuous exposure following the capping to the toxic components of crude, weathered crude and dispersed oil are still being evaluated (Silliman et al. 2012; Williams et al. 2011; Dubansky et al. 2013). Researching the long-term effects of the DHOS on individual Gulf species as well as trophic interactions between species is crucial to understanding the impact on the natural resources that are economic drivers in the Gulf communities (Coglianese 2010). It can be argued that underlying long term effects' following a large oil spill on fishery yields and population dynamics begins at the cellular level within individuals. Histopathology is an ideal technique to study exposures to contaminants because of its "intermediate location with regard to the level of biologic organization" (Bernet et al. 1999). Histopathology is useful to assess the severity of sub-lethal lesions over a range of exposure scenarios and is applicable to both field and laboratory studies.

Understanding lesions at a cellular level is important to assess the potential impact to individuals in a population and can be used to extrapolate effects at the population level following environmental disasters such as the DHOS. Gill lesions, such as telangiectasis (lamellar aneurysm due to rupture of structural pillar cells), epithelial lifting, and secondary lamellar hyperplasia, have been recorded in previous laboratory studies of crude oil exposure in teleosts (Agamy 2013). These lesions all contribute to decreased functionality of the gills by impairing oxygen exchange, osmoregulation, and ion transport. Ventricular and olfactory necrosis with an associated inflammatory response were seen in previous studies of *Menidia menidia* exposed to the water accommodated fraction of oil (Gardner 1975) indicating that multiple organs can be affected by these types of exposures. Based on the nature of the spill, along with the chronic natural seeps in the Northern Gulf of Mexico, it would not be surprising to see gill lesions (such as epithelial hyperplasia, epithelial lifting and telangiectasis) in fish collected from areas associated with the spill as well as lesions associated with chronic exposure in wild populations of fish collected in later years. In any field population similar lesions can be a result of different etiologies, but through special stains and multiple sections these other causes can be considered unlikely. It was hypothesized that histological evaluation of wild Gulf menhaden collected from waters overlapping with areas affected and not affected by the DHOS would reveal a spectrum of reversible to permanent histological lesions.

2.2 METHODS

2.2.1 Study site and sample collection

Over the course of the present study, Gulf menhaden were collected off the coast of Louisiana from two separate sites: Vermilion Bay, Louisiana (VB) and Grand Isle,

Louisiana (GI) which are separated by approximately 250 miles (Figure 2.1). The reference site, VB, is located in the south-central region of coastal Louisiana and was unaffected by the contamination associated with the DHOS based on oil transport models, satellite imaging, and the lack of shore oiling (Operational Science Advisory 2010; Norse and Amos 2010). The experimental site, GI, is located off the southeastern coast of Louisiana and the area was heavily oiled as a result of the spill (Norse and Amos 2010; Operational Science Advisory 2010). This oiling led to the assumption that fish from the GI would be affected by the weathered surface slick and remediation efforts associated with the spill, while menhaden from VB would not be impacted.

Pre-spill menhaden samples (N=3) from 2009 were analyzed for PAH content. The samples consisted of processed fish meal and fish oil collected by a Louisiana menhaden processing plant in July 2009. These values were used for comparison to post-spill menhaden samples.

During the fall of 2010, 39 menhaden were collected from near GI to develop a baseline for acute toxicological responses to the original spill. In later years, fish were collected approximately monthly between July until October from both GI and VB waters for histological analysis. In the summer of 2011 approximately 25 menhaden were collected on each trip using a standard 5-panel gill net approximately 200m in length. In the summer of 2012, 20 fish were collected from each site on monthly trips from July through September. The sampling locations for GI were as follows: 29^{o} 17’ 48.12” N 89^{o} 41’ 47.01” W; 29^{o} 15’ 58.27” N 89^{o} 56’ 34.31” W; 29^{o} 10’ 35.74” N 90^{o} 3’ 41.34” W and for VB were as follows: 29^{o} 33’ 30.64” N 92^{o} 1’ 1.63” W; 29^{o} 34’ 54.00” N 92^{o} 5’ 36.00” W; 29^{o} 28’ 20.93” N 91^{o} 49’ 57.77” W. Fish collected from the same date and

location are assumed to belong to the same school due to the nature of the fish (Lassuy 1983) and were analyzed as cohorts according to these schools. Additional menhaden were collected from these schools for GC-MS PAH analysis (section 2.5). These *Brevoortia patronus* collections are likely from the northern Gulf of Mexico menhaden population that reproduces off shore in the winter and are comprised of schools from their respective bays and estuaries (Anderson and Karel 2014).

2.2.2 Histological preparation and processing

Fish for histopathological analysis were sacrificed using tricaine methanosulfonate (MS222) in sea water or died during collection, peritoneal cavity opened, fixed in 10% buffered formalin, stored in plastic sample bags, and shipped to Rutgers University in New Brunswick, New Jersey. Samples were received, assigned accession numbers, and transferred to 70% ethanol for storage. After storage in ethanol, total lengths were taken to the nearest mm and weights were recorded to the nearest gram. Not all fish received were used for histopathology. Table 2.1 shows the numbers of fish that were processed for histopathology from each date and location as well as the average length and weight for each school. There was a preference for the selection of smaller fish to reduce variability in age and exposure history.

2.2.3 Dissections

Vital organs of the selected fish were removed including sections of gill, heart, stomach, intestine, liver, pancreas, muscle, kidney, nares, and gonad tissue if they were recovered. Gross observations of the external condition and internal organs of the fish such as tissue discoloration, the presence of visible residues or sheens, and organ malformations were noted during dissection. Organs were carefully removed and placed in appropriately

labeled cassettes, decalcified if necessary (Cal-Ex decalcifier, Fisher Scientific, Nazareth, PA USA), embedded in paraffin, sectioned at 4-6 µm, and mounted on glass slides. Slides were stained with hematoxylin and eosin. Grocott's methenamine silver stain and Masson's trichrome stain were used to distinguish the presence of fungi and connective tissue in select slides. Undissected fish were saved for future evaluation and not included in statistical analyses.

2.2.4 Histopathology analysis

Analysis of histology slides included a preliminary screening to observe morphology of tissues and organs. General observations of histopathological conditions were recorded for future reference. A second evaluation of histology was conducted to quantify lesions present in the preliminary screening. Gill, heart, and stomach lesions were quantified using an index ranging from 1 to 4. The score of 1 was given if the lesion was not present, 2 if the lesion was described as mild, 3 if the lesion was described as moderate, and 4 if the lesion was described as severe. Due to small sample size, statistical comparisons were made with regards to the prevalence of these lesions in the fish collected from Louisiana waters. The histopathologic results presented in this paper are limited to the gill, heart, and stomach.

2.2.5 Synchronous fluorescence scanning (SFS) of PAH standards and heart extracts

SFS was used for determining multicomponent PAH analysis from heart tissue as previously described (Patra 2003). For this procedure, a 0.5 g of heart tissue from menhaden containing black particulates collected from GI, Louisiana on July 27, 2011 was homogenized in 75% EtOH. An aliquot (100 µl) of homogenate was then added to 75% EtOH (900 µl). The resulting suspension was vortexed for one continuous minute in order

to extract PAH-like compounds. The extract was then centrifuged for 20 minutes at 13,000 rpm in order to pellet tissue. The supernatant was analyzed for fluorescent compounds using a Fluorolog 4 (model FL-1000, Horiba Jobin Yvon, Edison, NJ). FluorEssence software (V3.5) was used to collect data. Scanning parameters included masking 1st and 2nd order Rayleigh lines, 3D acquisition, integration time 0.1 sec, excitation start 315:end 410, 5 nm increments, side entrance/exit slits of 1 nm, grating density 1200 (Blaze 330), emission start 420:end 520, 5 nm increments, side entrance/exit slits of 1 nm, grating density 1200 (Blasé 500). Signal was read as S1/R1. PAH standards, 500 ng/ml of benzo [a] pyrene and benzo [b] fluoranthene, (Sigma-Aldrich Chemical Co.), were run using the same parameters.

2.2.6 GC/MS analysis of heart particulates and fish tissue

Black particulates were collected and pooled from approximately 25 menhaden hearts showing black particulate masses on the subsurface of the heart. Particulates were visibly identified, removed using sterilized tweezers, and combined into a composite sample for analysis. Samples of unweathered crude Macondo Prospect oil (MC252) and weathered oil samples collected in Bay Jimmy were also tested using the same methods for comparison to the extracted material. Extraction of PAHs and alkanes from the isolated particles followed methods outlined in EPA Method 8270 series. This method can detect 43 different PAHs including alkylated PAHs often found in crude oil extracts. The following method was used for GC/MS instrument analyses and has been previously reported by Olson et al. (Olson, Meyer, and Portier 2014). The method is briefly described below. After addition of internal standards, extracts were analyzed using an Agilent 7890A GC fitted with a 0.25 mm ID × 30 m HP-5MS column and an Agilent 7683B autosampler.

The injector was set to 250°C and the detector to 280°C. The column was held at 60°C for 1 min and then ramped at 25°C/min to 160°C followed by 3°C/min to 268°C and 12°C/min to 300°C, where it was held for 8 min. Detection of analytes involved the utilization of a HP 5975C Inert XL Series Mass Selective Detector operating in the Selected Ion Monitoring mode. Concentrations of parent PAHs were calculated based on calibrations using a five-point curve which were checked for each batch of extracts analyzed. Concentrations were reported on a dry weight basis. Approximate alkylated PAH concentrations were calculated assuming the same response factors for each parent and corresponding alkylated analogues. For alkylated phenanthrene/anthracenes, the results were reported as pairs to incorporate the uncertainty of the measurements and quantification based on the average response factor of the individual parent PAHs.

2.2.7 Statistical analysis

Means and standard deviations were calculated for lengths and weights of each school of menhaden. Comparisons between the prevalence of different lesions in fish at different locations and years were tested using a non-parametric Chi-Square analysis at a significance level of $p<0.05$. Yearly concentrations of TPAHs were compared using a Kruskal-Wallis One Way Analysis of Variance on Ranks at a significance level ($p<0.05$). All statistics were run using SigmaPlot 11.0 (Systat Software Inc.) commercially available software.

2.3 RESULTS

2.3.1 Initial metrics and gross observations

Menhaden collected in this study were determined to be age 1 fish in their second year of development based on the size of fish collected (Lassuy 1983) (Table 2.1). One

outlier school (8/24/2011) was in their third year of development and was not included in statistical comparison, but was included in the histological analysis. Observations during dissections of fish collected in 2011 included fin fraying, the presence of black residues, as well as visible oil sheens in the body cavity. Slight fin fraying was present in 73% of fish collected from VB while only 19% of fish from GI showed any fin fraying. Black residue within the body cavity was present in 52% of fish from GI and only 6% of fish from VB. Visible oil sheens were observed in the body cavity of 42% of fish from GI and 18% of fish from VB.

Menhaden collected in 2012 were of comparable sizes to fish collected in 2011 (Table 2.1). During dissections the presence of abnormal black coloration in the gastrointestinal tract was present in both GI and VB menhaden, 87% and 83%, respectively. Black areas along the surface of the ventricle were observed upon removal of the heart from the pericardial cavity (Figure 2.2B), while non-affected hearts were non-mottled and cream colored.(Figure 2.2A). Menhaden collected from GI had 33% and VB had 43% with distinct black areas.

2.3.2 Histopathology

Seven histological parameters were examined for severity in gill (Table 2.2), heart (Table 2.3) stomach tissue (Table 2.4) and yearly prevalence (Tables 2.5 & 2.6). The severity score for each lesion was determined and the average for each school is presented in Tables 2.2-2.4. There were distinct variations in lesion presence between schools, but lesions within schools were typically consistent. Differences in the prevalence of two lesions between GI and VB schools were found to be significant in 2012.

Gill tissue containing uniformly spaced secondary lamellae, thin epithelial layers, and minimal evidence of epithelial lifting or epithelial hyperplasia was considered normal (Figure 2.3A). Gill lesions including telangiectasis and epithelial hyperplasia were observed at both sites in both 2011 and 2012 menhaden (Figure 2.3B). Lifting of the secondary lamellae epithelium was also present in the majority of fish collected (Table 2.2). Telangiectasis was present in 49% of fish gill tissue from VB in 2011 and 39% of fish from GI in 2011. These values decreased to 13% for VB and 3% for GI in 2012. The lifting of lamellar epithelium was present in 91% percent of VB fish and 89% of GI fish in 2011. Epithelial lifting decreased substantially in both sites to 43% (VB) and 6% (GI) from 2011 to 2012. In 2012 epithelial lifting was significantly different between the two sites ($p=0.003$), Epithelial hyperplasia was present in the majority of primary gill lamellae. In severe cases, fusion of secondary lamellae was present in addition to hyperplasia.

Heart ventricle tissue and stomach muscle from menhaden collected in the northern Gulf of Mexico after the DHOS showed a substantial accumulation of black particulates (Table 2.5). Due to the limited sampling in 2010, only one school was analyzed for a limited number of fish. Hearts were isolated for histology from 16 fish of the 39 collected. Black particulates were present in 38% of fish hearts from 2010 (6 of 16). All schools from 2011 and 2012 had at least one fish with particulates present in heart or stomach muscle; 75% of menhaden had some level of black particulates in their hearts while 88% of menhaden had black particulates in their stomach muscularis over the entire study (2011-2012). The overall presence and average severity of the black particulates increased in each location from 2011 to 2012. These particulates range from approximately 1 to 4 µm in diameter and also existed as larger black emboli within the small blood vessels of the

heart epicardium (Figure 2.3D, Figure 2.4A), and stomach of menhaden (Figure 2.3F and Figure 2.4B). The dark particulates were in the same locations as observed upon necropsy (Figure 2.2B). The edges of particulates were birefringenet with polarized light and appeared as white needle-like crystals. The middle of the particulates was dark brown to black in areas where significant overlapping and clustering of the crystals was present. There were no significant differences in the severity or presence of black particulates between VB and GI in 2011 and 2012.

Other lesions in menhaden hearts included the degeneration of myocardium based on altered staining characteristics, degradation of the muscle fibers, and the presence of eosinophilic material in the interstitium (Figure 2.4 A). There were no significant differences in this degradation between sites or years, but it was present in the majority of fish. Inflammation was present in both stomach and heart muscle (data not shown) but with varying severity and appeared to be associated with connective tissue damage rather than the mere presence of the black particulate material, therefore, we cannot make a definitive statement on the association between particulates and inflammation. Masson's trichrome stain indicated the abnormal presence of collagen in both heart (Figure 2.4C, D) and stomach muscle. The deposition of collagen in stomach muscle was lower in VB schools in 2012 compared to GI ($p=0.044$). Evidence of fibrosis in the myocardium was present in 15 fish from 2011 and 8 fish from 2012 (data not shown).

2.3.3 Year to year comparison of Gulf menhaden

To determine any chronological trends in the prevalence of lesions the northern GOM menhaden were combined into two cohorts, 2011 and 2012 (Table 2.6). The prevalence of black particulates (p 0.017) and collagen tissue in the stomach muscularis

(p=0.009) increased significantly from 2011 to 2012 (Figure 2.3 E, F). Telangiectasis and epithelial lifting decreased significantly from 2011 to 2012 (both $p \leq 0.001$).

2.3.4 TPAH menhaden tissue concentrations

Fish from the same collections as were analyzed for histopathology were separately analyzed for TPAH concentrations as a function of dry fish weight using GCMS and compared to 2009 pre-spill menhaden samples (Figure 2.5) (Olson, Meyer, and Portier 2014). Chemical data from menhaden in 2010 was minimal due to sampling constraints placed on sampling after the spill. However, three composites of 2 fish each from VB were analyzed and found to have an average TPAH concentration of 1400+/- 160 ng/g dry menhaden tissue. These represented only a single collection and were not included in statistical analysis due to the small, unrepresentative sample. TPAH concentrations in menhaden were determined to be 3100 +/- 470 ng/g in 2009 (pre-spill), 6700 +/- 4300 ng/g in 2011, and 5600 +/- 1900 ng/g in 2012. There was a statistically significant increase in TPAH concentration in the 2 years post-DHOS when compared to the 2009 pre-spill menhaden sample ($p \leq 0.012$).

2.3.5 Heart particle chemical analysis

Two methods (SFS and GCMS) were used to help characterize the chemical composition of the particles present in the menhaden tissue. Using SFS, a qualitative PAH signature was obtained from extracts of heart particulates (Figure 2.6A) from 2011 GI menhaden. The extract from menhaden heart tissue with dark particulates showed intense fluorescence within the range of the high molecular weight PAH BbF (Figure 2.6B). Excitation ranged from 350-410 nm and emission ranged from 430-470 nm. Therefore, the SFS gave a qualitative measure and indicated that the heart tissue containing particulates

from menhaden collected from GI, Louisiana in the summer of 2011 had a fluorescence signal consistent with PAH-like compounds. In order to better asses the organic constituents of the particles, GCMS was run on isolated particles dissected from the heart tissue.

Chemical analysis via GCMS of black particulates from menhaden hearts (Figure 2.7A) and weathered oil samples collected in Bay Jimmy (Figure 2.7B), produced chromatograms with signatures representative of crude oil PAHs. Selected ion monitoring (SIM) chromatograms were evaluated for the black particulates, weathered crude oil from Bay Jimmy, and Crude MC252 Oil. Individual peaks and retention times were recorded for each of the three samples (Table 2.7) and compared to USEPA archives (Wang et al. 2003). Ion 220, commonly associated with trimethlyated phenanthrene, had a nearly identical signature and peak ratios across all three samples. Other ions showed very similar peak patterns over the same retention times.

2.4 DISCUSSION

A variety of histopathological lesions that have been associated with crude oil exposure (epithelial lifting and telangiectasis) were present in Gulf menhaden collected from the northern Gulf of Mexico, yet there were minimal differences in the presence of lesions between schools from the reference site, VB and the visibly oiled site, GI. There is consistency within each school based on the lesions observed and their severity, indicating that schools of fish likely share similar exposure histories to contaminants. Based on these trends it seems that the assumed classification of VB menhaden as reference cohorts could be inaccurate due to school migration, storm events, or an elevated background occurrence of lesions in this region. Movement of menhaden schools along

shorelines may contribute to the mixing of fish between sites in the northern Gulf of Mexico, thus skewing our results. The two sites that were sampled are relatively close in proximity (~ 250 miles), and are not separated by any physical boundaries, allowing VB and GI menhaden schools to move freely between sites. Menhaden that could have been impacted by the DHOS may have been collected at VB or fish from the open ocean with no significant exposure history could have been collected at GI. There could also be an increase in background lesions due to the presence of a variety of environmental factors in the northern Gulf of Mexico including natural oil seeps, the introduction of nutrients and xenobiotic compounds from the Mississippi river, as well as expanding dead zones in the northern Gulf of Mexico (Diaz and Rosenberg 2008; Peterson et al. 1996). The need for a more thorough understanding of the background levels of lesions in Gulf menhaden and other organisms inhabiting this multi-stress environment is apparent based on the findings of this study.

The histopathological gill lesions described in this research, including telangiectasis, epithelial lifting, and epithelial hyperplasia of lamellae, have been previously described in both field and laboratory studies examining fish exposed to crude oil (Agamy 2013; Haensly et al. 1982; Khan and Kiceniuk 1984). Epithelial lifting has been considered an adaptive response to acute chemical exposure and could serve as a defense mechanism to reduce the uptake of xenobiotic compounds across the gill epithelium (Agamy 2013; Simonato, Guedes, and Martinez 2008). The development of telangiectasis (lamellar aneurysm) has been attributed to the destruction of pillar cells after acute chemical or physical insult. These lesions reduce the efficiency of gill tissue to perform designated functions including respiration and ion exchange (Al-Kindi, Brown,

and Waring 2000; Au et al. 2004; Engelhardt, Wong, and Duey 1981). Similar lesions were observed in the 2010 GI collected menhaden and the extent of gill involvement (hyperplasia and lamellar fusion) was more extensive than that observed in the 2011 and 2012 fish (Bentivegna 2012). The significant decrease in the prevalence of epithelial lifting and telangiectasis in gill lamellae from 2011 to 2012 could indicate a return to background levels of xenobiotics in the environment or other etiologies that could result in similar effects. Varying degrees of hyperplasia were found in this study and prevalence was consistent between sites and years. Hyperplasia is a cellular response to continuous exposure of any variety of chemicals and physical irritants; if the insult is removed cellular structures will return to normal. In severe cases hyperplasia and lamellar adhesion can lead to lamellar fusion which greatly reduces surface area and thus respiratory capacity in fish (Au et al. 2004). It cannot be ruled out that the background presence of PM or xenobiotics in the Gulf of Mexico contributes at least partially to chronic hyperplasia observed in menhaden gill epithelium, especially at the base of the primary lamellae.

In fish, heart effects as a result of PAH exposure have become an organ of interest due to several field and laboratory studies demonstrating damage due to crude oil exposure or specific PAHs ((Khan and Kiceniuk 1984), Incardona et al. 2010; Zhang et al. 2013; Zhang et al. 2012). Earlier histological studies on cod exposed to Hibernia crude oil showed edema and evidence of fibrosis in hearts, but these observations were rare and not further explored (Khan and Kiceniuk 1984). Cardiac effects have reemerged as a target organ in part due to studies reporting deformities and decreased physiological efficiency of the heart following crude oil exposure from the DHOS. Crude oil from the DHOS caused significant heart malformations and impaired swimming ability in predatory fish

(Mager et al. 2014; Incardona et al. 2014). At a mechanistic level, it has been shown that crude oil components can also impair cardiac contraction in large pelagic species such as tuna (Brette et al. 2014).

Developmental effects were not examined in our study, but it should be pointed out that exposure during embryonic development of pelagic fish embryos could be an important life-stage for acute toxic effects and the presence of organ malformations in later life-stages. Although not examined in our study, DHOS exposure to menhaden pelagic eggs would likely result in similar toxic effects as reported in other teleosts exposed under laboratory conditions. Developmental lesions including pericardial edema, looping defects, and elongated or rounded ventricles have been observed in embryonic fish exposed to crude oil (µg/l), suggesting that the developing heart is significantly affected by PAH exposure (Incardona, Collier, and Scholz 2004, 2010; Zhang et al. 2012; Zhang et al. 2013). Exposure of zebrafish to phenanthrene induced significant looping defects in the heart and increased interstitial fibrosis that coincided with upregulation of MMP-9 and transforming growth factor β (TGF-β) (Zhang et al. 2013). Embryonic zebrafish exposed to low levels of weathered PAHs have shown decreases in heart efficiency and slower swimming ability after reaching adulthood (Hicken et al. 2011), reducing the fitness of these fish if they were in the wild

While our study did not specifically look at developmental heart malformation or cardiac output, there was a significant accumulation of PAH derived particulates in heart vasculature which was associated with myocardial lesions that we believe would likely decrease fitness. Black particulates were found in over three quarters of menhaden heart and stomach muscle collected in 2011 and 2012, and their prevalence significantly

increased in stomach muscle over those two years. Initial analytical methods show that particulates isolated from heart are PAH derived. The physical aggregation and accumulation of black particulates in the heart, as well as the stomach, has not been previously described in the literature and may indicate a specific response of menhaden to crude/dispersed oil or other fine suspended particulates.

The unique filter feeding used by menhaden and high lipid content may make menhaden more susceptible to this type of contaminant due to lipophilic compounds being readily absorbed into the vasculature through fish gills (Randall et al. 1998). Filter feeding fish, like menhaden, may also experience significant exposures from contaminated phytoplankton and zooplankton (Graham et al. 2010; Mitra et al. 2002). These black particulates in the tissue sections are typically 1-4 µm in diameter. The particulates themselves or tiny oil micelles are likely absorbed through the gill and/or digestive tract epithelium and then enter into the blood stream. Due to their size it is possible that the particles or micelles aggregate and form emboli in the capillary beds. Some of the largest aggregates of particulates observed histologically are approximately 40 µm. Gross observations of hearts show even larger aggregates of particulates just under the ventricle epicardium (Figure 2.2B). These PAH derived particulates appear to act as emboli and have shown to become trapped in capillaries of the muscle tissue in the stomach and heart. In a number of fish there was evidence of necrosis and cardiac muscle tissue replacement with collagen. This cardiac lesion is similar to what is observed following a myocardial infarction in higher vertebrates. More research is needed to determine the specific processes affecting the distribution and pathology of these PAH particulates in menhaden

tissue and the effects they may have on fitness of the species after exposure to crude oil components.

Other lesions identified in the study include the increased collagen in stomach muscle and presence of eosinophilic material in the heart interstitium. Collagen is a structural protein normally found in muscle tissue (Sikorski and Borderias 1995) that can be deposited in excess as a response to chronic inflammation. The presence of collagen in the stomach muscularis using special stains increased significantly from 2011 to 2012 and was associated with the presence of black particulates. Particulates were embedded in the collagenous tissue as well as in the blood vessels of the stomach. The entrapment of these embolic particulates may induce chronic inflammation causing this lesion, but to our knowledge there has been no previous research on this response. The presence of eosinophilic material within the heart ventricle could represent degradation of cardiac muscle, as was seen previously in *Menidia menidia* exposed to water accommodated fractions of crude oil (Gardner 1975). Previous studies also report muscle degradation (Haensly et al. 1982) in lateral and abdominal muscle from plaice exposed to crude oil indicating that multiple muscle types may be targets for crude oil exposure. Studies involving exposure of fish to PAHs during embryonic development have reported heart defects; however, the specific mechanism(s) are likely to be quite different from those reported in this study (Incardona, Collier, and Scholz 2010; Incardona et al. 2013; Zhang et al. 2013; Zhang et al. 2012).

Overall, the lack of differences between the designated exposure site and "reference" site may indicate the far-reaching potential of the DHOS or chronic release of South Louisiana crude oil from multiple sources. The lesions presented in this study are at

times severe and likely impact the fitness of Gulf menhaden, potentially decreasing commercial yields and altering population dynamics if large schools were affected. From 2011 to 2012 there is a statistically significant decrease in gill lesions (lamellar fusion and epithelial lifting) and there is a consistent prevalence of more severe long-lasting lesions (cardiac scarring and collagen deposition). The proportionate increase in the prevalence of collagen deposition in menhaden stomach muscle from 2011 to 2012 is consistent with the claim that fish will show a progression of tissue involvement dependent on exposure dose and extent of damage. A recent study of fish in the Gulf of Mexico (Murawski et al. 2014) reported a similar temporal trend of lesions and PAH concentrations in fish tissue following the DHOS.

The accumulation of black particulate in blood vessels has not previously been documented and could be significant in understanding how crude oil constituents from spills or other sources negatively affect aquatic biota. The Gulf menhaden collected in this study were from wild populations and it is plausible to assume that fish with increased lesion presence and severity may have been more affected and thus selected out of the population by predators and other environmental factors. Regardless, our studies are the first to evaluate the possible histopathological effects of the DHOS on menhaden, a wild pelagic fish species in the Gulf over multiple years. This study also demonstrates in the presence of particulates in the heart which have a PAH signature. Particulate toxicity associated with occlusion of capillaries should be examined further in this and other fish species for impacts on the health of the organisms.

Although inconclusive as to the specific source of the oil and exposure route, our studies have shown that PAH particulates are resulting in structural damage to the gill,

heart and stomach in this commercially important species. There is also no doubt that the area affected by the DHOS overlapped with productive menhaden habitat off coastal Louisiana during sensitive life stages. Exposure to DHOS during egg and embryonic stages would likely have caused similar lesions as reported by many other researchers (Brette et al. 2014; Incardona et al. 2014; Incardona et al. 2013). In addition, the migratory pattern, the filtering capacity and high lipid content of menhaden would expose them to suspended PAHs in the water column and through contaminated prey species. Similar particle accumulation as reported by our group has been reported previously in *Menidia menidia* following oil exposure (Gardner 1975). Based on our findings to date it would appear that there is a decrease in TPAHs in the 2012 menhaden and there are declines in several of the histological lesions examined. Our own preliminary laboratory studies are currently ongoing and have confirmed the movement of DHOS oil into blood vessels of the gills and gill rakers in YOY menhaden following non-filtered WSF exposure (data not presented). There is a large data gap in our understanding of impacts of particulates and associated contaminants both short term and long-term on finfish and especially on filter feeding species like the menhaden.

ACKNOWLEDGMENTS

We would like to thank Louisiana Fish and Wildlife, Louisiana State University, Seton Hall University, Sea Grant, the Rutgers New Jersey Agriculture Experiment Station, and the New Jersey Department of Environmental Protection for the funding, resources, and collaboration that support this research project. LDWF-LSU contract 69670; NJAES-Rutgers NJ01201.

Table 2.1. Mean morphometric values of fish collected for histopathology from Northern Gulf of Mexico Waters in 2011 and 2012.

Location	Date	N	Mean Total Length (mm)	Mean Weight (g)
Grand Isle	8/23/2011	10	149 +/- 7	35 +/- 5
	*8/24/2011	5	220 +/- 14	134 +/- 13
	9/13/2011	11	157 +/- 13	42 +/- 9
	10/11/2011	5	174 +/- 10	53 +/- 13
Vermilion Bay	7/6/2011	10	138 +/- 11	28 +/- 7
	8/23/2011	8	159 +/- 3	43 +/- 3
	9/15/2011	10	146 +/- 17	34 +/- 15
	9/21/2011	5	156 +/- 10	38 +/- 8
Grand Isle	7/17/2012	10	173 +/- 9	55 +/- 8
	8/21/2012	10	174 +/- 7	56 +/- 5
	9/11/2012	10	166 +/- 7	55 +/- 6
Vermilion Bay	7/12/2012	10	158 +/- 12	41 +/- 8
	8/22/2012	10	194 +/- 4	82 +/- 6
	9/18/2012	10	182 +/- 16	69 +/- 18

*=Older age class not included in statistical evaluations; +/- (Standard deviation)

Table 2.2 Average severity score of gill lesions identified in wild Gulf menhaden schools.

			Gill		
Location	Date	N	Telangiectasis	Epithelial lifting	Hyperplasia
Grand Isle	8/23/2011	10	2.0	2.4	3.3
	*8/24/2011	5	2.0	1.0	2.8
	9/13/2011	11	1.4	3.7	2.9
	10/11/2011	5	1.4	3.4	2.0
Vermillion Bay	7/6/2011	10	1.4	2.7	2.6
	8/25/2011	8	2.4	2.1	3.4
	9/15/2011	10	1.8	3.6	2.5
	9/21/2011	5	1.0	2.4	2.2
Grand Isle	7/17/2012	10	1.0	1.2	3.0
	8/21/2012	10	1.0	1.0	2.5
	9/11/2012	10	1.2	1.2	3.1
Vermillion Bay	7/12/2012	10	1.1	2.0	3.1
	8/22/2012	10	1.1	1.3	3.0
	9/18/2012	10	1.4	1.4	3.0

*=Older age class not included in statistical evaluations; Severity scores were calculated on a 1-4 scale (1 being not present, 2 being mild, 3 being moderate, and 4 being most severe) for each organ of each fish. The average score is displayed for each school of fish.

Table 2.3. Average severity score of particulate accumulation and myocardial degeneration
identified in wild Gulf menhaden schools.

			Heart	
Location	Date	N	PAH Particulate Accumulation	Myocardial Degeneration
Grand Isle	8/23/2011	10	1.1	3.1
	*8/24/2011	5	3.2	4.0
	9/13/2011	10	2.8	2.7
	10/11/2011	5	2.0	2.0
Vermillion Bay	7/6/2011	10	2.1	2.4
	8/25/2011	7	1.6	2.1
	9/15/2011	10	1.6	3.2
	9/21/2011	5	2.8	1.4
Grand Isle	7/17/2012	10	2.4	3.2
	8/21/2012	10	2.1	1.8
	9/11/2012	10	3.3	2.6
Vermillion Bay	7/12/2012	10	1.2	1.3
	8/22/2012	10	3.2	2.8
	9/18/2012	10	2.9	2.3

*=Older age class not included in statistical evaluations; Severity scores were calculated on a 1-4 scale (1 being not present, 2 being mild, 3 being moderate, and 4 being most severe) for each organ of each fish. The average score is displayed for each school of fish.

Table 2.4. Average severity score of particulate accumulation and collagen deposition identified in wild Gulf menhaden schools.

			Stomach	
Location	Date	N	PAH Particulate Accumulation	Collagen
Grand Isle	8/23/2011	10	1.5	1.7
	*8/24/2011	5	2.6	1.8
	9/13/2011	11	2.8	3.1
	10/11/2011	5	2.2	1.2
Vermillion Bay	7/6/2011	10	1.6	1.2
	8/25/2011	8	1.8	1.6
	9/15/2011	10	1.8	1.9
	9/21/2011	5	2.8	1.8
Grand Isle	7/17/2012	10	2.8	2.2
	8/21/2012	9	2.1	1.8
	9/11/2012	9	2.8	2.2
Vermillion Bay	7/12/2012	9	1.6	1.4
	8/22/2012	10	3.2	1.9
	9/18/2012	9	2.3	2.0

*=Older age class not included in statistical evaluations; Severity scores were calculated on a 1-4 scale (1 being not present, 2 being mild, 3 being moderate, and 4 being most severe) for each organ of each fish. The average score is displayed for each school of fish.

Table 2.5. Prevalence of black particulates in heart and stomach vasculature.

Location	Date	N	Heart	Stomach
Grand Isle	8/23/2011	10	10%	40%
	*8/24/2011	5	100%	100%
	9/13/2011	10/11	80%	100%
	10/11/2011	5	100%	100%
Vermilion Bay	7/6/2011	10	90%	40%
	8/23/2011	7/8	43%	63%
	9/15/2011	10	50%	70%
	9/21/2011	5	100%	100%
Grand Isle	7/17/2012	10	80%	100%
	8/21/2012	10/9	90%	89%
	9/11/2012	10/9	100%	100%
Vermilion Bay	7/12/2012	10/9	20%	56%
	8/22/2012	10	100%	100%
	9/18/2012	10/9	90%	89%

*=Older age class not included in statistical evaluations; N Values: (Heart)/(Stomach)

Table 2.6. Comparison of combined lesion prevalence in menhaden collected from Louisiana waters between 2011 and 2012.

Tissue	Lesion Present	N	2011	2012
Gill	*Telangiectasis	64/60	44%	8%
	*Epithelial lifting	64/60	90%	25%
	Hyperplasia	64/60	97%	95%
Heart	Black Particulates	62/60	63%	80%
	Myocardial Degeneration	62/60	90%	80%
Stomach	*Black Particulates	64/56	70%	89%
	*Collagen Presence	64/56	56%	80%

* = Significant difference between years sampled (Chi Square; $p<0.05$); N Values: (2011)/(2012)

Table 2.7. Presence of peaks from specific Selected-ion monitoring chromatograms (SIM) of black particulates isolated from hearts, fresh Macondo oil (MC252) and weathered oil collected from Bay Jimmy.

Associated Compound	Ion	Retention Time	Particulate	MC252 Oil	Bay Jimmy Oil
Naphthalene	128	13.4	S	S	S
		14.0	S		
Fluorene	166	21.2	S		S
Methyl Fluorene	180	21.2	S		
		26.4		S	S
		26.6		S	S
Phenanthrene	178	28.2	S	S	S
		28.9			S
		29.1			D
		29.3			S
		31.0	M		M
Methylphenanthrene	*192	30.7	D	D	D
		31.2	D	D	D
		31.6	S		
Dimethylphenanthrene	*206	33.0	D	D	D
		33.3	S	S	S
		33.4	S	S	S
		33.5	S	S	S
		33.7	D	D	D
		38.9	S		
Trimethylphenanthrene	*220	35.4	D	D	D
		35.5	S	S	S
		35.8	D	D	D
		36.1	D	D	D
Dibenzothiophene	184	25.8		S	S
		28.3	S		
		29.9	S		
Methyldibenzothiophene	198	29.7	D	S	S
		30.1	D	S	S
		30.7	D	S	S
Dimethyldibenzothiophene	*212	31.8	S	S	S
		32.2	M	M	M
		32.6	D	D	D
		33.0	D	D	D
Pyrene/Flouranthene	202	34.8	S	S	S
Methylpyrene/Flouranthene	216	36.6	S	S	S
		37.0	S	D	D
		37.6	D	D	D
		37.7	S	S	S
Dimethylpyrene/Flouranthene	227	40.7	S	S	S

* Indicates multi-peak SIM profiles match with South Louisiana Oil extracted ion chromatograms from EPA Archives (Wang et al. 2003). S indicates single peak at a centered retention time, D indicates double

peaks at a centered retention time, and M indicates multiple overlapping peaks present at a centered retention time.

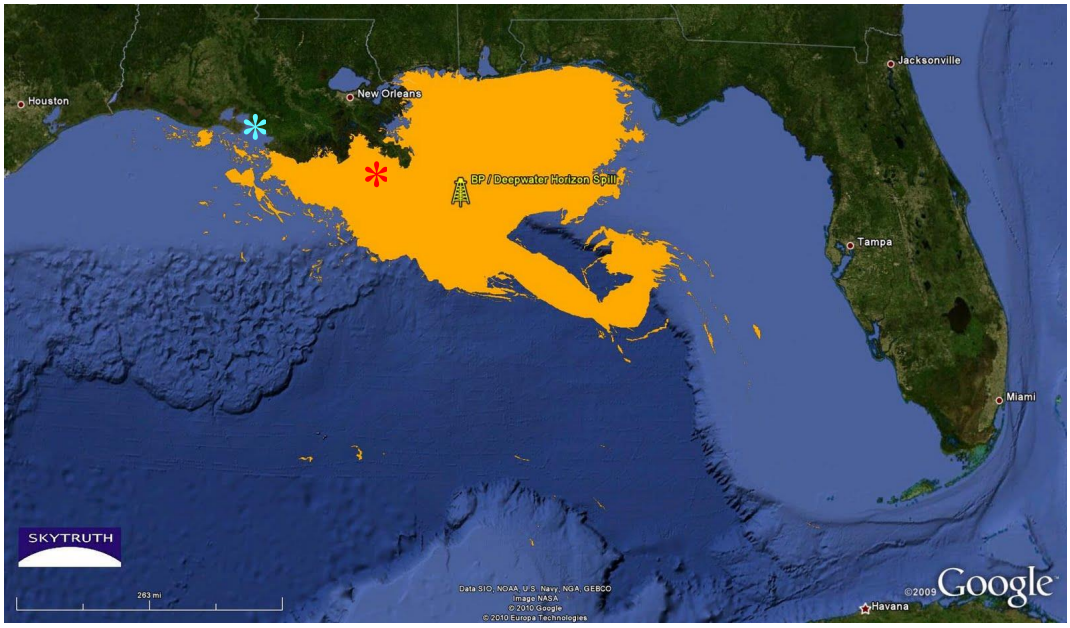

Figure 2.1 Total area affected by Deepwater Horizon oil surface slick (orange). (*) indicate approximate sampling locations (Blue: Vermilion Bay; Red: Grand Isle). Map created by Skytruth using NOAA data; blog.skytruth.org/2010/09/bp-gulf-oil-spill-cu

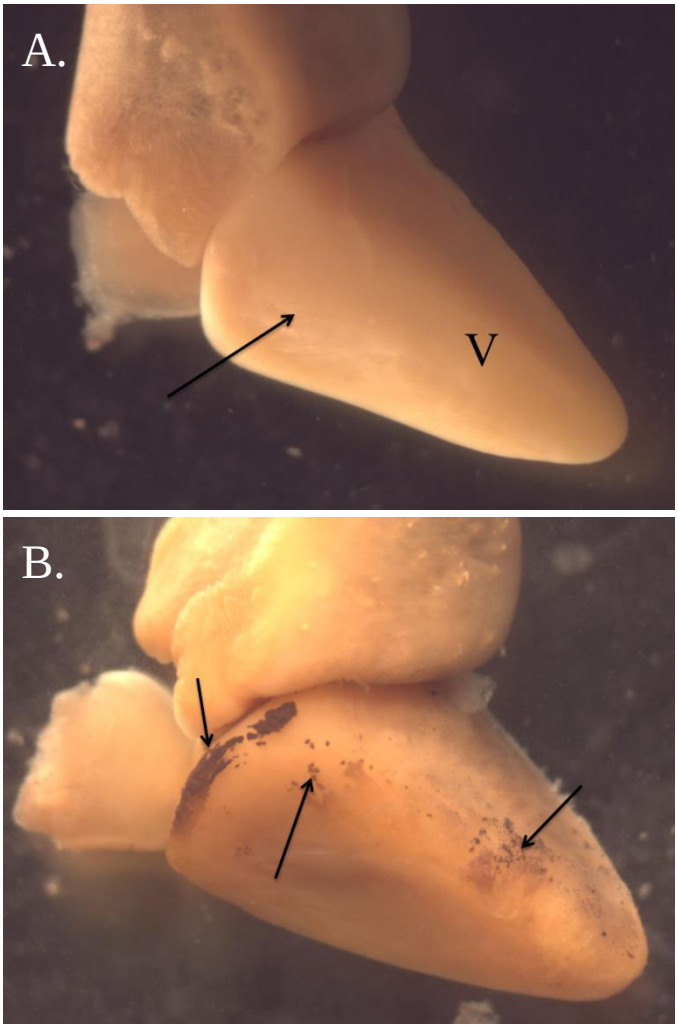

Figure 2.2 (A) Normal menhaden heart with creamy complexion of the ventricle (V) and smooth surface (arrow). (Stereomicroscope, Low magnification). (B) Abnormal menhaden heart with patches of surface pigmentation (arrows). (Stereomicroscope, Low magnification)

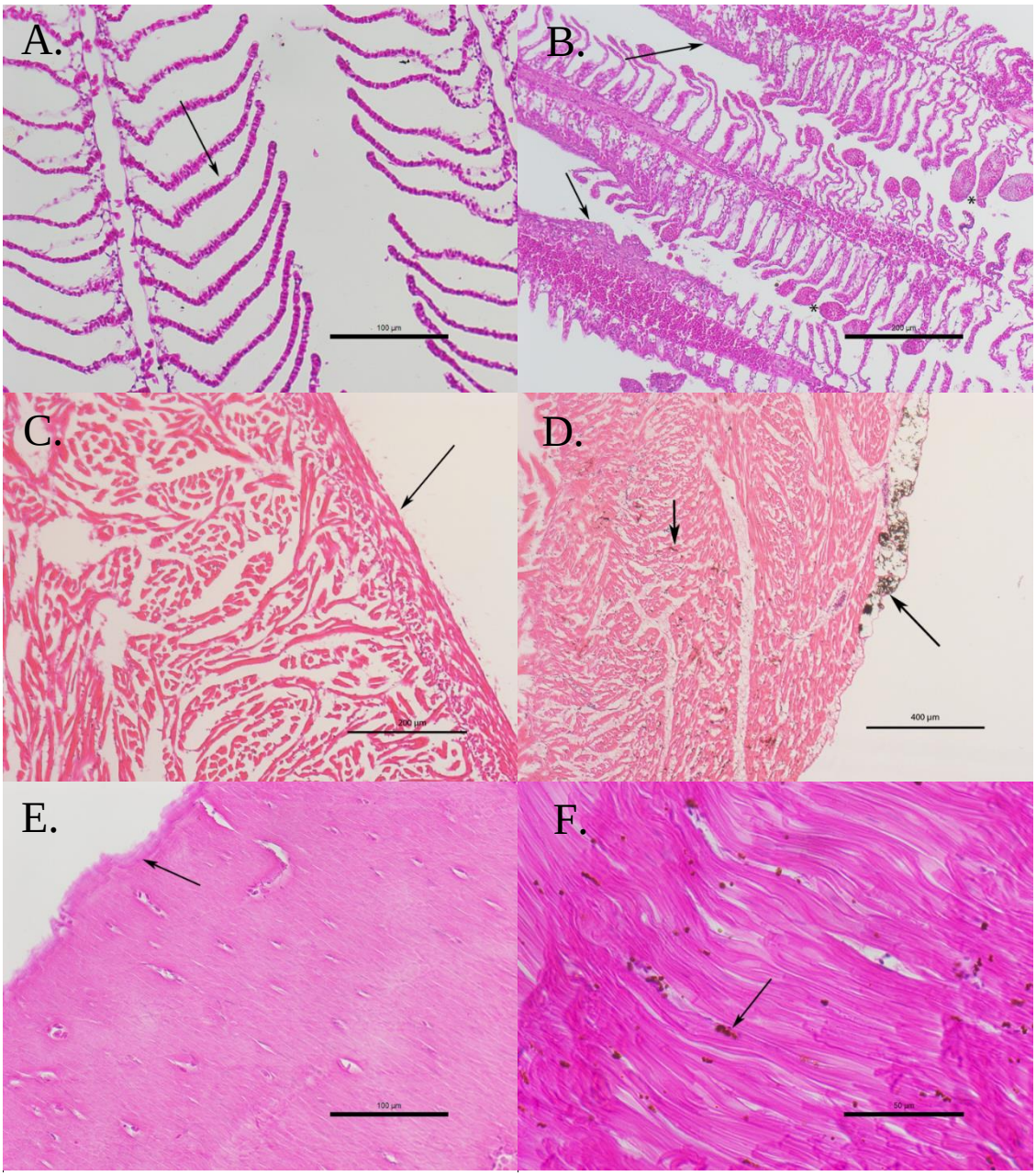

Figure 2.3 (A.) Normal secondary lamellae (arrow) in menhaden. (H&E, 200x) (B.) Moderate hyperplasia and minor fusion of secondary gill filaments (arrows) with secondary lamellar telangiectasia (*). (H&E, 100x) (C.) Normal menhaden ventricle with thin ventricle walls and uniform myocardium. (H&E, 100x). (D.) Lesioned menhaden ventricle with abundant black particulate in myocardium and ventricle wall (arrows). (H&E, 50x) (E.) Normal menhaden stomach muscularis, note smooth edge (arrow) of serosa and lack of pigmented material in capillaries and between muscle fibers. (H&E, 200x) (F.) Menhaden stomach muscularis with abundant particulate in muscle fibers. (H&E, 400x)

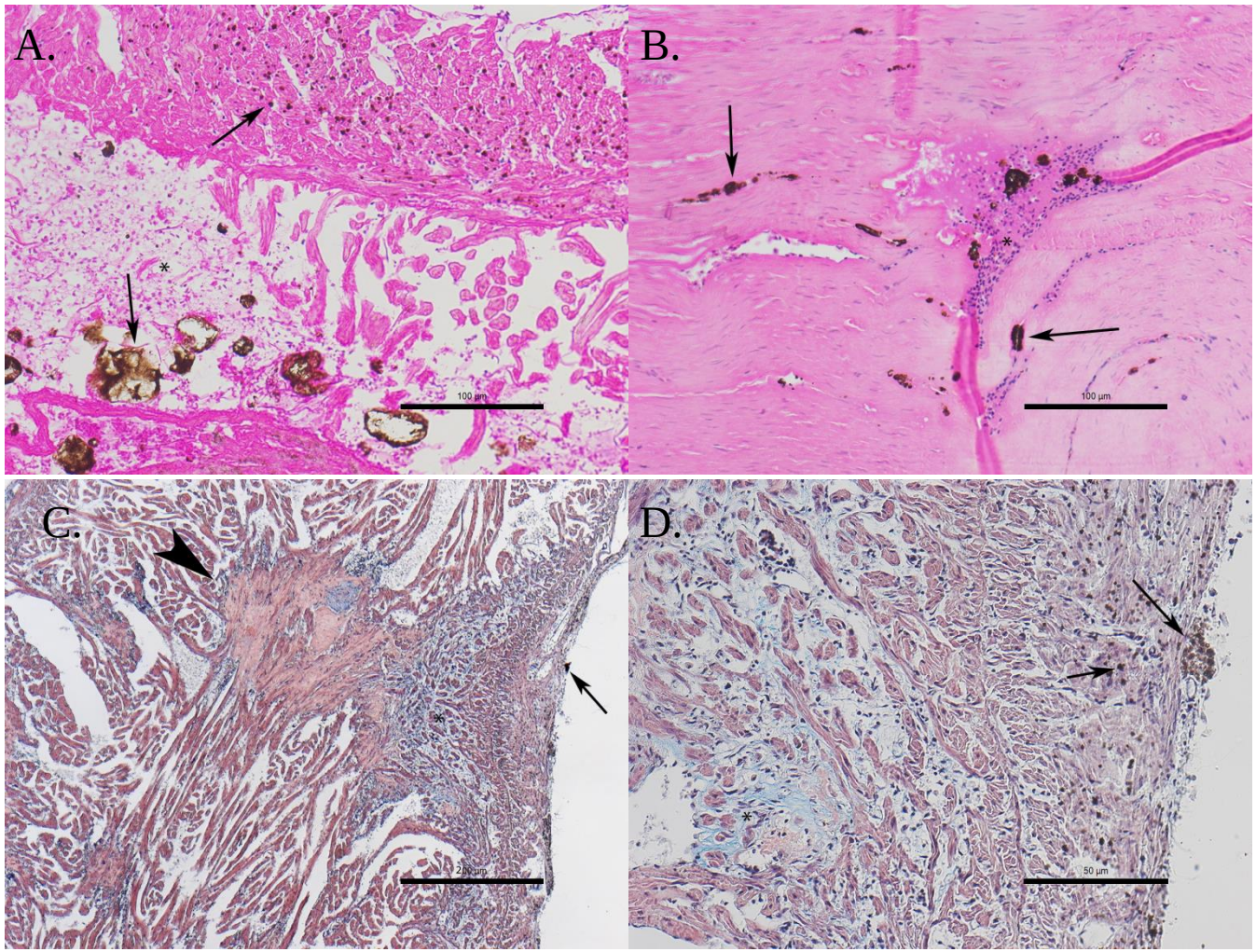

Figure 2.4 (A) Large accumulation of eosinophilic and degenerative material (*) in myocardium; abundant black particulates are also present (arrows). (H&E, 200x). (B) Stomach muscularis with focal necrosis and immune response (*). Also note particulates embedded

between muscle fibers (arrows). (H&E, 200x). (C) Abnormal ventricle with collagen deposition (*) and damaged ventricular wall (arrow). (Masson's Trichrome, 100x). (D) Higher power of Figure 4C showing collagen deposition (*) and black particulate (arrows). (Masson's Trichrome, 400x)

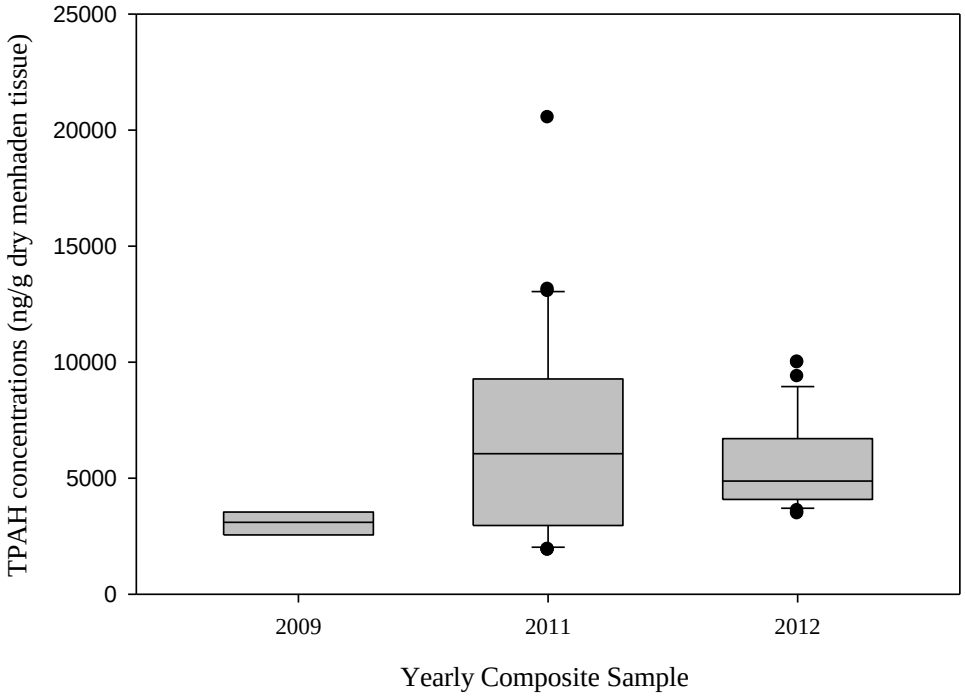

Figure 2.5 Composite TPAH concentrations in menhaden samples from summers of 2009, 2011, and 2012 with error bars representing standard deviation. There was a significant increase in TPAH concentration in 2011 and 2012 cohorts when compared to the reference sample (p=0.012). Due to sampling restraints in 2010, only 6 fish collected were able to be used for chemical analysis, resulting in an average TPAH concentration of 1443.00 +/- 159.54 ng/g dry menhaden tissue based on 3 composites (2 fish per composite). These results were omitted from the graph due to the small sample size not believed to be representative of the conditions in the Gulf at the time.

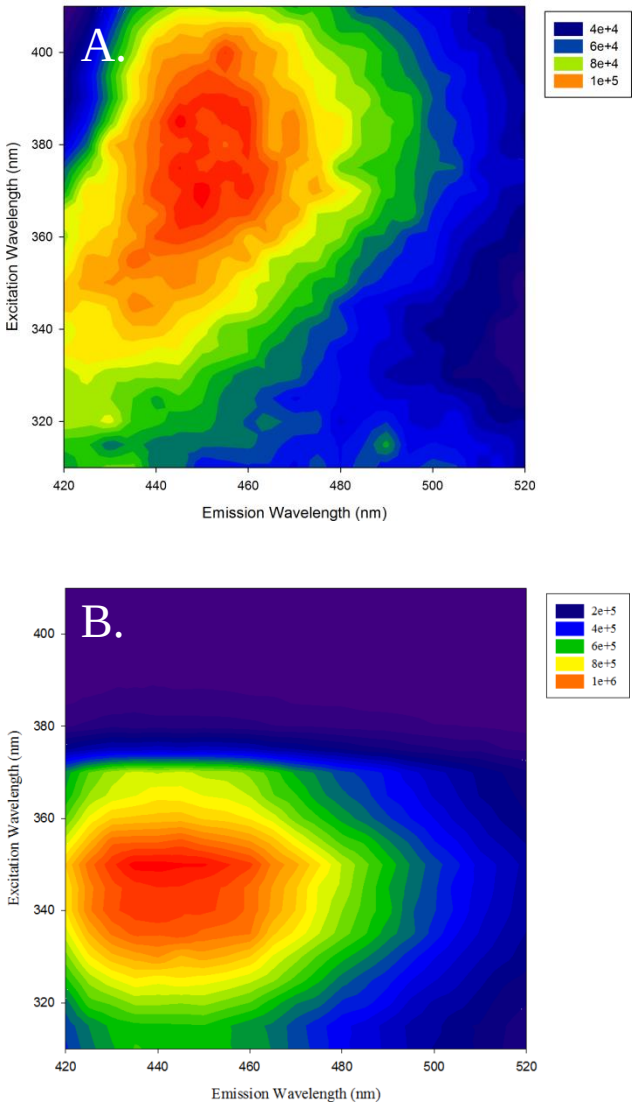

Figure 2.6 Excitation and emission wavelengths for particulates isolated from heart tissue of Gulf menhaden collected in Grand Isle 2011 (A) and a PAH reference, BbF (B).

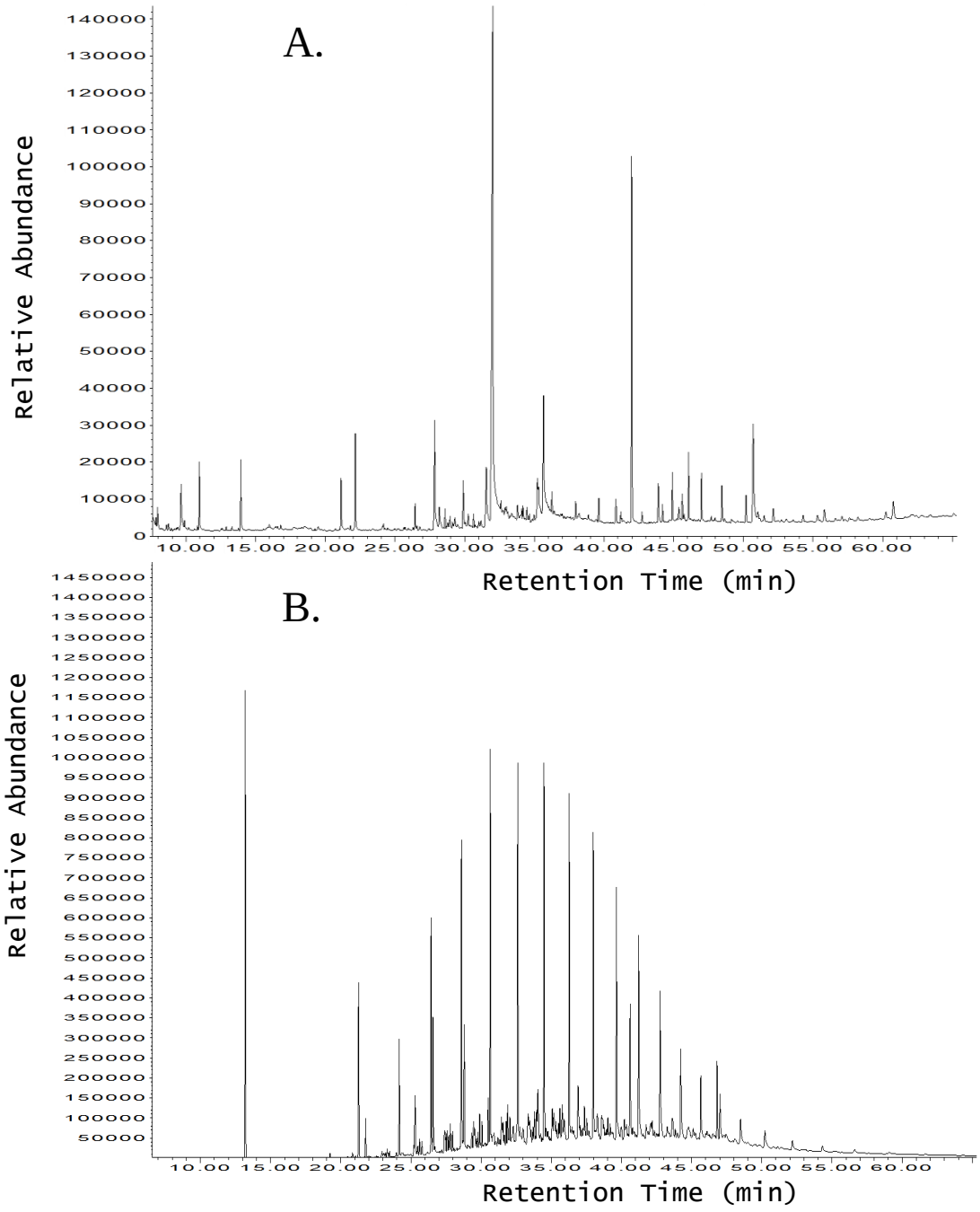

Figure 2.7 Total ion column chromatograms (TIC) from (A) black particulate isolated from Gulf menhaden hearts from the northern Gulf of Mexico and (B) weathered MC252 crude oil collected from Bay Jimmy.

CHAPTER 3: ACUTE EXPOSURE TO A CHEMICALLY DISPERSED SWEET CRUDE OIL CAUSES NEUROSENSORY LESIONS IN BREVOORTIA PATRONUS AND GILL LESIONS IN TRACHINOTUS CAROLINUS

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* NOTE: Methods 3.2.1 through 3.2.5 were conducted at the LUMCON facility and at Louisiana State University by the collaborators, acknowledged as co-authors. This chapter has been written in the format for submission to a journal in the field by the primary author, with methods input and editing assistance from collaborators.

Abstract

The toxicological effects following a 24 hour exposure of COREXIT 9500A dispersed Macondo crude oil to wild caught juvenile Gulf menhaden (*Brevoortia patronus*) and Florida pompano (*Trachinotus carolinus*) were examined. We hypothesized that the sensory and gill tissue of menhaden would be more sensitive to exposure based on anecdotal reports of unusual schooling behavior in previous studies of this species. The LC50s for menhaden and pompano were calculated to be 0.5 (95% CI=0.46-0.55) ppm and 3.5 (95% CI= 3.0-4.0) ppm total petroleum hydrocarbons (TPH), respectively. Hemorrhage at the base of the olfactory lamellae was prevalent in chemically enhanced water

accommodated fraction (CEWAF) exposed menhaden compared to control. Degeneration of olfactory lamellae and neuromast tissue was also more common in exposed menhaden. No significant differences in gill lesions were observed in menhaden. No significant olfactory/neurosensory lesions were observed in the pompano, but exposed fish did show a higher prevalence of secondary lamellar adhesion and gill epithelial hyperplasia.

Highlights

- Comparison of CEWAF oil toxicity to juvenile Gulf menhaden and Florida pompano.
- Gulf menhaden are more sensitive than Florida pompano to the CEWAF of a Louisiana sweet crude oil based on 24 hour LC50.
- Neurosensory tissue was damaged in treated Gulf menhaden; gill tissue was damaged in treated Florida pompano.

3.1 INTRODUCTION

Mangroves, fish, corals, and shellfish have all been shown to be negatively affected by oil exposure and oil dispersant mixtures (Tansel et al. 2015; Tjeerdema, Bejarano, and Edge 2013; Goodbody-Gringley et al. 2013; Vignier et al. 2015). Protection of these vulnerable resources as well as coastal habitats from dispersants is typically regulated at the state level; however, the Deepwater Horizon oil spill (DHOS) was a unique case where federal agencies implemented responses as deemed necessary. The EPA mandated that no more than 10,000 gallons of dispersant be used per day in the months following DHOS. Despite this regulation, the length of the spill created a scenario where the amount of dispersant intentionally released was greater than most other accidental spills in the United States. Approximately 2.1 million gallons of oil dispersant (COREXIT 9527 and COREXIT 9500) were applied in combination at the wellhead and on the surface slicks after the spill (Kujawinski et al. 2011). COREXIT application led to an increased penetration of oil material into the water column, increasing the chance for exposure to fish species inhabiting 1 meter beneath the slick. TPH concentrations ranging from background levels (< 0.01 ppm TPH) to 1.6 ppm TPH were detected at this depth after dispersant use (BenKinney et al. 2011). Field studies conducted after the DHOS found that some pelagic fish species had increased PAH concentrations in their tissues relative to previous years or reference sites, which indicates an exposure in excess of the background contributions from natural seeps (Murawski et al. 2014; Ylitalo et al. 2012; Bentivegna et al. 2015).

Gulf menhaden (*Brevoortia patronus*) and Florida pompano (*Trachinotus carolinus*) are species of fish native to the Gulf of Mexico that are abundant in areas impacted by the DHOS. Gulf menhaden are seasonally migratory and move from estuaries

and coastal waters to deeper open waters for spawning from October to March (Lassuy 1983). Movement inshore of their planktonic larval stage coincided with the DHOS (April 20th, 2010 - September 19th, 2010) and juvenile menhaden development occurred throughout the duration of the spill. The maximum density for menhaden populations is in the north central Gulf, primarily off the coast of southeastern Louisiana (Lassuy 1983; Vaughan, Shertzer, and Smith 2007) in the areas significantly affected by the spill and ensuing remediation efforts. Gulf menhaden are planktivorous filter-feeders capable of filtering millions of l of water on a yearly basis, which may contribute to a reported increase in sensitivity to crude oil exposures (Fucik, Carr, and Balcom 1995). Measurements in Atlantic menhaden (*Brevoortia tyrannus*), a similar species, showed a filtration rate of 15.2 l per minute or 3.9 million l in a 180 day period per fish, assuming constant filtration (Lippson 1991). Another study determined a maximum filtration rate of 24.8 l per minute in adult fish and a maximum particle size of greater than 1 mm (Durbin and Durbin 1975). Previous studies have shown that menhaden diets include a variety of zooplankton, phytoplankton, and detrital matter from multiple sources of varying sizes (Deegan, Peterson, and Portier 1990; Durbin and Durbin 1975). Due to this filtering capacity and range in food sources, it is likely that menhaden in the Gulf were exposed to crude oil constituents in the water column, including the soluble fraction, dispersed oil droplets, contaminated plankton (Almeda et al. 2014), and other detrital debris associated with the DHOS (marine snow) (Passow et al. 2012). Menhaden play a key role as forage fish in marine food webs and are involved in the redistribution of nutrients and energy from estuaries to near shore waters (Deegan 1993; Robinson et al. 2015). Consequently, as these fish school near the surface to avoid predators or to feed they interact with the micro-

surface layer of the ocean which may contain elevated levels of contaminants (Wurl and Obbard 2004). Commercially, Gulf menhaden make up the second largest fishery (by weight) in the United States and commercial landings contributed to an average revenue of 163 million dollars between 2000 and 2005 (McCrea-Strub et al. 2011; Vaughan, Shertzer, and Smith 2007). The total catch in 2014 was 391,854 metric tons, compared to the previous 5-year mean of 505,262 metric tons (National Oceanic and Atmospheric Administration (NOAA) 2015). The negative impacts of oil spills on Gulf menhaden and their fishery become difficult to quantify due to the challenges of laboratory rearing, field sampling, and defining of exposure histories.

Florida pompano inhabit similar coastal habitats as menhaden but prefer the shallow surf zone of the barrier island beaches. As adults they are commonly found further offshore and sought out by recreational fisherman for both sport and consumption. Florida pompano command high dockside prices due to their prized taste and limited adaptation for aquaculture (Main et al. 2007). Spawning behaviors have not been readily observed in the wild; however, pompano breed offshore in the spring and again in the late summer based on the capture of pelagic larvae 24 km off the west coast of Florida (Finucane 1969) and the presence of juveniles in nearshore estuarine and intertidal zones throughout the summer and into the fall (Main et al. 2007; Gilbert 1986). These young-of-the-year (YOY) fish feed on a variety of available prey items including post-larval shrimp, crabs, and small mollusks (Bellinger and Avault Jr 1971). Juvenile menhaden and pompano occurrence along the beaches and within the coastal estuaries would have overlapped with the DHOS shoreline oiling, possibly causing exposure during these sensitive early life stages (McCrea-Strub et al. 2011).

Gill tissue is commonly used for histological and molecular biomarkers to assess fish health (along with liver and kidney) (Bernet et al. 1999; Bentivegna et al. 2015), especially in crude oil and contaminated sediment exposures (Spies et al. 1996; Costa et al. 2009). Fish gills are essential for respiration, pH balance, and nitrogenous waste elimination, so any evidence of impaired function could negatively alter the fitness of the organism (Evans 1987). Olfactory and neurosensory tissues are not as commonly assessed in toxicological studies. Recent data has supported the use of these neurosensory tissues as a toxicological endpoint for metal exposures (Froehlicher et al. 2009), but there is a dearth of recent data on olfactory and lateral line toxicity as a result of oil exposures. Gardner (1975) reported an impaired lateral line (neuromast) and olfactory tissues in field populations of menhaden and laboratory reared *Menidia* exposed to crude oil constituents, but laboratory studies have not pursued this observation.

Understanding the toxicity of chemically dispersed crude oil in native species with distinct ecological roles becomes important when conducting ecological risk assessments, modeling population effects, and determining remediation approaches. Laboratory reared finfish may not be the best surrogates for determining the sensitivity and toxicological responses of wild caught species. Some species of fish, including Gulf menhaden and Florida pompano, can be challenging to maintain in captivity and thus it has been difficult to assess pollution impacts. To further assess the potential impacts of chemically dispersed crude oil on pelagic fish from different trophic levels, wild caught Gulf menhaden and Florida pompano were investigated for their response to the chemically enhanced water accommodated fraction (CEWAF) of a Louisiana sweet crude oil. It was hypothesized that sensory and gill tissue of Gulf menhaden would be impacted to a greater extent than Florida

pompano due in part to their feeding mechanisms. The present studies' goal was to determine the dose response and LC50s for the CEWAF of Louisiana sweet crude oil and to determine the possible target organs of this exposure to juvenile Gulf menhaden (*Brevoortia patronus*) and Florida pompano (*Trachinotus carolinus)*. These studies compare the toxicity of a chemically dispersed crude oil to two native Gulf species not readily kept in captivity. Our results demonstrate the importance of examining neurosensory endpoints in CEWAF exposure studies, especially in the native filter feeder Gulf menhaden.

3.2. METHODS

3.2.1 Organism Collection and Laboratory Conditions:

YOY Gulf menhaden were collected the night before experiments between 8 pm and 12am by 3/8" square mesh cast net near the LUMCON facilities (Cocodrie, LA). They were held overnight in a 200 gallon recirculating system at 20 practical salinity units (psu) without food. YOY Florida pompano were collected by 3.5 m beach seine from West Timbalier Island, Louisiana and transported back to the marine center by boat in an oxygen aerated transport tank. These fish were first given a freshwater dip, and then were quarantined for two weeks with copper (Cutrine-Plus) levels at 0.25 mg/l to minimize external parasites. Temperature, salinity, and pH were monitored daily. Ammonia levels were only checked if fish were found dead or appeared stressed. An electric feeder on a timer provided 0.3 mm dry feed (Cargill, Minneapolis) multiple times a day.

In preparation for each treatment, 10 fish were placed into 76 liter circular fiberglass tanks containing 30 l of filtered seawater (5μm; 20 psu). There was a total of 6

menhaden control tanks, 18 menhaden exposure tanks, 4 pompano control tanks and 12 pompano exposure tanks. Fish were starved for at least 24 hours prior to exposure. Two grams of ChlorAm-X and 25 g of sodium bicarbonate were added to each tank to aid in controlling ammonia levels and buffering pH for the duration of the experiment. Water quality parameters including DO, temperature, and pH were monitored and maintained (DO $\geq$ 4 mg/l, temperature 24 $\pm$ 1°C, pH 8.4 $\pm$ 0.4) throughout the duration of each exposure.

3.2.2 Exposure Solution

Multiple CEWAFs of crude oil were prepared following the methods by Singer et al (2000) with the exception of using freshwater in lieu of saltwater. Different volumes of Macondo 252 Louisiana sweet crude oil (BP Energy Company) were added to 1800 ml of freshwater in a 2 l aspirator bottle to create a nominal range of exposure solutions. Corexit 9500 was gradually added at a 20:1 oil to dispersant ratio via syringe while being stirred at 220-230 RPMs with a 2 inch stir bar for 21 hours. Solutions were allowed to sit for 3 hours after mixing and 1700 ml of the aqueous layer was drained and added to exposure tanks. Control tanks received 1700 ml of freshwater to compensate for the extra volume of water. These nominal test solution concentrations were designed to produce mortality in 24-48 hours and were based on previously conducted range finding experiments. Actual concentrations determined via GC-MS and fluorescence spectroscopy are presented in the results section.

Water samples (450 ml) from exposure and control tanks were recovered and combined with their respective duplicate at the beginning and at the end of each trial for hydrocarbon analysis using GC-MS. Additional water samples (3 ml) were taken at the

beginning and end of each trial for fluorescence analysis to correlate GC-MS determined TPH concentrations and fluorescence. The initial fluorescence measurements were fitted to a standard curve of TPH concentrations to determine LC50 values for each species.

3.2.3 Exposure Scenario

Fish were typically exposed to test solutions for 24 hours. Tanks were designated as control or exposure, with two replicates being carried out for each treatment or control. Exposure concentrations ranged from 0.012 ppm to 1.45 ppm TPH for menhaden studies (total of 18 exposure tanks, 6 control tanks) and 1.5 ppm to 7 ppm TPH for pompano studies (total of 12 exposure tanks, 6 control tanks). Sub-surface water samples were collected into I-CHEM glass containers to determine TPH water concentrations using GC/MS and fluorescence spectroscopy. At the end of each 24 hour trial, 3 fish from each tank were preserved for histology.

3.2.4 Fluorescence Determined Water Concentrations

Water samples were collected for fluorescence analysis at the beginning of the exposure and at the termination. These samples were collected below the surface of the water by pipet in an attempt to prevent any surface oil from contaminating the sample. Samples were read with a Trilogy Laboratory Fluorometer (Turner Designs; model #7200-000) using the Crude Oil Fluorescent Module (model# 7200-063) according to methods reported by Booksh et al (1996). In order to establish a standard curve for the fluorescent units, the split samples were run for GC-MS analysis. Alkane and PAH concentrations were determined via GC-MS for water samples collected from holding tanks immediately after introduction of test solution as well as at the conclusion of the experiment, the same as for fluorescence analysis.

3.2.5 GC-MS Extraction Method and Instrumental Analysis

Extraction of PAHs and alkanes in CEWAFs followed methods outlined in EPA Method 8270D series and as described by Olson et al (2014). For analysis, flasks were rinsed with dichloromethane (DCM) to ensure the complete solubilization of all oil into the final, extractable liquid fraction. A 30 ml aliquot of DCM spiked with a known amount of standard surrogate was then added to 200 ml of water in a separatory funnel, mixed, and allowed to settle. The solvent layer was drained through an anhydrous sodium sulfate funnel into a flat bottom flask. The solvent addition and draining step were repeated twice. The solvent fraction was placed on a rotary evaporation system and concentrated to a volume between 5-10 ml DCM, transferred to a calibrated extraction thimble, and further concentrated to a volume of 1.0 ml using a nitrogen blow down evaporator. The DCM extract was then exchanged to hexane. The remaining hexane extract was reduced to a volume between 1-2 ml before using the nitrogen blow down evaporator to achieve a final volume of 1.0 ml hexane with extracted sample.

Analysis of samples, in addition to internal standards, was conducted using an Agilent 7890A GC fitted with a 0.25 mm ID × 30 m HP-5MS column and an Agilent 7683B autosampler. Parent PAH concentrations were calculated based on calibrations using a five-point curve which was repeated for each batch of samples analyzed. Alkylated PAH concentrations were calculated assuming the same response factors for each parent and corresponding alkylated analogues. The results for alkylated phenanthrene and anthracenes were reported as pairs to incorporate the uncertainty of the measurements and quantification based on the average response factor of the individual parent PAHs (EPA 1998). Data from each category were combined into TPH for comparison with fluorescence

data. A standard curve was generated using TPH and relative fluorescence to back calculate the initial TPH concentrations used in the determination of 24-hour LC50s for Gulf menhaden and Florida pompano.

3.2.6 Histology:

After the 24 hour exposure period, 3 fish from each trial were collected for histology. Fish were anesthetized with MS222 (Pharmaq, Hampshire, United Kingdom) and had their abdomen slit open to increase penetration of the fixative. Sacrificed fish were preserved and stored in 10% percent buffered formalin prior to being shipped to Rutgers University. Upon arrival fish were transferred to 70% ethanol and assigned accession numbers for tracking. Necropsy was performed and transverse section of fish snouts were taken to examine craniofacial nervous tissue and olfactory sinuses. The second gill arch was also removed and sectioned (laterally).

Lesion severity and prevalence were recorded in a non-blind preliminary evaluation. After all samples were examined and notable lesions determined, a randomized blind re-evaluation was conducted as recommended in Wolf et al (2015) . Accession numbers were hidden and slides were given new identification numbers by a separate researcher using a random number generator. Lesion severity was scored using a semi-quantitative grading system (not present, minimal, mild, moderate, and severe). Gill endpoints included secondary lamellar adhesion, secondary lamellar epithelial hyperplasia, lamellar epithelial lifting, telangiectasis, and any evidence of parasitism. Secondary lamellar adhesion was also quantified for each fish by counting the number of individual lamellae affected in 5 fields at 400x. Neurosensory lesions included cranial neuromast degeneration, olfactory lamellar degeneration, alterations in olfactory lamellar architecture

(spacing or adhesion), olfactory hemorrhage, craniofacial sinus exudate, as well as any evidence of craniofacial parasitism. After lesion grading, the severity scores for lesions were matched with their original accession numbers and analyzed for trends using Fisher's exact test and Chi-square.

3.2.7 Data Analysis

Water samples taken at the beginning and end of each trial were evaluated for TPH via GC-MS and fluorescence. A standard curve relating relative fluorescence and TPH concentrations was used to generate measured TPH in exposure tanks. This standard curve equation was determined to be $y = 0.3057\ln(x) + 3.5028$ (R^2=0.9). Survival data was fitted to log probit paper according to Litchfield and Wilcoxon (1948) and LC50 values were calculated. The concentration response curve for calculating the LC50 and 95% confidence interval (CI) used a modified Litchfield and Wilcoxon method (Litchfield and Wilcoxon 1948). The average percentage of dead fish was graphed against the measured TPH concentration for each cohort, and the statistically best fit line applied to the data.

Severity scores were tabulated for species and treatment. For statistical analysis of lesion prevalence, all fish exposed to test solution were grouped into a single "exposed" cohort due to small sample sizes and variability in the measured concentrations for each treatment. Lesion prevalence was compared using Fisher's exact test to determine significance ($p<0.05$) between control and treated fish. The number of secondary gill lamellae affected by adhesion in each fish (5 fields at 400x) were compared using Chi-square ($p<0.05$). SigmaPlot 11.0 software was used in statistical calculations.

3.3 RESULTS

3.3.1 Water Chemistry and Effects on Survival

Water quality parameters including DO, temperature, and pH were maintained at appropriate levels (DO ≥ 4 mg/l, temperature 24 ± 1°C, pH 8.4 ± 0.4) throughout the duration of the experiment. Control tanks were determined to have average TPH concentrations of less than 3 ppb which is consistent with background levels in the Gulf (Wade et al. 2016). The exposure concentrations ranged from 0.012 ppm to 1.45 ppm TPH for menhaden studies (total of 18 exposure tanks) and 1.2 ppm to 7 ppm TPH for pompano studies (total of 12 exposure tanks). Twenty-four hour CEWAF LC50 values were determined to be 0.5 (95% CI= 0.46-0.55) ppm TPH for Gulf menhaden and 3.5 (95% CI= 3.0-4.0) ppm TPH for Florida pompano based on the median concentrations determined for each exposure. There was 100% survival in the control fish.

3.3.2 Morphology and Histopathology

The mean total length of Gulf menhaden collected was 79 mm and mean wet weight was 4.9 grams. The mean total length of Florida pompano collected was 61 mm and mean wet weight was 2.8 grams. The mean total length for menhaden used for histopathology was 84 mm (std +/- 11) and their mean wet weight was 6.1 g (std +/- 2.0). The mean total length for pompano used for histopathology was 58 mm (std +/- 7) and their mean wet weight was 2.7 g (std +/- 0.8). No severe external lesions were observed in any of the fish preserved for histology.

Severity scores for each cohort were tabulated and are presented in Table 3.1. Control menhaden and pompano were determined to have minimal lesions to their olfactory lamellae (Figure 3.1A), lateral line (Figure 3.1C), and gill structures (Figure 3.2

A). Exposed Gulf menhaden suffered from a higher prevalence of olfactory lamellar hemorrhage (Figure 3.1B) as well as lateral line degeneration; in severe cases the neuromast was completely necrotic (Figure 3.1D). Olfactory associated hemorrhage was significantly more common in exposed Gulf menhaden compared to controls ($P<0.001$). There was an increase in the prevalence and severity of olfactory lamellar degeneration in exposed menhaden. The prevalence of exudate and cellular debris in craniofacial sinuses also increased in exposed menhaden (Table 3.2). Neuromast tissue associated with the lateral line system of the cranium was also severely affected in Gulf menhaden from the exposure cohort based on the increase in severity scores and prevalence. The average number of secondary lamellae involved in lamellar adhesion in Gulf menhaden was not statistically significant between control and exposure fish (out of approximately 150 counted per fish).

Pompano exposed to the CEWAF of crude oil displayed an increased prevalence of lamellar adhesion (Figure 3.2B, 3.2C). The occurrence of this adhesion increased from 44% of fish in the control group to 71% in the treatment (Table 3.3) and had multiple moderate and severe cases (Table 3.1). The average number of secondary lamellae involved in lamellar adhesion in Florida pompano also increased significantly ($P<0.001$; Chi-square), from 1 lamellae in controls to 96 in exposure (out of 180 counted per fish). Other lesions such as lifting of the epithelium of secondary lamellae and gill secondary lamellae epithelial hyperplasia were present in the majority fish regardless of exposure.

3.4 DISCUSSION

Gulf menhaden were more sensitive than Florida pompano to the CEWAF of Louisiana sweet crude oil based on a 7-fold decrease in LC50 (0.5 ppm vs 3.5 ppm).

Previous studies have determined LC50s of similar magnitude including 5.4 ppm TPH (48 hours) for Mysid shrimp and 7.6 ppm TPH (96 hours) for larval inland silverside minnows exposed to a CEWAF (Hemmer, Barron, and Greene 2011). The relatively higher sensitivity of menhaden to chemically dispersed Western Gulf of Mexico crude oil (96 hour LC50= 22.2 ppm TPH; embryonic study) has also been shown by Fucik et al. (1995) relative to other species including red drum (>100 ppm TPH), spot (68.2 ppm TPH), and inland silverside (59.4 ppm TPH) exposed to the same dispersed oil. This may be due to characteristics specific to menhaden and other clupeids. Clupeids include primarily planktivorous species and many of them, such as menhaden, are filter-feeders. This aforementioned extensive filtering, interaction with the neuston, and seasonal accumulation and storage of lipid (Deegan 1986; Dubrow, Hale, and Bimbo 1976), may make them particularly susceptible to oil droplets found in the upper water column (or on the surface) both before and after dispersant use.

Hemorrhage associated with the olfactory lamellae was present in 45% (N=41) of exposed menhaden in this study. An increased prevalence of degenerative and necrotic neuromast tissue was also evident. Pompano showed no indication of impaired neurosensory tissues after the exposure. Previous reports by Gardner (1975) described the presence of “crazy” or “spinning” menhaden near a nuclear generating station at Millstone Point, Connecticut as well as other areas of the Chesapeake and coastal New York and New Jersey. Some of their reported lesions from these field collections match those seen in this study, including olfactory hemorrhage and the presence of proteinaceous exudate in craniofacial sinuses. At the time, however, these effects were attributed to metals or some mixture of effluent in these urban watersheds and not to crude oil. Anecdotal reports from

after the DHOS include observations of disoriented menhaden; this study provides evidence of impairment to the neurosensory tissues responsible for orientation and schooling behavior after exposure to a dispersed crude oil. CEWAF exposed menhaden did show altered swimming behavior which was displayed as a loss of normal schooling as well as disoriented individuals swimming with the current as opposed to against the current as was observed in the control fish. DiMichele and Taylor (1978) also reported nasal and lateral line necrosis in 90% of their test animals *(Fundulus heteroclitus)* exposed to 10 mg/l naphthalene, a constituent of many crude oils, for 24 hours. Evidence collected in our and other studies indicate that the olfactory and lateral-line systems are important tissues that should be examined following xenobiotic exposures because of their apparent sensitivity and important role in fish behavior.

Gill lesions were elevated in Florida pompano exposed to the crude oil CEWAF, while menhaden had no significant trends in gill lesions as a result of the acute exposure. Fish gill epithelium serves as the main site for gas exchange, ion balance, pH regulation, and the elimination of nitrogenous waste (Evans 1987), which are all necessary processes to maintain homeostasis. Gill lesions such as epithelial hyperplasia, secondary lamellar fusion, and telangiectasis are commonly assessed as endpoints in crude oil exposure studies (Khan and Payne 2005; Bentivegna et al. 2015). It has been well documented that gill lesions are usually non-specific and can occur after exposure to a variety of compounds including metallic salts and organic contaminants (Mallatt 1985). Contaminants such as crude oil (both dissolved and droplets) can penetrate gill epithelium and lead to an imbalance in homeostasis or even death in acute exposure studies (Ramachandran et al. 2004). Gill epithelial tissue is readily repaired after removal of a suspected irritant;

however, it is important to document and characterize the effects seen in studies especially if the concentrations of concern are causing mortality. In our study we believe, the extent of the gill damage in the Florida pompano was sufficient to disrupt normal gill functions that could result in the observed lethality.

Dispersants have been shown to be effective in remediating oil spills by increasing the surface area of oil droplets thus maximizing the metabolic potential of oil degrading bacteria (Prince and Butler 2013; Prince et al. 2013). It is clear that the situational use of dispersants can be beneficial, and there is a common understanding that consequences of dispersed oil in pelagic ecosystems would be less severe than the direct coastline oiling (George-Ares and Clark 2000). Regardless, sensitive life stages of menhaden, as well as other commercially important species of fish such as snapper, grouper, and tuna, were likely present in areas affected by the remedial efforts of the spill (National Oceanic and Atmospheric Administration (NOAA) 2010). Limited studies have been conducted on commercially important pelagic fish and their response to CEWAF fractions of crude oil. Menhaden and pompano have not been extensively examined for toxicological effects following oil spills and the subsequent actions taken, and thus remain relatively understudied members of the pelagic community. This study demonstrates that differences in lifestyle (Gulf menhaden, a filter feeding planktivore and Florida pompano, a higher trophic level consumer) of the organism likely alters the target organ system primarily affected by the CEWAF of crude oil.

Menhaden and pompano collected in this study were from wild populations and tested after a 24-hour acclimation period, which likely explains the occurrence of lesions in the control cohorts. Lesions that occurred with no statistical differences between

treatments are indicative of baseline lesions present in the wild caught population. We have previously reported the occurrence of lesions in wild Gulf menhaden from the northern Gulf of Mexico with varying severities between dates and location of collection (Bentivegna et al. 2015). Understanding an organism's or a group of organisms' exposure history is pivotal in determining the severity of effects seen. It is also important to note that for histopathological analysis the different exposures were collapsed into "exposed" versus "non-exposed" groups for prevalence analysis due to the small number of fish provided per treatment group. Organisms that did not survive the exposure were not included in histopathological analysis, therefore skewing the results towards less impacted surviving individuals. The observed mortality could be due to the anesthetic effects of PAH components, damage to the gill, damage to the lateral line systems, or other factors.

The observed organ systems in Gulf menhaden and Florida pompano responded differently to CEWAF exposures. Our lab reports that the prevalence of neurosensory lesions increased in menhaden exposed to the CEWAF of crude oil. The nervous tissue of the lateral line enables menhaden to properly school for protection; failure in the wild to orient to their surroundings could result in affected individuals being removed from the population by predators. These lesions occurred at TPH concentrations lower than used in the Pompano exposure, supporting our hypothesis that menhaden are more susceptible to CEWAF oil exposure, despite an apparent difference in target organ systems. Gill tissue in Florida pompano was significantly impacted by exposure to a crude oil CEWAF, likely resulting in an impaired ability to maintain homeostasis. There is a need to better characterize the impacts of TPH and crude oil CEWAF on clupeids because of their unique feeding behavior and important role in food-web dynamics.

Acknowledgements

The authors would like to thank members of Louisiana Fish and Wildlife, Louisiana State University, Louisiana University Marine Consortium, Sea Grant, the Rutgers New Jersey Agriculture Experiment Station, and the New Jersey Department of Environmental Protection for the funding, resources, and collaboration that support this research project. Funding for this work was provided by LDWF-LSU contract 69670; LSU Project Number 36469-3; NJAES-Rutgers NJ01201; NJWRRI FY2015 Program Project ID 2015NJ369B (USGS Grant Number G11AP20094).

All animals were handled humanely following LSU IACUC protocol 13-087.

The authors declare they have no conflict of interest.

Table 3.1. Summary of lesion prevalence and severity in YOY Florida pompano and Gulf menhaden exposed to the CEWAF of Macando 252 crude oil. Number examined is the N for the control/treatment; numbers in the row of the lesion type are the number of individuals showing the lesion which are listed again by severity down the column.

		Pompano		Menhaden	
Measured concentration (ppm TPH)		Control	1.2 - 4.5	Control	0.012 - 0.84
Gills	Number examined	18	24	18	41
	Lamellar Adhesion	8	17	3	7
	Minimal	3	5	2	7
	Mild	3	1	1	-
	Moderate	1	8	-	-
	Severe	1	3	-	-
	Lamellar Epithelial Hyperplasia	15	22	5	10
	Minimal	13	14	5	10
	Mild	-	7	-	-
	Moderate	2	1	-	-
	Severe	-	-	-	-
	Lamellar Epithelial Lifting	17	19	0	2
	Minimal	10	5	-	2
	Mild	4	7	-	-
	Moderate	3	7	-	-
	Severe	-	-	-	-
	Lamellar Telangiectasis	1	3	1	2
	Minimal	1	3	1	2
	Mild	-	-	-	-
	Moderate	-	-	-	-
	Severe	-	-	-	-
	Evidence of Parasitism	3	4	4	18
	Minimal	1	1	2	17
	Mild	-	1	2	1
	Moderate	2	2	-	-
	Severe	-	-	-	-
Transverse Anterior Cranium	Number examined	18	25	17	40
	Olfactory Associated Hemorrhage	2	1	0	13
	Minimal	2	-	-	2
	Mild	-	1	-	2
	Moderate	-	-	-	4
	Severe	-	-	-	5
	Olfactory Lamellar Degeneration	4	6	6	25
	Minimal	3	5	5	8
	Mild	1	-	-	10
	Moderate	-	1	1	4
	Severe	-	-	-	3
	Olfactory Spacing/Adhesion	1	3	3	17
	Minimal	1	1	1	7
	Mild	-	-	-	6
	Moderate	-	2	2	3
	Severe	-	-	-	1
	Lateral Line Neuromasat Degeneration	7	2	2	15
	Minimal	3	1	1	9
	Mild	4	1	1	3
	Moderate	-	-	-	-
	Severe	-	-	-	3
	Frontal Sinus Exudate	0	8	8	26
	Minimal	-	4	4	12
	Mild	-	3	3	4
	Moderate	-	1	1	5
	Severe	-	-	-	5
	Evidence of Parasitism	0	11	11	25

Minimal	-	6	6	9
Mild	-	4	4	14
Moderate	-	1	1	2
Severe	-	-	-	-

Table 3.2. Comparison of the prevalence of cranium and gill lesions in control and CEWAF exposed Gulf menhaden. *****indicates statistical significance (Fisher's Exact Test; $P < 0.05$). N=18 for gill control, 17 for cranium control, 41 for gill exposure, and 40 for cranium exposure.

	Menhaden Control %	Menhaden Exposure %	P-Value
Gill			
Lamellar Adhesion	17	17	1.000
Epithelial Hyperplasia	28	24	0.758
Epithelial Lifting	0	5	1.000
Telangiectasis	6	5	1.000
Evidence of Gill Parasitism	22	44	0.149
Transverse Anterior Cranium			
*Olfactory Associated Hemorrhage	0	45	0.001
Olfactory Lamellar Degeneration	35	63	0.083
Olfactory Spacing/Adhesion	18	43	0.128
Lateral Line Neuromast Degeneration	12	38	0.064
Frontal Sinus Exudate	47	65	0.247
Evidence of Cranial Parasitism	65	63	1.000

Table 3.3 Comparison of the prevalence of cranium and gill lesions in control and CEWAF exposed Florida pompano. * indicates statistical significance (Fisher's Exact Test; $P < 0.05$). N=18 for both gill and cranium control, 24 for the gill exposure, and 25 for the cranium exposure.

	Pompano Control %	Pompano Exposure %	P-Value
Gill			
Lamellar Adhesion	44	71	0.117
Epithelial Hyperplasia	83	92	0.638
Epithelial Lifting	94	79	0.214
Telangiectasis	6	13	0.623
Evidence of Gill Parasitism	17	17	1.000
Transverse Anterior Cranium			
Olfactory Associated Hemorrhage	11	4	0.562
Olfactory Lamellar Degeneration	22	12	0.427
Olfactory Spacing/Adhesion	6	8	1.000
Lateral Line Neuromast Degeneration	39	36	1.000
Frontal Sinus Exudate	0	0	1.000
Evidence of Cranial Parasitism	6	0	0.419

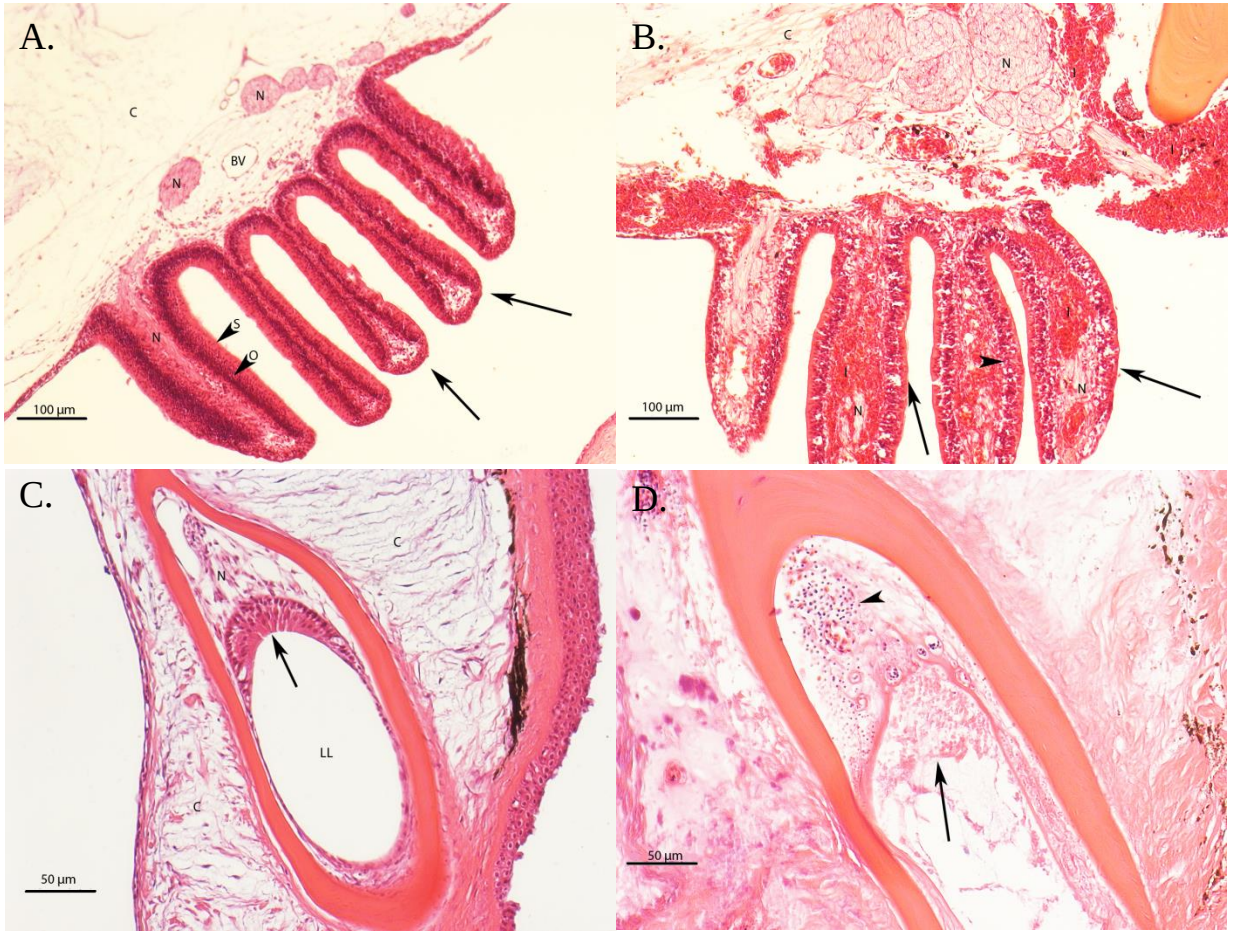

Figure 3.1 A. Evenly spaced menhaden olfactory lamellae (arrows) with well-defined sustentacular (S arrowhead) and olfactory receptor (O arrowhead) cell layers. Note clear demarcation of nerve bundles (N), blood vessel (BV) and healthy connective tissue (C) (H&E, 50x; Menhaden

Control). B. Hemorrhagic menhaden olfactory lamellae (arrows). Loss of sustentacular/olfactory organization (arrowhead) with blood infiltration (I) into the nerve bundles (N) and connective tissue (C) (H&E, 50x; Menhaden Exposure). C. Normal menhaden lateral line (LL) with well-defined neuromast cells (arrow), nerve fibers (N), and normal connective tissue (C) (H&E, 100x; Menhaden Control). D. Necrotic menhaden lateral line with completely degenerative neuromast; sloughing of neurosensory epithelium (arrow) into the lateral line canal and inflammation associated with nerve fibers (arrowhead) is present (H&E, 100x Menhaden Exposure).

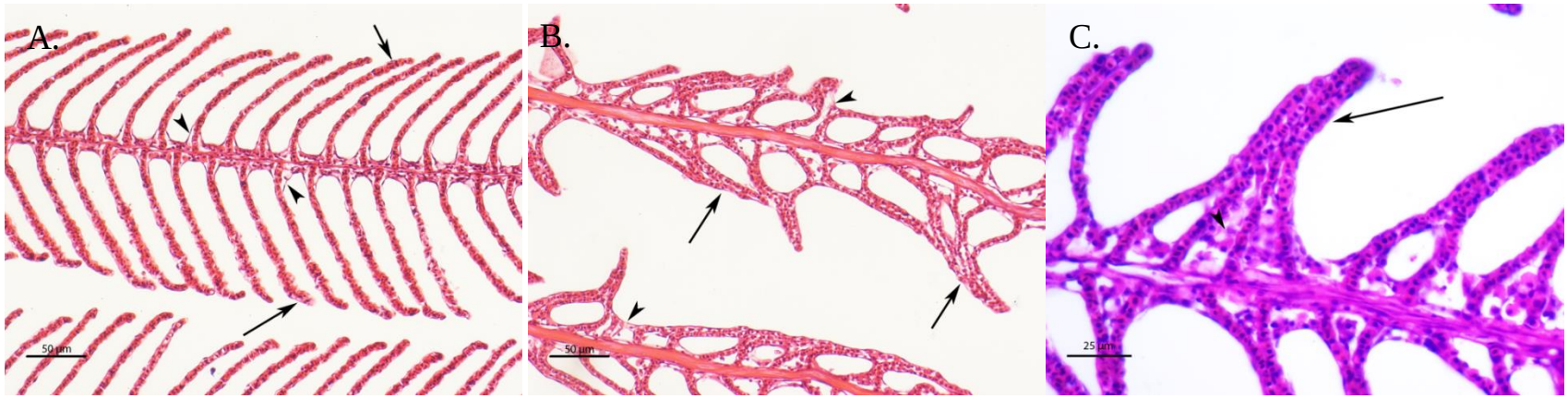

Figure 3.2 A. Consistent and evenly spaced pompano gill secondary lamellae (arrows). Slight lifting of epithelial cells at base of secondary lamellae (arrowheads); possibly an artifact of fixation (H&E, 50x: Control Pompano). B. Severe adhesion of secondary lamellae with complete loss of normal spacing (arrows). Severe epithelial lifting evident (arrowheads). (H&E, 50x Pompano Exposure). C. Higher magnification of fully adhered lamellae (arrow) and hyperplasia associated with the loss of epithelium due to extensive adhesion (arrowhead) (H&E, 100x Pompano Exposure).

CHAPTER 4: UPTAKE AND DISTRIBUTION OF MICROPARTICLES IN THE ZEBRAFISH (*DANIO RERIO*) AND ATLANTIC KILLIFISH (*FUNDULUS HETEROCLITUS*)

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Completed with fund from NJWRRI Project ID 2015NJ369B (USGS Grant Number G11AP20094); formatted for submission to a journal in the field.

Abstract:

The uptake and distribution of suspended particulates by aquatic organisms is not well characterized. Our recent research has shown that there can be physical mechanisms for particulate toxicity in wild fish collected from the Gulf of Mexico. Particulates derived from PAHs and microplastics are of specific concern because of their ubiquitous presence in urbanized environments and potential to store and desorb lipophilic pollutants over time. Our previous findings prove that particulates are present in the microvasculature, heart, stomach, and gills of wild menhaden. We hypothesize that particulates in the relevant size range (0.2-5 µm) will cross gastrointestinal and gill epithelium leading to accumulation within the tested fish species, zebrafish and mummichog. These findings could be used to further asses the toxicological potential of these particulates as well as contribute to knowledge of the fate of particulate loadings into the environment. There is a dearth of information on small particulates crossing respiratory and digestive mucosa, their toxicodynamics within the blood stream, and deposition into capillary beds within tissues.

4.1 INTRODUCTION

Organisms in aquatic environments are susceptible to a variety of pollutants from anthropogenic sources, especially in urbanized areas of freshwater and coastal watersheds. Increases in land development, impervious surfaces, and channelized waterways are known to increase urban run-off and significantly degrade aquatic habitats. Waterways and estuaries in New Jersey are severely impacted by the development of this infrastructure as is seen in the lower Passaic River and Barnegat Bay (Conway 2007; Walker, McNutt, and Maslanka 1999; Conway and Lathrop 2005; Kennish et al. 2007; Iannuzzi et al. 2004). Urban run-off contains a myriad of chemicals including heavy metals, pesticides, polychlorinated biphenyls, and PAHs (Walker, McNutt, and Maslanka 1999) which can create significant exposure scenarios in local wildlife. The acute/subchronic toxicity of these pollutants has primarily been studied as individual compounds; however, many laboratory studies overlook the physical exposure to particulates present in the environment.

Particulate matter, as an atmospheric pollutant, has been a concern in environmental toxicology for several decades (EPA Clean Air Act). These particles are classified by having a diameter less than 10 μm, with sub classifications for particles less than 5 μm and 2.5 μm in diameter. These size classifications are important because they differentiate between the particles' ability to penetrate and embed within the lungs of terrestrial mammals, most notably humans. Different size categories of PM are regulated differently based on this ability to penetrate the human respiratory system. Particles less than 2.5 μm are specifically known to embed deep in the lung and have been well-documented in clinical research to cause impaired pulmonary and cardiac function (EPA 2009). Particulate

composition, however, is believed to drive toxicological responses in environmental settings (Grantz, Garner, and Johnson 2003). Compounds ranging from metals, chlorinated hydrocarbons, and PAHs are present in varying concentrations on PM (Cormier et al. 2006). Concerns over the levels of PM in the atmosphere have come to the forefront of exposure science and human health studies; however, less attention has been paid to these particles in aquatic environments. Atmospheric deposition of PM directly into waterways and onto impervious surfaces (Kam et al. 2012) may result in their introduction into aquatic habitats.

Large scale events, such as oil spills, introduce massive volumes of heterogeneous hydrocarbon material that over time can become particulate in nature as a result of weathering and remediation processes. Burning and dispersant use will remove the smaller, more reactive and volatile components but leave behind residues comprised of larger, typically more toxic, PAHs. These weathered components of crude oil can assimilate into the food chain (Mitra et al. 2012; Graham et al. 2010) and accumulate in species from higher trophic levels. Suspended PM, specifically particulates derived from PAHs, has been overlooked as a significant source of toxic exposure to aquatic organisms.

The effects of suspended PM on aquatic species are not well characterized. The aforementioned EPA report on PM states that “a causal relationship is likely to exist between deposition of PM and a variety of effects on individual organisms and ecosystems” but goes on to explain that these data are limited and there are gaps in the research of these effects in aquatic environments (EPA 2009). Evidence of PM having ecological significance is focused primarily on plant physiology and other terrestrial studies, most notably in soil processes (Grantz, Garner, and Johnson 2003). Some research has been

conducted on the response of aquatic organisms to suspended solids, however, suspended solids are typically characterized as larger than PM and are more applicable to scenarios in which sediment is perturbed (Bilotta and Brazier 2008). Fish in these studies responded primarily by avoiding the disturbed sediment or showing physical signs of irritation to sensitive organ structures such as the gills (Robertson, Scruton, and Clarke 2007; Lake and Hinch 1999).

Studies on the aquatic toxicity of PM have not yet been conducted and a more thorough understanding of this toxicity is necessary to address ongoing concerns in ecosystem functioning (Grantz, Garner, and Johnson 2003). Our lab has shown that Gulf menhaden (*Brevoortia patronus*) from the northern Gulf of Mexico accumulated PAH based particulates in heart ventricle vasculature, and Florida pompano accumulated particles in the gill lamellae (Figure 4.1 A, B, & C). This accumulation occurred in a temporal pattern consistent with the DHOS, suggesting a mechanism by which PAH derived suspended particulates are created (by atmospheric deposition or crude oil weathering) and then taken up by aquatic organisms. Our finding shows a direct correlation to the particulates and focal lesions such as necrosis due to the blocking of microcapillaries and perhaps oxidative stress resulting from desorption of reactive PAHs. This finding is supported by a study not directly related to PM; Woodhead (1981) showed that black carbon (as India ink) was absorbed through the lower gastrointestinal track of the Amazon molly (*Poecilia formosa*) and distributed to the heart ventricle and kidney (Woodhead 1981). That study, although not specifically examining PM, determined that inert carbon

was absorbed into the fish, likely by macrophages. The proposed study will demonstrate a specific exposure scenario in which toxic particulates are absorbed into teleost species.

Our recent studies on aquatic pollutants have suggested that PAH-derived particulates may enter menhaden vasculature and deposit into capillary beds. Microplastics are also of great interest because of their ubiquitous distribution, persistence, and potential as carriers for lipophilic xenobiotics. Previous work on aquatic PM would indicate that acetylene black and plastic microspheres less than 2.5 μm will cross over gill and gastrointestinal epithelial tissue and enter the vascular system where they may become embedded in small capillary beds. In the current study, acetylene black was used as a surrogate for urban PM (Busby Jr and Newberne 1995) and fluorescent plastic microspheres were used to aid in the visualization of the uptake and distribution of particulate material.

Based on our previous works, particulate material will cross epithelial barriers of the gastrointestinal tract and gill lamellae, resulting in distribution to vital organs (kidney, heart) of the selected fish species. This accumulation can result in focal damage due to ischemia and desorption of reactive compounds from particulate surfaces. Two fish species, the zebrafish (*Danio rerio*) and the mummichog (*Fundulus heterclitus*), will be tested to examine the possible effect of feeding mechanism on particulate accumulation and determine the LC50 of the selected PM material. Zebrafish are a selective carnivorous fish commonly used as a model organism for toxicological research due to their easy maintenance and high fecundity in captivity. Atlantic killifish are a primarily detritivorous species with more significant applications to the natural estuarine environments found in

urban environments. Atlantic killifish have direct applications to field studies, and thus could be used in future biomonitoring projects.

This study's goal was to identify the potential toxic impacts of PM on aquatic organisms, specifically a relevant fish species. These particulates are environmentally relevant due to their deposition into the environment as a result of anthropogenic activities as well as potential for creation from larger scale events such as oil spills. Ultimately this project provides evidence of PM toxicity to aquatic organisms in a way that is meaningful and applicable to water resource management. There could be direct applications to regulate particulate loadings in the receiving waters of urbanized watersheds and as well as applications to post oil spill exposure assessments of affected wildlife. A better understanding of particulate toxicity in aquatic organisms is needed due to their formation, presence, and persistence in aquatic environments.

4.2 METHODS

All zebrafish handling methods were approved by the Rutgers IACUC guidelines. AB and Fli strain zebrafish were maintained on an Aquatic Habitats (Apopka, FL) Stand-Alone System (10% daily water change, 22–25 ◦C) with a 14:10 light:dark photoperiod. Adults were fed brine shrimp (*Artemia salina*) and TetraMin Flakes. Embryos were raised at 26◦C in embryo media.

4.2.1 Gavage Exposure

Two types of Fluoresebrite multifluorescent microspheres™ (0.2 μm and 1 μm in diameter; excitation maxima of 377, 517 and 588nm, and emission maxima of 479, 546 and 612nm) and acetylene black (a diesel soot surrogate (Busby Jr and Newberne 1995))

were used to assess the possible translocation of particulates across the gastrointestinal epithelium. Five to ten adult AB-strain zebrafish (approximately 24 months old) were acclimated to 10 gallon tanks for 48 hours prior to their first gavage. Zebrafish were fed throughout the course of the gavage studies with standard flake formulation in the evenings 3 hours after gavage recovery. Solutions containing 2.5% weight/volume of each of these materials were used to gavage individual adult fish with methods modified from Collymore et al (2013) (Collymore, Rasmussen, and Tolwani 2013). Acetylene black exposure solution and the respective controls also contained 0.4% Tween 20 surfactant to assist in the suspension of the particles. Prior to gavage, fish were each anesthetized with 200 mg/l of MS222 until they no longer responded to physical stimulus (pinching of the tail). Five µl of gavage solution was administered and fish were placed into an aerated recovery tank with fresh system water before being returned to the original holding tank. This procedure was carried out every other day for a week for a total of three oral gavages. After one additional day of depuration, fish were sacrificed for blood analysis and histopathology.

On day 6, fish were anesthetized with 200 mg/l of MS222 to enable collection of blood. The caudal fin was removed with an industrial razor blade and a smear was created with a drop blood collected from the caudal vein. At this point, the spine was severed and a cross section of the fish was taken from behind the pectoral fin, through the anterior body cavity and used to create and impression smear of the viscera, kidney, and muscle tissue onto slides coated with poly-L-lysine to promote the retention of muscle and viscera. Remaining tissues were frozen (fluorescent microspheres) or fixed in 10% buffered formalin (acetylene black) for additional histological analysis. Formalin fixed tissue

followed typical histological processing, was sectioned at 4-5 µm, and stained with hematoxylin and eosin for light microscopy.

4.2.2 Plate Exposure

Two-week old AB and Fli-strain zebrafish larvae were placed randomly into the first, third, and fifth rows of a clear polyethylene 48-well plate prior to exposure to the aforementioned Flouresebrite microspheres™ to test the ability of these plastic microspheres to cross gill or gastrointestinal epithelium in a static bath exposure. The zebrafish rearing solution was carefully removed from each well until 200 uL remained, at which point 800 µl of exposure solution or fresh rearing solution (for controls) was added giving a total volume of 1 ml and a concentration of 50 mg/l of fluorescent microspheres. Row one contained the control fish, row three contained the 0.2 µm microspheres and row 5 contained the 1.0 µm microspheres. Multiple replicates were conducted without feeding to maintain water quality in the small volumes. After 24 hours, fish were transferred to clean rearing solution to rinse off any remaining microspheres adhered to the surface epithelia, sacrificed with a lethal dose of MS222 and fixed in 10% buffered formalin. Due to the loss of fluorescence over time, fish were examined within 24 hours of the conclusion of the exposure to identify the location of fluorescent microspheres with an inverted microscope fitted with a fluorescent laser light source using a Trit C filter.

4.2.3 Acetylene Black Feed study

Twelve AB strain zebrafish, approximately 30 days post fertilization (dpf), were acclimated in 10 gallon aquaria for 24 hours prior to being fed a 2% mass/mass mixture of acetylene black in standard fish feed to assess the impact of acetylene black under chronic conditions. Each tank was given either 40 mg of control or spiked feed once per day for 60

days prior to sacrifice with an MS222 overdose. Water changes were conducted weekly to control for ammonia and nitrate levels. Temperature was maintained between 22 and 26 ºC throughout the duration of the experiment. Sacrificed fish were measured to assess growth over the 60-day exposure period and fixed in 10% buffered formalin. Histological processing was conducted as previously described.

4.2.4 Pulse-Dip Exposure

Adult zebrafish and mummichog were used to determine the penetration of particulate material after an acute, high dose exposure to acetylene black. Adult AB zebrafish (approximately 2 years old) were taken from our system after being retired from active reproduction. Adult mummichog were collected from Tuckerton, New Jersey and maintained in the laboratory at 22 degrees Celsius and 20 psu. Five fish of each species were exposed for 6 hours to 50 mg/l of acetylene black in a 2 L beaker to replicate conditions similar to a stormwater pulse event with elevated concentrations of suspended solids. The control and test solutions also contained 0.02% tween to assist in the initial suspension of the acetylene black particles. After six hours, the fish were placed into 1 liter of clean water to recover from the exposure and eliminate any surface adhesion of the particles. After this recovery fish were sacrificed with a lethal dose of MS222, fixed in 10% buffered formalin, and processed for histology using methods previously described.

4.2.5 Data analysis

The primary endpoint in these studies was to determine the presence of particles within the vasculature of zebrafish (*Danio rerio*) using microscopy and light imaging techniques. Fluorescebrite multifluorescent microspheres™ (0.2 µm and 1 µm in diameter; excitation maxima of 377, 517 and 588nm, and emission maxima of 479, 546 and 612nm)

were detected with a TRIT C filter. Histopathology was used to assess the accumulation of acetylene black particulates into fish gastrointestinal and gill epithelium. Organ systems were analyzed for abundance and severity of lesions associated with particulate exposure as well as any other lesions of note using guidelines and methods recommended by Wolf et al (2015). Prevalence data was assessed and where appropriate Chi-Square was used to determine statistical significance.

4.3 RESULTS

4.3.1 Gavage Exposure

There were a total of 10 fish gavaged with control material and 10 fish gavaged with 2.5% acetylene black. Five fish were examined for each of the fluorescent microsphere gavage cohorts. All fish survived the repeated gavage procedure. There was no conclusive evidence of accumulation of black particulate through the gastrointestinal tract in zebrafish after gavage. There appeared to be some accumulation in the gastrointestinal epithelium as well as some pigmented sections of the parietal pericardium, however this pigmented material was not overly abundant and could not be differentiated from the natural occurrence of melanin in zebrafish tissues.

Zebrafish gavaged with fluorescent microspheres had blood smears and imprint smears prepared the day after final gavage. There was no fluorescence detected in blood collected from the caudal vein relative to controls for either of the size classes of microspheres (Figure 4.2 A, B, & C). Imprint smears did show abundant fluorescent in the gastrointestinal tract, but there was no detectable fluorescence in other tissues.

4.3.2 Acetylene Black Feed study

There was 100 percent survival of fish fed a spiked acetylene black feed for 60 days. There was no significant difference in the growth of exposed fish compared to control fish based on the weight/length ratios and comparison (Figure 4.3). Particulates also did not appear to cross the gastrointestinal epithelium based on the histological analysis. Similar to the acetylene black gavage, areas of possible particulate accumulation could not be definitively differentiated from natural melanin deposits.

4.3.3 Plate Exposure

There were no differences in mortality of fourteen dpf zebrafish exposed for 24 hours to 50 mg/l of fluorescent microspheres relative to controls. Exposed larvae actively ingested and appeared to accumulate particulates within the gastrointestinal tract; there was no fluorescence present in controls (Figure 4.4 A & B). Using the plane of focus, it was determined that any particles present outside of the gastrointestinal tract were adhered to the surface of the fish. Flourescent particles within the fish would dampen and lose "resolution" if there was tissue blocking the light.

4.3.4 Pulse-Dip Exposure

There was 100% survival in zebrafish exposed to 50 mg/l acetylene black with 0.02 % tween, with minimal accumulation of particulate material on the external epidermis. Four out of 8 mummichog died as a result of the same exposure conditions while there was 100% survival in the controls. There was abundantly more acetylene black material associated with the external epidermis and gill tissue. There was a concentrated accumulation of black particulate beneath the operculum, on the ventral side of the gills. This collection of material appeared to be organized based on some type of removal

mechanism from the gills. Histology results indicate that there is acetylene black present in the buccal cavity and between gill lamellae, but there is no evidence of particulate material within gill vasculature.

4.4 DISCUSSION

The initial findings of this study suggest that larval zebrafish ingest particulate material in bath exposures and this material likely enters other organ systems as well. Fluorescent microspheres are ingested and concentrate in the gastrointestinal tract, with some occurrence of fluorescence in other tissues. The lack of fluorescent particles in the blood of zebrafish gavaged with 2.5% solutions of fluorescent particles indicates that there is minimal uptake via the gastrointestinal tract, at least that results in the blood from the caudal vein. Imprint smears also support this finding; there was no fluorescent particles in tissues other than the gastrointestinal tract. In mammalian studies, uptake of fluorescent beads (and other PM) appears to occur through the Peyer's Patches: lymphoid nodules associated with the lower gastrointestinal tract. Teleost species lack this lymph tissue and thus it is not surprising to find minimal evidence of particulate uptake. Previous studies have stated that microplastics can accumulate in the liver after absorption through the gastrointestinal tract, although it remains unclear as to whether the gills play an important role in particulate uptake (Lu et al. 2016; Avio, Gorbi, and Regoli 2015).

Zebrafish may not be the best model to determine particulate accumulation based on their lack of a stomach and relatively short gastrointestinal tract compared to species that are classified as herbivores and detritivores. Amazon mollies, a similar teleost species with regards to ecological niche, showed negligible uptake of particulate material as well based on the resemblance to melanin (Woodhead 1981). The results of the acetylene black

bath exposure to mummichog further led us to believe that particulates are not likely able to cross epithelial barriers alone.

Additional studies should further examine the presence of particulate material in teleost vasculature and how this material is deposited there. Natural conditions are also difficult to replicate in microcosms and may play a role in an advanced mechanism of uptake Our earlier field studies (Millemann et al. 2015) indicate that PAH derived particulates are present in menhaden hearts, but it is not known whether these particles enter the vascular system or if they are formed within the tissues as concentrations of PAHs accumulate. Prolonged exposures to low doses of PAHs may elucidate a mechanism behind this formation, or if particles can cross epithelial barriers due to secondary damage. Stressful conditions in urban environments contribute to secondary gill or gastrointestinal damage due to concurrent exposures. Chemicals like dispersants increase membrane permeability which would increase particulate uptake. Gill and intestinal parasites may also play a large role in the accumulation of particulates in fish vasculature based on an increased immune response or other pathology.

Suspended PM toxicity is rarely examined in aquatic toxicology and could greatly impact the critical water resources. It has been demonstrated that atmospheric deposition of soot particulates (Gigliotti et al. 2002) as well as the weathering of crude oil contaminants (Passow et al. 2012) are at least partly responsible for the introduction of these particulates into aquatic ecosystems. Other particles, such as microplastics, have been identified and characterized in multiple surface waters. The accumulation of these particles in fish species has not been quantified and is not well understood. These studies lay a

foundation to further test the potential for particulates (both PM and plastics) to cross gastrointestinal or gill epithelia under a variety of exposure conditions.

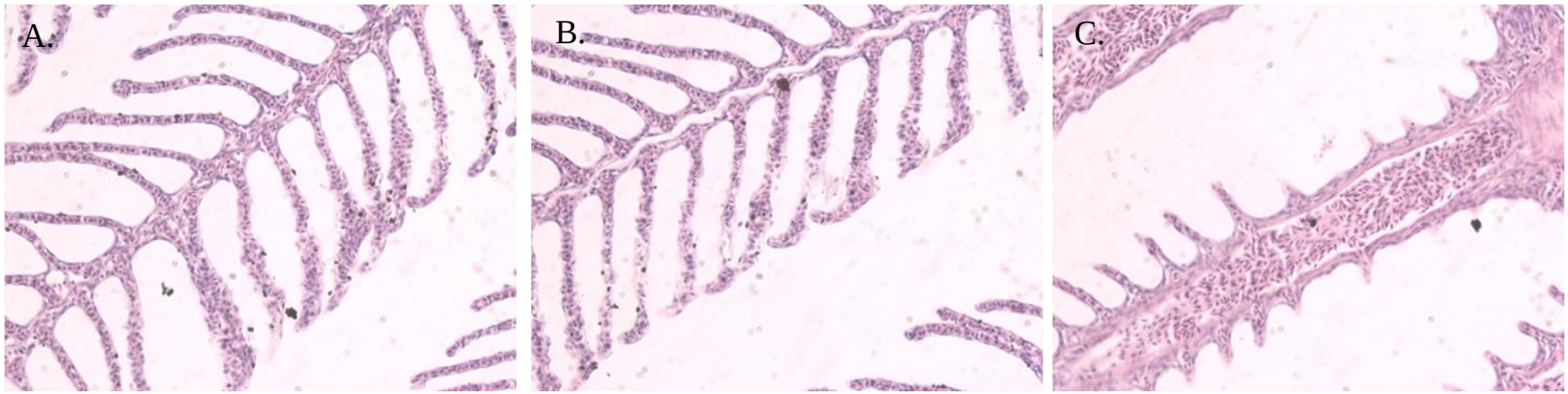

Figure 4.1 Particulate material entering vasculature in Florida pompano (A, B, C) through primary gill lamellae. H&E 100x

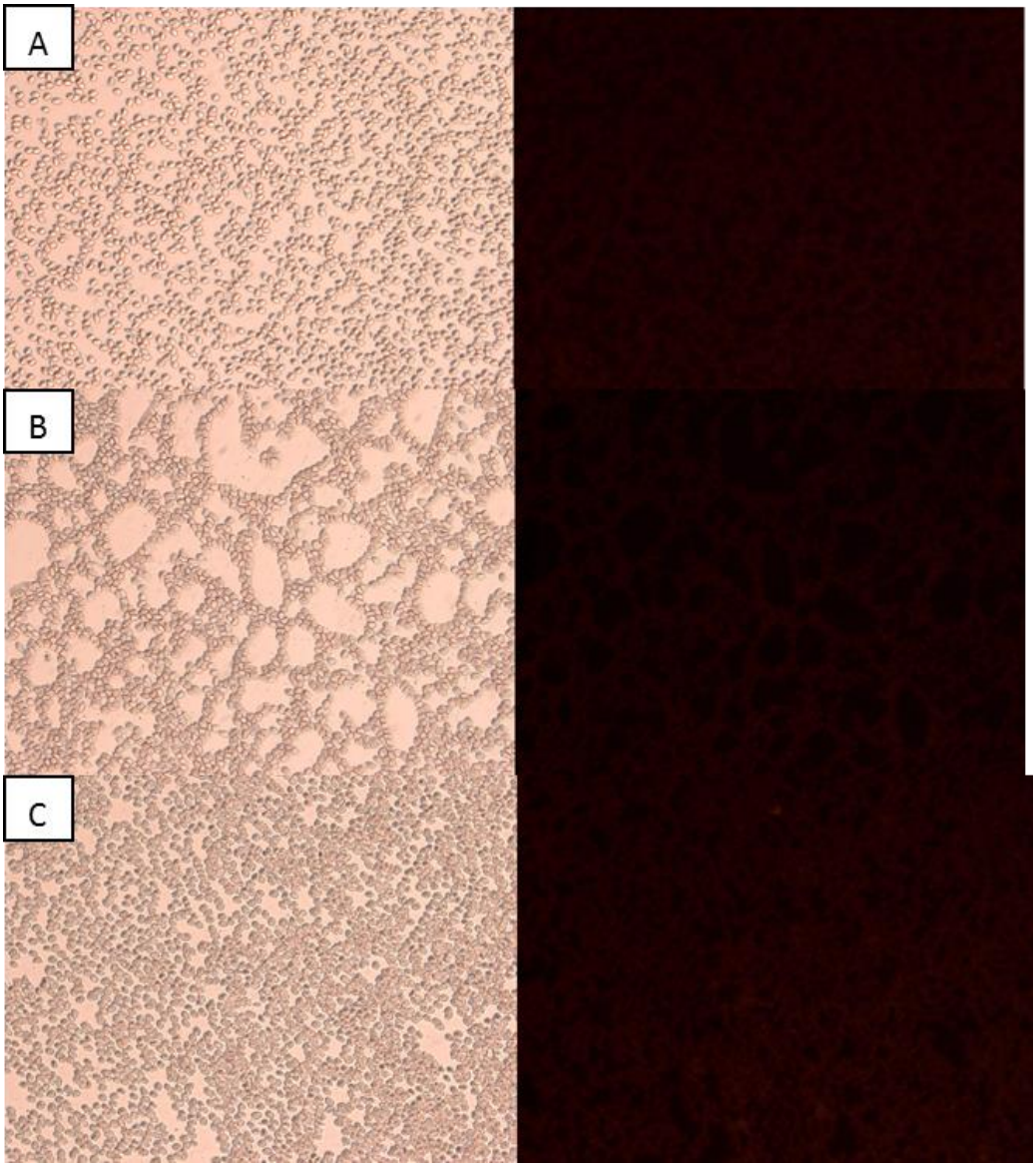

Figure 4.2 Comparison of blood smears of control (A), 0.2 µm (B) and 1.0 µm (C) microsphere gavaged fish. Left column is light microscopy, right column is fluorescent light with TRIT C filter.

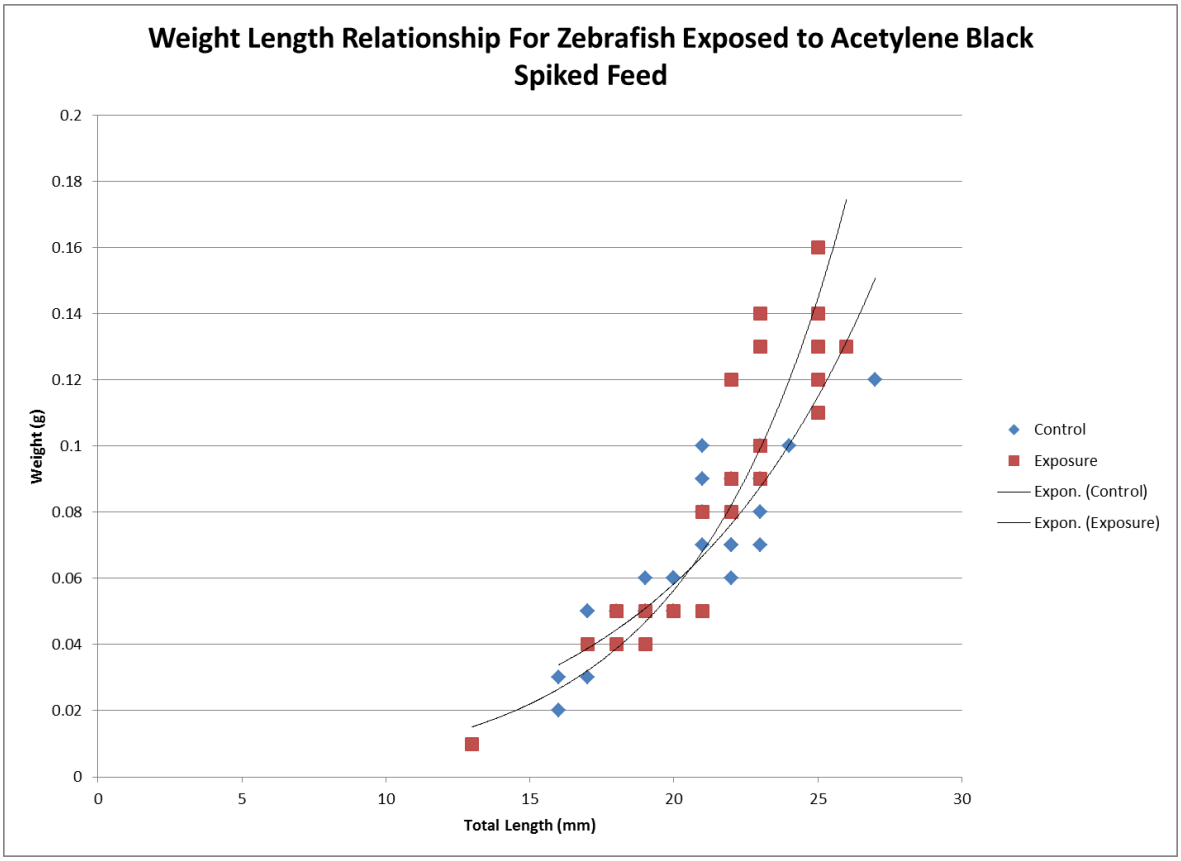

Figure 4.3 Comparison of the weight and length of fish exposed to acetylene black spiked feed relative to controls.

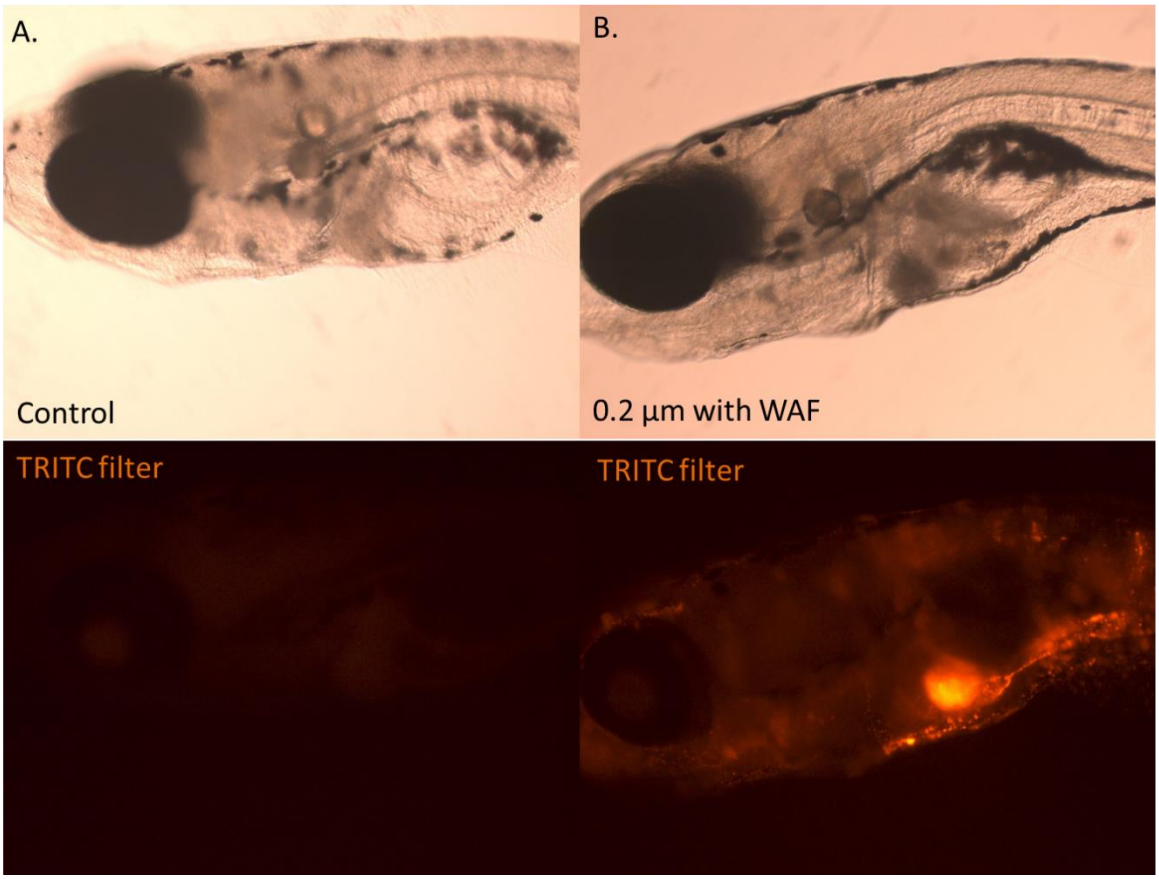

Figure 4.4 Fluorescent microspheres present in the gastrointestinal tract of larval zebrafish as well as in other tissues. Additional methods are being tested to eliminate dampening of accumulated particulates from the larger accumulation in the gastrointestinal tract to identify potential sinks for particulate material.

CHAPTER 5: DISCUSSION, SUMMARY OF THE DISSERTATION, AND FUTURE STUDIES

On April 20th, 2010, the largest marine oil spill in United States history began. Approximately 4.9 million barrels of crude oil were released into the northern Gulf of Mexico as a result of the Deepwater Horizon disaster (McNutt et al. 2012). In an effort to diminish the mass of oil, remediation techniques including dispersant application and burning were implemented. While these actions may have affected both offshore and inshore species, no massive fish kills were reported and the oiling of coastal wetlands and pelagic cetaceans and fish was believed to be reduced. In situ burning and dispersant applications altered the natural state of the oil and may have provided more widespread contamination in the air and water, but reduced the amount of oil potentially reaching sensitive wetlands. These remediation efforts did contribute to deposits of weathered oil that were observed along coastlines and within the water column, possibly contributing to chronic exposures in sensitive habitats or increasing the bioavailability of certain crude oil components.

This dissertation examined the possible acute and chronic impacts of the DHOS to a filter feeding species of fish based on field observations, collections, and an acute dispersed oil exposure. It was hypothesized that the filter feeder, Gulf menhaden (*Brevoortia patronus*), exposed to crude oil will show lesions in the gills and heart representative of acute and chronic exposures, based on the time of collection and exposure scenario. Lesions observed in Gulf menhaden from laboratory exposures (Chapter 3) and field collections (Chapter 2) were typical of what might be expected after an acute exposure to crude oil and/or dispersant materials, thus supporting our initial hypothesis. Comparative

studies with a carnivorous species of fish, Florida pompano, further investigated species sensitivity and differences in target organ systems between menhaden and pompano. These results have implications in how responders may approach remedial strategies based on the species present and supports identifying the most sensitive species affected for risk assessment purposes. Additional findings from our field studies of menhaden indicated that particulate material derived from PAHs accumulate in the vasculature of this fish species. These particulates, when present in tissue, appear to result in localized damage either from physical blockage resulting in focal ischemic areas (Figure 2.4) or from the reactivity of absorbed chemicals desorbing from the particulate. Laboratory exposures were used to demonstrate possible uptake mechanisms in zebrafish and mummichog exposed to particulate material, to further investigate target tissues for this uptake and distribution. These model organisms are more readily available and are able to be housed in typical laboratory settings without large-scale commercial aquaculture systems used to maintain larger marine species. Acetylene black, a diesel particulate surrogate, and fluorescent polystyrene microspheres (Fluoresbrite®) were used to examine particulate uptake and ability to penetrate epithelial barriers.

One of the major drawbacks of the data presented in Chapter 2, and in most oil spill events, is the lack of a reliable baseline or background data. We were able to obtain some samples of Gulf menhaden, our species of interest, to analyze for PAHs from before the spill, and did see significant increases in PAH concentrations when those were compared to later years (Figure 2.5). Despite this, there has been very limited work on menhaden histopathology, especially in the gulf and the ubiquitous nature of PAHs makes any conclusions with regards to the specific toxicological impacts due solely to the DHOS of

the spill uncertain. PAHs can enter the water through atmospheric deposition or runoff as well as directly from natural oil seeps or small spills in the Gulf. The background occurrence of the observed lesions is not well documented in the northern Gulf of Mexico, and it is quite possible that exposures to PAHs from natural seeps or other sources occur regularly for coastal species. The best approach to study chemical exposures to natural populations of fish is to examine temporal trends and find reference sites outside of the region of interest, but even these may not be reliable if prior conditions were not similar between sites (See Figure 5.1).

5.1 Observations of wild caught menhaden in years following Deepwater horizon

The goal of this chapter was to evaluate the histopathological condition of Gulf menhaden from the northern Gulf of Mexico in the years following the DHOS. It was hypothesized that histological evaluation of wild Gulf menhaden collected from waters overlapping with areas affected by the DHOS would reveal a spectrum of reversible to permanent lesions in the years following the spill. Gulf menhaden collected from VB (a reference site) and GI (a heavily oiled site) did not show any significant differences in the prevalence of lesions typical in fish exposed to crude oil. Fish were collected on multiple dates throughout the sampling season (late summer/early fall), and it appears that individual schools had similar histopathological conditions regardless of site (Tables 2.2, 2.3, 2.4). This is not surprising given the fact that menhaden schools travel along the coast and exposure history is not necessarily dependent on the location of capture. Each school likely has a very specific and unique exposure history, which is dependent on the movement of the school between different habitats. These movements are based on a number abiotic, biotic, and anthropogenic conditions, such as temperature, time of day,

turbidity, and fishing pressures (Kemmerer 1980). The small subset of menhaden collected in 2010 had low concentrations of PAHs, likely due to a lack of exposure from the actual spill itself. A defined exposure history would be useful to pinpoint the etiology of the observed lesions and concentrations of PAHs in schools of Gulf menhaden.

Telangiectasia and epithelial lifting of the secondary lamellae were the primary lesions identified in Gulf menhaden, in addition to the accumulation of black particles visible at necropsy and in micrographs of heart and stomach tissue (Figures 2.3 and 2.4). Analytical analysis of these particulates indicated that they comprised of primarily petrogenic PAHs (Table 2.7 and Figure 2.7), although due to the abundance of natural seeps in the Gulf of Mexico a definitive source could not be identified. These particles, when trapped in capillaries, can cause local ischemia and may also release reactive aromatic hydrocarbons to adjacent cells. It is very difficult to determine source oil from weathered oil, especially after any amount of biotransformation or partitioning occurs.

The observed gill lesions (telangiectasia, epithelial lifting) significantly decreased from 2011 to 2012. Gills actively repair and recover from physical insult, indicating that there is recovery taking place in the Gulf menhaden populations we sampled. The identified stomach lesions (collagen deposition, black particulate) significantly increased from 2011 to 2012, indicating that there is a continuous exposure, or perhaps that these effects are not readily repaired after the initial exposures in previous years. Heart lesions also showed no significant difference between the years sampled, indicating that these lesions are also not recovering in the populations, or there is a continuous source of exposure resulting in these lesions. Other observed lesions of the heart included fibrosis and looping defects, which may be attributed to the particulate accumulations in heart vasculature or developmental

exposure to crude oil components. There were no differences in the two sites examined, but there were lesions present that are representative of the expected timeline of exposure and recovery in the northern Gulf of Mexico following the DHOS.

5.2 Acute responses to dispersed crude oil may be species-specific

The goal of this chapter was to determine the acute sensitivity of Gulf menhaden and Florida pompano, two economically and ecologically relevant Gulf of Mexico species, to dispersed crude oil. It was hypothesized that Gulf menhaden will be more sensitive than Florida pompano to dispersed crude oil exposure, and that sensory tissue (neuromast and olfactory epithelium) of Gulf menhaden will be more sensitive than gill tissue to a dispersed crude oil exposure.

A 24-hour acute exposure to the CEWAF of Macondo crude oil produced significant mortality in Gulf menhaden and Florida pompano in the range of TPH concentrations reported from the Gulf of Mexico. Menhaden were determined to have an LC50 of 0.5 ppm TPH while pompano had a calculated LC50 of 3.5 ppm TPH. The histopathological results of this study indicate that filter feeders such as Gulf menhaden have a different target organ than Florida pompano in dispersed crude oil exposures.

Analysis of the organ systems of these fish led us to believe that the primary contributor to the death seen in this study was likely due to degeneration of neurosensory tissue, or adhesion of secondary gill filaments for menhaden and pompano, respectively. Narcosis effects following crude oil exposure have been attributed in part to peripheral and central nervous system membrane solvent effects; however, this study demonstrates that the disorientation seen in menhaden after an oil spill event could also be attributed to damage to their lateral line and olfactory epithelium. Previous studies have reported that

the gills and lamellar epithelial cells are targets of acute oil and dispersant exposures, but unless severely damaged these tissues can undergo repair.

Lamellae involved in lamellar adhesion increased significantly in exposed Florida pompano and Gulf menhaden. Dispersed crude oil exposures caused degenerative lesions in the olfactory lamellae and neuromast tissue of Gulf menhaden. Florida pompano exposed to similar mixtures (at a nearly 4-fold increase in concentration) were found to have abundant and severe gill adhesions and epithelial lifting. These apparent and extreme differences in target organ have been typically overlooked in toxicological studies, and have only recently begun to be determined. Field studies of fish species should take into account all possible target organ systems, especially neuromast tissue that is in direct contact with the surrounding water.

5.3 Particulate material does not appear to readily cross gastrointestinal epithelium

The goal of the 4th chapter was to determine if particulate material of different size and composition can cross epithelial barriers of zebrafish and mummichog. It was hypothesized that acetylene black and fluorescent microspheres will cross gastrointestinal and gill epithelium and enter the vascular system of zebrafish and mummichog.

This chapter examined the potential for uptake different particulate types, ranging in size from 0.2 μm up to 5 μm. Our previous field observations proved that macroscopic particles enter the blood stream of Gulf menhaden, likely across the gill or gastrointestinal epithelium. To test this, zebrafish and mummichog were exposed to acetylene black, a common diesel fuel particulate surrogate, as well as fluorescent plastic particles using static exposures and gavage studies to test for uptake and distribution.

Zebrafish and mummichog exposed to high concentrations of particulates in a static exposure readily ingest the particles; however, there was no evidence in these studies that showed definitive uptake into the vasculature based on histology of gill and gastrointestinal tissues. Previous studies showed that black carbon, in the form of india ink, exposed to Amazon mollies did accumulate through the gills and gastrointestinal tract, but this accumulation was very low and not quantifiable based on the presence of melanin, which cannot be differentiated when observed via microscopy. Blood smears taken after our exposure did not show any signs of fluorescence, indicating that these particles were likely not absorbed through the epithelium. One key question with regards to any particulate toxicity is whether or not the particles can cross epithelial barriers and enter circulation, where they would have potential to significantly impact tissues by blocking capillaries or desorbing reactive compounds. There is also the possibility that the particulates do not actually cross epithelium themselves, but rather enter the organism as dissolved molecules. Due to the hydrophobic nature of PAHs, they would associate in the lipid of fish, and then may crystalize as that lipid storage is used up. Menhaden have a very drastic seasonal accumulation and reduction of lipid content, which may explain the overall formation of the needle/spindle shaped crystals examined in the second chapter of this dissertation.

5.4 Conclusions

The main question asked after any large disaster, natural or anthropogenic, is what will be the long term impacts of that event to the environment and to the natural resources present in the affected region. The DHOS was no exception, being the largest marine oil spill in the United States and resulting in an estimated $36.7 billion to BP, and another $12.32 billion to economic sectors in the northern Gulf of Mexico (Smith, Smith, and

Ashcroft 2011). In addition to the sheer size of the spill, the location of the release at the bottom of the ocean was also a major concern, approximately 1500m below the surface 66 km from shore. The unique conditions surrounding the spill created much uncertainty in the eventual outcome, this dissertation investigated the impacts of the DHOS on an economically and ecologically important filter feeder, Gulf menhaden. Ultimately, this research supports the importance for understanding the organismal effects of key species in chemical spills. These data can be used to assist in risk assessment and remedial decisions. The DHOS was an unprecedented spill, and had many unique characteristics leading to novel exposure scenarios and studies in the fate and transport of crude oil. The fact that Louisiana sweet crude oil is, by definition, a lighter crude oil and contained a relatively high gas content contributed to the initial explosion as well as the resultant BTEX subsurface plumes. This type of spill (very large, deep, offshore) may have created ideal conditions for particulates to accumulate in the food chain due to the formation of marine snow and dissolution of crude oil components at depth. The heavier PAHs that are more resistant to weathering persisted in the surface slicks and created a new habitat for organisms to interact with. Oil degrading microbes could thrive in the surface emulsions that were created, which was believed to be a major factor associated with the formation of marine snow.

This research also reports the accumulation of PAH derived PM in Gulf menhaden, an understudied and uncommon observation in ecological toxicology. Filter feeding species, like menhaden, were actively feeding throughout the duration of the spill in overlapping areas and would actively eat marine snow aggregates. In addition to actively feeding, menhaden spawn throughout the winter and larvae are transported to coastal

estuaries throughout the early spring. This larval transport coincided with the spill, as did the larval recruitment of numerous species of pelagic fish. Developmental exposure to crude oil components, specifically PAHs, are well documented for malformation of heart tissue at low ppb concentrations.

The results presented are important because they demonstrate the unique response of a filter feeder, and highlight the importance of understanding species differences in the endpoints affected to similar exposures. This dissertation also reports the presence of particulate PAHs in the vasculature of heart tissue, which is a novel impact of what is likely to be a particulate based exposure. These results could have direct applications to other scenarios, such as to regulate particulate loadings in the receiving waters of urbanized watersheds and as well as applications to post oil spill exposure assessments of affected wildlife. A better understanding of particulate toxicity in aquatic organisms is needed due to their formation, presence, and persistence in aquatic environments.

5.5 Future Studies

This dissertation examined the impacts of crude oil on fish species, and also reports the accumulation of particulate PAHs in menhaden hearts and ventricles. Observations from field collected samples and comparative studies of native species led to additional testing for particulate accumulation in more readily handled model species. The following future directions may prove to elucidate and further contribute to crude oil science and the toxic nature of particulates:

1. The use of accepted model species to test the accumulation of particulates may have limited our findings. Use of alternate species, specifically a known filter feeder may more accurately represent the findings we saw in previous studies. Gulf menhaden

are a novel choice for field studies and incorporating methods derived in this study on a more similar species may provide the answers to what we are looking for. Filter feeders, like Gulf menhaden, have specialized gill rakers for ingesting particles as well as an extensive gastrointestinal tract with pyloric caeca, intestinal folds common in some fish species. Zebrafish and mummichogs have relatively short gastrointestinal tracts, compared to menhaden.

2. The role of other damage, from secondary exposures to crude oil components or even parasites, in particulate uptake may also be a major contributor. Crude oil and dispersants are known to disrupt membranes and may increase permeability. In severe cases, hyperplasia and hemorrhaging of thin epithelial tissues may trap or pick up particles, respectively. These particles will enter the vasculature after healing. Additional macrophage circulation in areas infected with parasites and any damaged epithelial areas are more likely to accumulate particles. Laboratory exposures with controlled settings and no secondary exposures are less likely to have these compromised epithelia.
3. Changing the lengths of exposure or particle types may cause a shift in the accumulation of particles. True PM (and not a surrogate) will have compounds that may activate the immune system or metabolic enzymes causing increased uptake. The continued exposure of reactive constituents (metals, PAHs) on particles that do enter may cause focal damage and lead to additional accumulation in damaged areas.
4. We determined in our static exposure studies that particles were ingested by fish species, even if they never entered the blood stream. The impacts of these ingested

particles have been shown to impact fish growth and behavioral responses. Particulates such as plastics could be inadvertently, or even intentionally ingested by juvenile fish species but these fish will receive no nutritional benefit from the particles. The time and energy spent foraging on particles could have more serious impacts on their development and growth relative to species that are more selective in their food choices. Determining the residence time of plastic particles in gastrointestinal tract relative to other food sources will also impact forage behavior, survivorship, and growth.

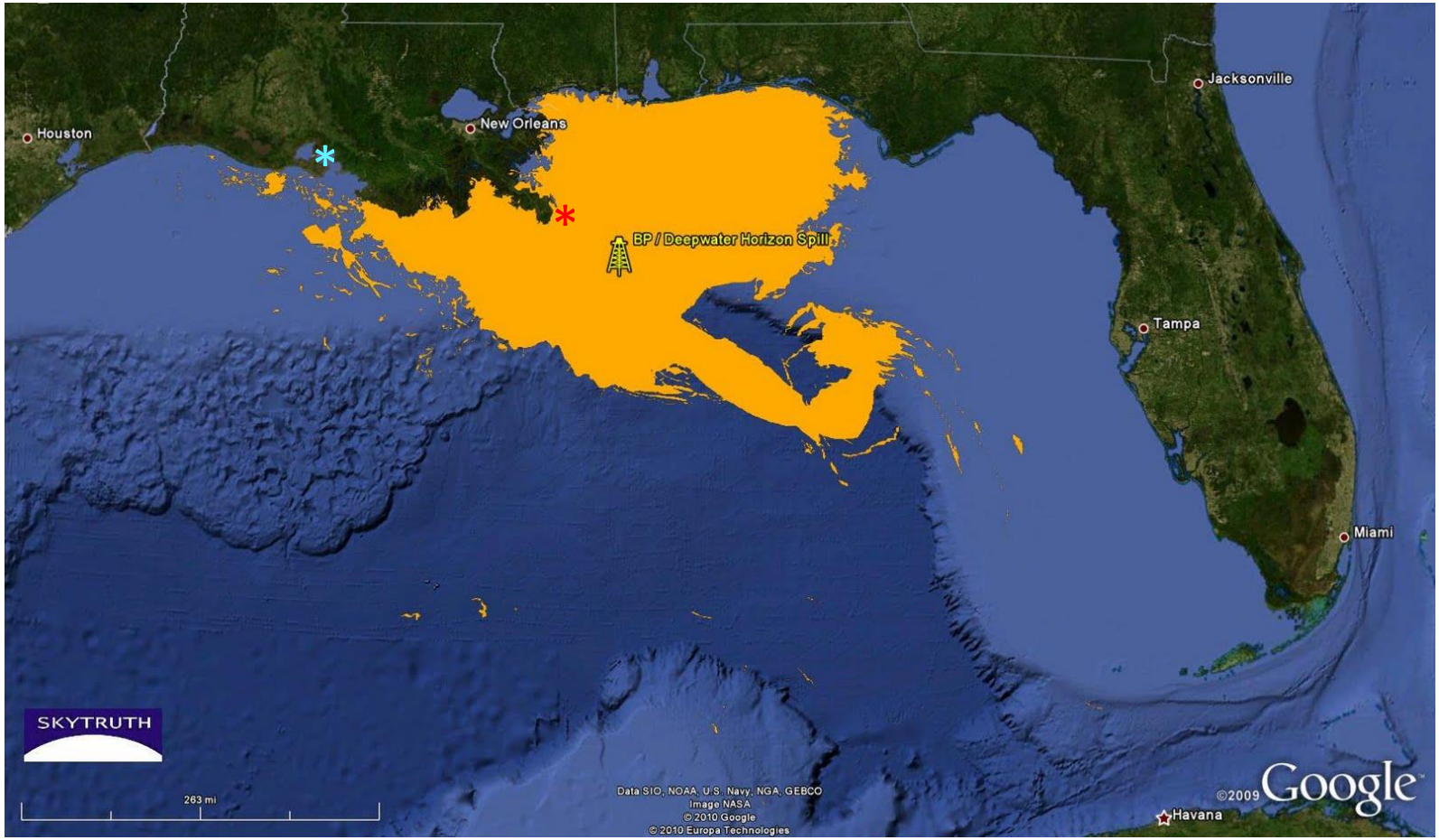

Figure 5.1 Site map showing extent of spill and areas without contamination. The exposure history of fish collected at either site (VB, Reference, Blue or GI, Oiled, Red) cannot be confidently described.

REFERENCES:

Adams, Charles M., Emilio Hernandez, and James C. Cato. 2004. 'The economic significance of the Gulf of Mexico related to population, income, employment, minerals, fisheries and shipping', *Ocean & Coastal Management*, 47: 565-80.

Aeppli, Christoph, Catherine A. Carmichael, Robert K. Nelson, Karin L. Lemkau, William M. Graham, Molly C. Redmond, David L. Valentine, and Christopher M. Reddy. 2012. 'Oil weathering after the Deepwater Horizon disaster led to the formation of oxygenated residues', *Environmental science & technology*, 46: 8799-807.

Agamy, Esam. 2012a. 'Histopathological changes in the livers of rabbit fish (*Siganus canaliculatus*) following exposure to crude oil and dispersed oil', *Toxicologic pathology*, 40: 1128-40.

———. 2012b. 'Histopathological liver alterations in juvenile rabbit fish (*Siganus canaliculatus*) exposed to light Arabian crude oil, dispersed oil and dispersant', *Ecotoxicology and environmental safety*, 75: 171-79.

———. 2013. 'Impact of laboratory exposure to light Arabian crude oil, dispersed oil and dispersant on the gills of the juvenile brown spotted grouper (*Epinephelus chlorostigma*): A histopathological study', *Marine Environmental Research*.

Ahrenholz, Dean W. 1991. "Population biology and life history of the North American menhadens, *Brevoortia spp*." In.

Al-Kindi, A. Y. A., J. A. Brown, and C. P. Waring. 2000. 'Endocrine, physiological and histopathological responses of fish and their larvae to stress with emphasis on exposure to crude oil and various petroleum hydrocarbons', *Science and Technology, Special Review*: 1-30.

Allan, Sarah E., Brian W. Smith, and Kim A. Anderson. 2012. 'Impact of the deepwater horizon oil spill on bioavailable polycyclic aromatic hydrocarbons in Gulf of Mexico coastal waters', *Environmental science & technology*, 46: 2033-39.

Alldredge, Alice L, and Mary W Silver. 1988. 'Characteristics, dynamics and significance of marine snow', *Progress in oceanography*, 20: 41-82.

Almeda, Rodrigo, Sarah Baca, Cammie Hyatt, and EdwardJ Buskey. 2014. 'Ingestion and sublethal effects of physically and chemically dispersed crude oil on marine planktonic copepods', *Ecotoxicology*, 23: 988-1003.

Almeda, Rodrigo, Zoe Wambaugh, Zucheng Wang, Cammie Hyatt, Zhanfei Liu, and Edward J. Buskey. 2013. 'Interactions between zooplankton and crude oil: toxic effects and bioaccumulation of polycyclic aromatic hydrocarbons', *PloS one*, 8: e67212.

Anderson, Joel D, and William J Karel. 2014. 'Limited genetic structure of Gulf Menhaden (*Brevoortia patronus*), as revealed by microsatellite markers developed for the genus *Brevoortia* (Clupeidae)', *Fishery Bulletin*, 112: 71-81.

Atlas, Ronald M, and Richard Bartha. 1992. 'Hydrocarbon biodegradation and oil spill bioremediation.' in, *Advances in microbial ecology* (Springer).

Atlas, Ronald M, and Terry C Hazen. 2011. 'Oil biodegradation and bioremediation: a tale of the two worst spills in US history', *Environmental science & technology*, 45: 6709-15.

Au, D. W. T., C. A. Pollino, P. K. S. Shin, S. T. F. Lau, and J. Y. M. Tang. 2004. 'Chronic effects of suspended solids on gill structure, osmoregulation, growth, and triiodothyronine in juvenile green grouper *Epinephelus coioides*', *Marine Ecology Progress Series*, 266: 255-64.

Avio, Carlo Giacomo, Stefania Gorbi, and Francesco Regoli. 2015. 'Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observations in commercial species from Adriatic Sea', *Marine Environmental Research*, 111: 18-26.

Baumann, Paul C, and John C Harshbarger. 1995. 'Decline in liver neoplasms in wild brown bullhead catfish after coking plant closes and environmental PAHs plummet', *Environmental Health Perspectives*, 103: 168.

Bejarano, Adriana C, Edwin Levine, and Alan J Mearns. 2013. 'Effectiveness and potential ecological effects of offshore surface dispersant use during the Deepwater Horizon oil spill: a retrospective analysis of monitoring data', *Environmental monitoring and assessment*, 185: 10281-95.

Bellinger, John W, and James W Avault Jr. 1971. 'Food habits of juvenile pompano, *Trachinotus carolinus*, in Louisiana', *Transactions of the American Fisheries Society*, 100: 486-94.

BenKinney, Marie, John Brown, Stephen Mudge, Marcus Russell, Andy Nevin, and Charles Huber. 2011. 'Monitoring Effects of Aerial Dispersant Application during the MC252 Deepwater Horizon Incident', *International Oil Spill Conference Proceedings*, 2011: abs368.

Bentivegna, Carolyn S, Keith R Cooper, Gregory Olson, Edwin A Pena, Daniel R Millemann, and Ralph J Portier. 2015. 'Chemical and histological comparisons between *Brevoortia sp*.(menhaden) collected in fall 2010 from Barataria Bay, LA and Delaware Bay, NJ following the DeepWater Horizon (DWH) oil spill', *Marine Environmental Research*, 112: 21-34.

Bentivegna, CS., Sowa, JR., Cooper, KR., Snow, NH. . 2012. "Trophic Transfer of Oil Contaminants from Menhaden Fish: Will the Gulf Oil Spill Effect NJ? ." In, edited by New Jersey Department of Environmental Protection.

Bernet, D., H. Schmidt, W. Meier, P. Burkhardt-Holm, and T. Wahli. 1999. 'Histopathology in fish: proposal for a protocol to assess aquatic pollution', *Journal of Fish Diseases*, 22: 25-34.

Bilotta, G. S., and R. E. Brazier. 2008. 'Understanding the influence of suspended solids on water quality and aquatic biota', *Water Research*, 42: 2849-61.

Booksh, Karl S, Allen R Muroski, and ML Myrick. 1996. 'Single-measurement excitation/emission matrix spectrofluorometer for determination of hydrocarbons in ocean water. 2. Calibration and quantitation of naphthalene and styrene', *Analytical Chemistry*, 68: 3539-44.

Brette, Fabien, Ben Machado, Caroline Cros, John P Incardona, Nathaniel L Scholz, and Barbara A Block. 2014. 'Crude oil impairs cardiac excitation-contraction coupling in fish', *Science*, 343: 772-76.

Busby Jr, William F, and Paul M Newberne. 1995. 'Diesel emissions and other substances associated with animal carcinogenicity', *Diesel Exhaust: A Critical Analysis*: 185-220.

Camilli, Richard, Christopher M. Reddy, Dana R. Yoerger, Benjamin A. S. Van Mooy, Michael V. Jakuba, James C. Kinsey, Cameron P. McIntyre, Sean P. Sylva, and James V. Maloney. 2010. 'Tracking hydrocarbon plume transport and biodegradation at Deepwater Horizon', *Science*, 330: 201-04.

Coglianese, Matthew P. 2010. 'The importance of determining potential chronic natural resource damages from the Deepwater Horizon accident', *Envtl.L.Rep.News & Analysis*, 40: 11, 100, 11, 01.

Collymore, Chereen, Skye Rasmussen, and Ravi J Tolwani. 2013. 'Gavaging adult zebrafish', *Journal of visualized experiments: JoVE*.

Conway, Tenley M, and Richard G Lathrop. 2005. 'Alternative land use regulations and environmental impacts: assessing future land use in an urbanizing watershed', *Landscape and Urban Planning*, 71: 1-15.

Conway, Tenley M. 2007. 'Impervious surface as an indicator of pH and specific conductance in the urbanizing coastal zone of New Jersey, USA', *Journal of Environmental Management*, 85: 308-16.

Cormier, Stephania A., Slawo Lomnicki, Wayne Backes, and Barry Dellinger. 2006. 'Origin and Health Impacts of Emissions of Toxic By-Products and Fine Particles from Combustion and Thermal Treatment of Hazardous Wastes and Materials', *Environmental Health Perspectives*, 114: 810-17.

Costa, Pedro M, Mário S Diniz, Sandra Caeiro, Jorge Lobo, Marta Martins, Ana M Ferreira, Miguel Caetano, Carlos Vale, T Ángel DelValls, and M Helena Costa. 2009. 'Histological biomarkers in liver and gills of juvenile *Solea senegalensis* exposed to contaminated estuarine sediments: a weighted indices approach', *Aquatic Toxicology*, 92: 202-12.

Daly, Kendra L, Uta Passow, Jeffrey Chanton, and David Hollander. 2016. 'Assessing the impacts of oil-associated marine snow formation and sedimentation during and after the Deepwater Horizon oil spill', *Anthropocene*.

Danovaro, Roberto, Cristina Gambi, Antonio Dell'Anno, Cinzia Corinaldesi, Simonetta Fraschetti, Ann Vanreusel, Magda Vincx, and Andrew J Gooday. 2008. 'Exponential decline of deep-sea ecosystem functioning linked to benthic biodiversity loss', *Current Biology*, 18: 1-8.

Das, Nilanjana, and Preethy Chandran. 2010. 'Microbial degradation of petroleum hydrocarbon contaminants: an overview', *Biotechnology research international*, 2011.

De Gouw, JA, AM Middlebrook, C Warneke, R Ahmadov, Elliot L Atlas, R Bahreini, DR Blake, CA Brock, J Brioude, and DW Fahey. 2011. 'Organic aerosol formation downwind from the Deepwater Horizon oil spill', *Science*, 331: 1295-99.

Deegan, L. A. 1986. 'Changes in body composition and morphology of young-of-the-year gulf menhaden, *Brevoortia patronus* Goode, in Fourleague Bay, Louisiana', *Journal of Fish Biology*, 29: 403-15.

Deegan, Linda A. 1993. 'Nutrient and energy transport between estuaries and coastal marine ecosystems by fish migration', *Canadian Journal of Fisheries and Aquatic Sciences*, 50: 74-79.

Deegan, Linda A, Bruce J Peterson, and Ralph Portier. 1990. 'Stable isotopes and cellulase activity as evidence for detritus as a food source for juvenile Gulf menhaden', *Estuaries*, 13: 14-19.

Diaz, Robert J., and Rutger Rosenberg. 2008. 'Spreading dead zones and consequences for marine ecosystems', *Science*, 321: 926-29.

Diercks, Arne- R., Raymond C. Highsmith, Vernon L. Asper, DongJoo Joung, Zhengzhen Zhou, Laodong Guo, Alan M. Shiller, Samantha B. Joye, Andreas P. Teske, and Norman Guinasso. 2010. 'Characterization of subsurface polycyclic aromatic hydrocarbons at the Deepwater Horizon site', *Geophysical Research Letters*, 37: L20602.

DiMichele, Leonard, and Malcolm H Taylor. 1978. 'Histopathological and physiological responses of *Fundulus heteroclitus* to naphthalene exposure', *Journal of the Fisheries Board of Canada*, 35: 1060-66.

Du, Mengran, and John D Kessler. 2012. 'Assessment of the spatial and temporal variability of bulk hydrocarbon respiration following the Deepwater Horizon oil spill', *Environmental science & technology*, 46: 10499-507.

Dubansky, Benjamin, Andrew Whitehead, Jeffrey Miller, Charles D. Rice, and Fernando Galvez. 2013. 'Multi-tissue molecular, genomic, and developmental effects of the Deepwater Horizon oil spill on resident Gulf killifish (*Fundulus grandis*)', *Environmental science & technology*.

Dubrow, David, Malcolm Hale, and Anthony Bimbo. 1976. 'Seasonal variations in chemical composition and protein quality of menhaden', *Mar.Fish.Rev*, 38: 12-16.

Durbin, Ann Gall, and Edward G. Durbin. 1975. 'Grazing rates of the Atlantic menhaden *Brevoortia tyrannus* as a function of particle size and concentration', *Marine Biology*, 33: 265-77.

Engelhardt, F. R., M. Pe Wong, and M. E. Duey. 1981. 'Hydromineral balance and gill morphology in rainbow trout *Salmo gairdneri*, acclimated to fresh and sea water. As affected by petroleum exposure', *Aquatic Toxicology*, 1: 175-86.

EPA. 1998. 'EPA Method 8270D (SW-846): Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC-MS)'.

EPA, December. 2009. 'Integrated science assessment for particulate matter', *US Environmental Protection Agency Washington, DC*.

Evans, David H. 1987. 'The fish gill: site of action and model for toxic effects of environmental pollutants', *Environmental Health Perspectives*, 71: 47.

Federal Interagency Solutions, Group. 2010. "Oil budget Calculator, Deepwater Horizon; Report to the National Incident Command." In.

Finucane, John H. 1969. 'Ecology of the pompano (*Trachinotus carolinus*) and the permit (*T. falcatus*) in Florida', *Transactions of the American Fisheries Society*, 98: 478-86.

Froehlicher, Mirjam, Anja Liedtke, Ksenia J Groh, Stephan CF Neuhauss, Helmut Segner, and Rik IL Eggen. 2009. 'Zebrafish (*Danio rerio*) neuromast: promising biological endpoint linking developmental and toxicological studies', *Aquatic Toxicology*, 95: 307-19.

Fucik, Kenneth W, Kelly A Carr, and Brian J Balcom. 1995. 'Toxicity of oil and dispersed oil to the eggs and larvae of seven marine fish and inverterbrates from the Gulf of Mexico', *ASTM Special Technical Publication*, 1252: 135-71.

Gardner, George R. . 1975. 'Chemically induced lesions in estuarine or marine teleosts.' in William E.; Migaki Ribelin, George (ed.), *The Pathology of Fishes* (The University of Wisconsin Press: Madison, Wisconsin).

George-Ares, A, and JR Clark. 2000. 'Aquatic toxicity of two Corexit® dispersants', *Chemosphere*, 40: 897-906.

Gigliotti, Cari L, Paul A Brunciak, Jordi Dachs, Thomas R Glenn, Eric D Nelson, Lisa A Totten, and Steven J Eisenreich. 2002. 'Air—water exchange of polycyclic aromatic hydrocarbons in the New York—New Jersey, USA, Harbor Estuary', *Environmental Toxicology and Chemistry*, 21: 235-44.

Gilbert, Carter. 1986. "Species Profiles: Life Histories and Environmental Requirements of Coastal Fishes and Invertebrates (South Florida)- Florida Pompano." In, 1-14. US Fish and Wildlife Service, 82(11.42). US Army Corps of Engineers, TR EL-82-4.

Goodbody-Gringley, Gretchen, Dana L. Wetzel, Daniel Gillon, Erin Pulster, Allison Miller, and Kim B. Ritchie. 2013. 'Toxicity of Deepwater Horizon Source Oil and the Chemical Dispersant, Corexit® 9500, to Coral Larvae', *PloS one*, 8: e45574.

Graham, William M., Robert H. Condon, Ruth H. Carmichael, Isabella D'Ambra, Heather K. Patterson, Laura J. Linn, and Frank J. Hernandez Jr. 2010. 'Oil carbon

entered the coastal planktonic food web during the Deepwater Horizon oil spill', *Environmental Research Letters*, 5: 045301.

Grantz, D. A., J. H. B. Garner, and D. W. Johnson. 2003. 'Ecological effects of particulate matter', *Environment International*, 29: 213-39.

Grassle, JF. 1977. 'Slow recolonisation of deep-sea sediment'.

Haensly, W. E., J. M. Neff, J. R. Sharp, A. C. Morris, M. F. Bedgood, and P. D. Boem. 1982. 'Histopathology of *Pleuronectes platessa* L. from Aber Wrac'h and Aber Benoit, Brittany, France: long-term effects of the Amoco Cadiz crude oil spill', *Journal of Fish Diseases*, 5: 365-91.

Hazen, Terry C., Eric A. Dubinsky, Todd Z. DeSantis, Gary L. Andersen, Yvette M. Piceno, Navjeet Singh, Janet K. Jansson, Alexander Probst, Sharon E. Borglin, and Julian L. Fortney. 2010. 'Deep-sea oil plume enriches indigenous oil-degrading bacteria', *Science*, 330: 204-08.

Hemmer, Michael J, Mace G Barron, and Richard M Greene. 2011. 'Comparative toxicity of eight oil dispersants, Louisiana sweet crude oil (LSC), and chemically dispersed LSC to two aquatic test species', *Environmental Toxicology and Chemistry*, 30: 2244-52.

Hicken, Corinne E., Tiffany L. Linbo, David H. Baldwin, Maryjean L. Willis, Mark S. Myers, Larry Holland, Marie Larsen, Michael S. Stekoll, Stanley D. Rice, and Tracy K. Collier. 2011. 'Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish', *Proceedings of the National Academy of Sciences*, 108: 7086-90.

Iannuzzi, Timothy J, Tina N Armstrong, John B Thelen, David F Ludwig, and Clifford E Firstenberg. 2004. 'Chemical contamination of aquatic organisms from an urbanized river in the New York/New Jersey Harbor estuary', *Human and Ecological Risk Assessment*, 10: 389-413.

Incardona, John P, Luke D Gardner, Tiffany L Linbo, Tanya L Brown, Andrew J Esbaugh, Edward M Mager, John D Stieglitz, Barbara L French, Jana S Labenia, and Cathy A Laetz. 2014. 'Deepwater Horizon crude oil impacts the developing hearts of large predatory pelagic fish', *Proceedings of the National Academy of Sciences*, 111: E1510-E18.

Incardona, John P., Tracy K. Collier, and Nathaniel L. Scholz. 2004. 'Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons', *Toxicology and applied pharmacology*, 196: 191-205.

———. 2010. 'Oil spills and fish health: exposing the heart of the matter', *Journal of Exposure Science and Environmental Epidemiology*.

Incardona, John P., Tanya L. Swarts, Richard C. Edmunds, Tiffany L. Linbo, Allisan Aquilina-Beck, Catherine A. Sloan, Luke D. Gardner, Barbara A. Block, and Nathaniel L. Scholz. 2013. 'Exxon Valdez to Deepwater Horizon: Comparable toxicity of both crude oils to fish early life stages', *Aquatic Toxicology*, 142: 303-16.

Jernelöv, Arne, and Olof Lindén. 1981. 'Ixtoc I: a case study of the world's largest oil spill', *Ambio*: 299-306.

Jones, David Alan, Miles Hayes, Friedhelm Krupp, Gino Sabatini, Iain Watt, and Lee Weishar. 2008. 'The impact of the Gulf War (1990–91) oil release upon the intertidal Gulf coast line of Saudi Arabia and subsequent recovery.' in, *Protecting the Gulf's Marine Ecosystems from Pollution* (Springer).

Joye, Samantha B., Ian R. MacDonald, Ira Leifer, and Vernon Asper. 2011. 'Magnitude and oxidation potential of hydrocarbon gases released from the BP oil well blowout', *Nature Geoscience*, 4: 160-64.

Kam, W, JW Liacos, JJ Schauer, RJ Delfino, and C Sioutas. 2012. 'Size-segregated composition of particulate matter (PM) in major roadways and surface streets', *Atmospheric Environment*, 55: 90-97.

Kemmerer, AJ. 1980. "Environmental preferences and behavior patterns of gulf menhaden (*Brevoortia patron*) inferred from fishing and remotely sensed data [collected in the northern Gulf of Mexico]." In *ICLARM Conference Proceedings (Philippines)*.

Kennish, Michael J, Suzanne B Bricker, William C Dennison, Patricia M Glibert, Robert J Livingston, Kenneth A Moore, Rachel T Noble, Hans W Paerl, Joy M Ramstack, and Sybil Seitzinger. 2007. 'Barnegat Bay-Little Egg Harbor Estuary: case study of a highly eutrophic coastal bay system', *Ecological Applications*, 17: S3-S16.

Kessler, John D., David L. Valentine, Molly C. Redmond, Mengran Du, Eric W. Chan, Stephanie D. Mendes, Erik W. Quiroz, Christie J. Villanueva, Stephani S. Shusta, and Lindsay M. Werra. 2011. 'A persistent oxygen anomaly reveals the fate of spilled methane in the deep Gulf of Mexico', *Science*, 331: 312-15.

Khan, R. A., and J. Kiceniuk. 1984. 'Histopathological effects of crude oil on Atlantic cod following chronic exposure', *Canadian journal of zoology*, 62: 2038-43.

Khan, RA. 1995. 'Histopathology in winter flounder, *Pleuronectes americanus*, following chronic exposure to crude oil', *Bulletin of environmental contamination and toxicology*, 54: 297-301.

Khan, RA, and JF Payne. 2005. 'Influence of a crude oil dispersant, Corexit 9527, and dispersed oil on capelin (*Mallotus villosus*), Atlantic cod (*Gadus morhua*), longhorn sculpin (*Myoxocephalus octodecemspinosus*), and cunner (*Tautogolabrus adspersus*)', *Bulletin of environmental contamination and toxicology*, 75: 50-56.

Khan, Rasul A. 2013. 'Effects of polycyclic aromatic hydrocarbons on sexual maturity of Atlantic cod, *Gadus morhua*, following chronic exposure', *Environment and Pollution*, 2: 1.

Kırman, Zeynep Dincer, José L Sericano, Terry L Wade, Thomas S Bianchi, Franco Marcantonio, and Alexander S Kolker. 2016. 'Composition and depth distribution of hydrocarbons in Barataria Bay marsh sediments after the Deepwater Horizon oil spill', *Environmental Pollution*, 214: 101-13.

Klaassen, Curtis, and John B Watkins III. 2015. *Casarett & Doull's essentials of toxicology* (McGraw Hill Professional).

Kujawinski, Elizabeth B., Melissa C. Kido Soule, David L. Valentine, Angela K. Boysen, Krista Longnecker, and Molly C. Redmond. 2011. 'Fate of dispersants associated with the Deepwater Horizon oil spill', *Environmental science & technology*, 45: 1298-306.

Kvenvolden, K. A., and C. K. Cooper. 2003. 'Natural seepage of crude oil into the marine environment', *Geo-Marine Letters*, 23: 140-46.

Lake, Randal G, and Scott G Hinch. 1999. 'Acute effects of suspended sediment angularity on juvenile coho salmon (*Oncorhynchus kisutch*)', *Canadian Journal of Fisheries and Aquatic Sciences*, 56: 862-67.

Lassuy, Dennis R. 1983. 'Species profiles-life histories and environmental requirements (Gulf of Mexico): Gulf menhaden'.

Leifer, Ira, William J Lehr, Debra Simecek-Beatty, Eliza Bradley, Roger Clark, Philip Dennison, Yongxiang Hu, Scott Matheson, Cathleen E Jones, and Benjamin Holt. 2012. 'State of the art satellite and airborne marine oil spill remote sensing: Application to the BP Deepwater Horizon oil spill', *Remote Sensing of Environment*, 124: 185-209.

Lewis, RM, and Ch M Roithmayr. 1980. "SPAWNING AND SEXUAL MATURITY OF GULF MENHADEN, *BREVOORTIA-PATRONUS*." In, 947-51. NATL MARINE FISHERIES SERVICE SCIENTIFIC PUBL OFFICE 7600 SAND POINT WAY NE BIN C15700, SEATTLE, WA 98115.

Lippson, Robert L. 1991. 'Atlantic menhaden *Brevoortia tyrannus*.' in Steven L; Mihursky Funderburk, Joseph A; Jordan, Stephen J; Riley, David (ed.), *Habitat Requirements for Chesapeake Bay Living Resources* (Chesapeake Research Consortium, Inc.: Solomons, Maryland).

Litchfield, JT, and F Wilcoxon. 1948. 'Simplified method of evaluating dose-effect experiments. ', *Journal of Pharmacology and Experimental Therapeutics*: 99-113.

Lu, Yifeng, Yan Zhang, Yongfeng Deng, Wei Jiang, Yanping Zhao, Jinju Geng, Lili Ding, and Hongqiang Ren. 2016. 'Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (*Danio rerio*) and Toxic Effects in Liver', *Environmental science & technology*, 50: 4054-60.

Mager, E. M., A. J. Esbaugh, J. D. Stieglitz, R. Hoenig, C. Bodinier, J. P. Incardona, N. L. Scholz, D. D. Benetti, and M. Grosell. 2014. 'Acute embryonic or juvenile exposure to Deepwater Horizon crude oil impairs the swimming performance of mahi-mahi (Coryphaena hippurus)', *Environ Sci Technol*, 48: 7053-61.

Main, Kevan L., Nicole Rhody, Michael Nystrom, and Matthew Resley. 2007. "Species Profile-Florida Pompano." In.: Southern Regional Aquaculture Center Publication 7206.

Mallatt, Jon. 1985. 'Fish gill structural changes induced by toxicants and other irritants: a statistical review', *Canadian Journal of Fisheries and Aquatic Sciences*, 42: 630-48.

Mascarelli, Amanda. 2010. 'Deepwater Horizon: After the oil', *Nature*, 467: 22.

McCrea-Strub, A, K Kleisner, UR Sumaila, W Swartz, R Watson, D Zeller, and D Pauly. 2011. 'Potential impact of the Deepwater Horizon oil spill on commercial fisheries in the Gulf of Mexico', *Fisheries*, 36: 332-36.

McNutt, Marcia K., Rich Camilli, Timothy J. Crone, George D. Guthrie, Paul A. Hsieh, Thomas B. Ryerson, Omer Savas, and Frank Shaffer. 2012. 'Review of flow rate estimates of the Deepwater Horizon oil spill', *Proceedings of the National Academy of Sciences*, 109: 20260-67.

Milinkovitch, Thomas, Hélène Thomas-Guyon, Christel Lefrançois, and Nathalie Imbert. 2013. 'Dispersant use as a response to oil spills: toxicological effects on fish cardiac performance', *Fish Physiology and Biochemistry*: 1-6.

Millemann, Daniel R, Ralph J Portier, Gregory Olson, Carolyn S Bentivegna, and Keith R Cooper. 2015. 'Particulate accumulations in the vital organs of wild *Brevoortia patronus* from the northern Gulf of Mexico after the Deepwater Horizon oil spill', *Ecotoxicology*, 24: 1831-47.

Mitra, Siddhartha, Thomas S. Bianchi, Brent A. McKee, and Martha Sutula. 2002. 'Black carbon from the Mississippi River: Quantities, sources, and potential implications for the global carbon cycle', *Environmental science & technology*, 36: 2296-302.

Mitra, Siddhartha, David G Kimmel, Jessica Snyder, Kimberly Scalise, Benjamin D McGlaughon, Michael R Roman, Ginger L Jahn, James J Pierson, Stephen B Brandt, and Joseph P Montoya. 2012. 'Macondo-1 well oil-derived polycyclic aromatic hydrocarbons in mesozooplankton from the northern Gulf of Mexico', *Geophysical Research Letters*, 39.

Montagna, Paul A, Jeffrey G Baguley, Cynthia Cooksey, Ian Hartwell, Larry J Hyde, Jeffrey L Hyland, Richard D Kalke, Laura M Kracker, Michael Reuscher, and Adelaide CE Rhodes. 2013. 'Deep-sea benthic footprint of the Deepwater Horizon blowout', *PloS one*, 8: e70540.

Muhling, B. A., M. A. Roffer, J. T. Lamkin, G. W. Ingram Jr, M. A. Upton, G. Gawlikowski, F. Muller-Karger, S. Habtes, and W. J. Richards. 2012. 'Overlap between Atlantic bluefin tuna spawning grounds and observed Deepwater Horizon surface oil in the northern Gulf of Mexico', *Marine Pollution Bulletin*, 64: 679-87.

Murawski, Steven A, William T Hogarth, Ernst B Peebles, and Luiz Barbeiri. 2014. 'Prevalence of external skin lesions and polycyclic aromatic hydrocarbon concentrations in Gulf of Mexico fishes, post-Deepwater Horizon', *Transactions of the American Fisheries Society*, 143: 1084-97.

National Marine Fisheries, Service. 2012. "Annual Commercial Landing Statistics." In.

National Oceanic and Atmospheric Administration (NOAA). 2010. "Fish Stocks in the Gulf of Mexico " In, 2 pp. US Department of Commerce.

———. 2015. "Forecast for the 2015 Gulf and Atlantic Menhaden Purse-Seine Fisheries and Review of the 2014 Fishing Season." In, 8 pp. Beaufort, North Carolina: National Oceanic and Atmospheric Administration.

Norse, Elliott A., and John Amos. 2010. 'Impacts, Perception, and Policy Implications of the Deepwater Horizon Oil and Gas Disaster', *Environmental Law Institute, Washington, DC*, 40: 11058.

NTP. 2011. "NTP 12th Report on Carcinogens." In *Report on carcinogens: carcinogen profiles/US Dept. of Health and Human Services, Public Health Service, National Toxicology Program*, iii. National Toxicology Program.

Olson, Gregory M., Buffy M. Meyer, and Ralph J. Portier. 2014. 'Adaptation of Sonication-Assisted Matrix Solid Phase Dispersion of Tissues for the Subsequent Extraction of Polycyclic Aromatic Hydrocarbons from Gulf Menhaden (*Brevoortia patronus*)', *Biochemistry Research International*, 2014: 7.

Operational Science Advisory, Team. 2010. "Summary report for sub-sea and sub-surface oil and dispersant detection: sampling monitoring." In.

Passow, U., K. Ziervogel, V. Asper, and A. Diercks. 2012. 'Marine snow formation in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico', *Environmental Research Letters*, 7: 035301.

Patra, Digambara. 2003. 'Applications and new developments in fluorescence spectroscopic techniques for the analysis of polycyclic aromatic hydrocarbons'.

Perring, AE, JP Schwarz, JR Spackman, R Bahreini, JA De Gouw, RS Gao, JS Holloway, DA Lack, JM Langridge, and J Peischl. 2011. 'Characteristics of black carbon aerosol from a surface oil burn during the Deepwater Horizon oil spill', *Geophysical Research Letters*, 38.

Peterson, Charles H, Stanley D Rice, Jeffrey W Short, Daniel Esler, James L Bodkin, Brenda E Ballachey, and David B Irons. 2003. 'Long-term ecosystem response to the Exxon Valdez oil spill', *Science*, 302: 2082-86.

Peterson, Charles H., Sean S. Anderson, Gary N. Cherr, Richard F. Ambrose, Shelly Anghera, Steven Bay, Michael Blum, Robert Condon, Thomas A. Dean, and Monty Graham. 2012. 'A tale of two spills: novel science and policy implications of an emerging new oil spill model', *BioScience*, 62: 461-69.

Peterson, Charles H., Mahlon C. Kennicutt Ii, Roger H. Green, Paul Montagna, Jr Donald E. Harper, Eric N. Powell, and Pasquale F. Roscigno. 1996. 'Ecological consequences of environmental perturbations associated with offshore hydrocarbon production: a perspective on long-term exposures in the Gulf of Mexico', *Canadian Journal of Fisheries and Aquatic Sciences*, 53: 2637-54.

Prince, Roger C, and Josh D Butler. 2013. 'A protocol for assessing the effectiveness of oil spill dispersants in stimulating the biodegradation of oil', *Environmental Science and Pollution Research*: 1-5.

Prince, Roger C, Kelly M McFarlin, Josh D Butler, Eric J Febbo, Frank CY Wang, and Tim J Nedwed. 2013. 'The primary biodegradation of dispersed crude oil in the sea', *Chemosphere*, 90: 521-26.

Rabalais, Nancy N, and R Eugene Turner. 2006. 'Oxygen depletion in the Gulf of Mexico adjacent to the Mississippi River.' in, *Past and Present Water Column Anoxia* (Springer).

Ramachandran, SD, CW Khan, PV Hodson, K Lee, and T King. 2004. "Role of droplets in promoting uptake of PAHs by fish exposed to chemically dispersed crude oil." In *ARCTIC AND MARINE OILSPILL PROGRAM TECHNICAL SEMINAR*, 765-72. Environment Canada; 1999.

Randall, D. J., D. W. Connell, R. Yang, and S. S. Wu. 1998. 'Concentrations of persistent lipophilic compounds in fish are determined by exchange across the gills, not through the food chain', *Chemosphere*, 37: 1263-70.

Randolph, R Cean, John T Hardy, Scott W Fowler, Andrew RG Price, and Walter H Pearson. 1998. 'Toxicity and persistence of nearshore sediment contamination following the 1991 Gulf War', *Environment International*, 24: 33-42.

Reddy, Christopher M, J Samuel Arey, Jeffrey S Seewald, Sean P Sylva, Karin L Lemkau, Robert K Nelson, Catherine A Carmichael, Cameron P McIntyre, Judith Fenwick, and G Todd Ventura. 2012. 'Composition and fate of gas and oil released to the water column during the Deepwater Horizon oil spill', *Proceedings of the National Academy of Sciences*, 109: 20229-34.

Reddy, Christopher M, Timothy I Eglinton, Aubrey Hounshell, Helen K White, Li Xu, Richard B Gaines, and Glenn S Frysinger. 2002. 'The West Falmouth oil spill after thirty years: The persistence of petroleum hydrocarbons in marsh sediments', *Environmental science & technology*, 36: 4754-60.

Rico-Martínez, Roberto, Terry W. Snell, and Tonya L. Shearer. 2013. 'Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A® to the *Brachionus plicatilis* species complex (Rotifera)', *Environmental Pollution*, 173: 5-10.

Robertson, Martha J, David A Scruton, and Keith D Clarke. 2007. 'Seasonal effects of suspended sediment on the behavior of juvenile Atlantic salmon', *Transactions of the American Fisheries Society*, 136: 822-28.

Robinson, Kelly L, James J Ruzicka, Frank J Hernandez, William M Graham, Mary Beth Decker, Richard D Brodeur, and Malinda Sutor. 2015. 'Evaluating energy flows through jellyfish and gulf menhaden (*Brevoortia patronus*) and the effects of fishing on the northern Gulf of Mexico ecosystem', *ICES Journal of Marine Science: Journal du Conseil*: 12 pp.

Rooker, Jay R., Larissa L. Kitchens, Michael A. Dance, R. J. David Wells, Brett Falterman, and Maelle Cornic. 2013. 'Spatial, Temporal, and Habitat-Related Variation in Abundance of Pelagic Fishes in the Gulf of Mexico: Potential Implications of the Deepwater Horizon Oil Spill', *PloS one*, 8: e76080.

Rose, WL, BL French, WL Reichert, and M Faisal. 2000. 'DNA adducts in hematopoietic tissues and blood of the mummichog (*Fundulus heteroclitus*) from a creosote-contaminated site in the Elizabeth River, Virginia', *Marine Environmental Research*, 50: 581-89.

Roth, Agatha-Marie F, and Donald M Baltz. 2009. 'Short-term effects of an oil spill on marsh-edge fishes and decapod crustaceans', *Estuaries and Coasts*, 32: 565-72.

Ryerson, Thomas B, Richard Camilli, John D Kessler, Elizabeth B Kujawinski, Christopher M Reddy, David L Valentine, Elliot Atlas, Donald R Blake, Joost De Gouw, and Simone Meinardi. 2012. 'Chemical data quantify Deepwater Horizon hydrocarbon flow rate and environmental distribution', *Proceedings of the National Academy of Sciences*, 109: 20246-53.

Schaefer, Jacob, Nkrumah Frazier, and Jonathan Barr. 2016. 'Dynamics of Near-Coastal Fish Assemblages following the Deepwater Horizon Oil Spill in the Northern Gulf of Mexico', *Transactions of the American Fisheries Society*, 145: 108-19.

Schaefer, Kurt M, Daniel W Fuller, and Barbara A Block. 2007. 'Movements, behavior, and habitat utilization of yellowfin tuna (*Thunnus albacares*) in the northeastern Pacific Ocean, ascertained through archival tag data', *Marine Biology*, 152: 503-25.

Schaum, John, Mark Cohen, Steven Perry, Richard Artz, Roland Draxler, Jeffrey B. Frithsen, David Heist, Matthew Lorber, and Linda Phillips. 2010. 'Screening level assessment of risks due to dioxin emissions from burning oil from the BP Deepwater Horizon Gulf of Mexico spill', *Environmental science & technology*, 44: 9383-89.

Shaw, Richard F., William J. Wiseman Jr, R. Eugene Turner, Lawrence J. Rouse Jr, Richard E. Condrey, and Francis J. Kelly Jr. 1985. 'Transport of larval gulf menhaden *Brevoortia patronus* in continental shelf waters of western Louisiana: a hypothesis', *Transactions of the American Fisheries Society*, 114: 452-60.

Sikorski, Zdzisław E., and Javier A. Borderias. 1995. 'Collagen in the muscles and skin of marine animals.' in, *Seafood proteins* (Springer).

Silliman, Brian R., Johan van de Koppel, Michael W. McCoy, Jessica Diller, Gabriel N. Kasozi, Kamala Earl, Peter N. Adams, and Andrew R. Zimmerman. 2012. 'Degradation and resilience in Louisiana salt marshes after the BP–Deepwater Horizon oil spill', *Proceedings of the National Academy of Sciences*, 109: 11234-39.

Simonato, Juliana Delatim, Carmen L. B. Guedes, and Cláudia B. R. Martinez. 2008. 'Biochemical, physiological, and histological changes in the neotropical fish *Prochilodus lineatus* exposed to diesel oil', *Ecotoxicology and environmental safety*, 69: 112-20.

Singer, MM, D Aurand, GE Bragin, JR Clark, GM Coelho, ML Sowby, and RS Tjeerdema. 2000. 'Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing', *Marine Pollution Bulletin*, 40: 1007-16.

Smith, Lawrence C, Murphy Smith, and Paul Ashcroft. 2011. 'Analysis of environmental and economic damages from British Petroleum's Deepwater Horizon oil spill', *Albany Law Review*, 74: 563-85.

Spier, Chelsea, William T. Stringfellow, Terry C. Hazen, and Mark Conrad. 2013. 'Distribution of hydrocarbons released during the 2010 MC252 oil spill in deep offshore waters', *Environmental Pollution*, 173: 224-30.

Spies, Robert B, John J Stegeman, David E Hinton, Bruce Woodin, Roxanne Smolowitz, Mark Okihiro, and Damion Shea. 1996. 'Biomarkers of hydrocarbon exposure and sublethal effects in embiotocid fishes from a natural petroleum seep in the Santa Barbara Channel', *Aquatic Toxicology*, 34: 195-219.

Stieglitz, John D, Edward M Mager, Ronald H Hoenig, Daniel D Benetti, and Martin Grosell. 2016. 'Impacts of Deepwater Horizon crude oil exposure on adult mahi-mahi (*Coryphaena hippurus*) swim performance', *Environmental Toxicology and Chemistry*.

Szeliga, Jan, and Anthony Dipple. 1998. 'DNA adduct formation by polycyclic aromatic hydrocarbon dihydrodiol epoxides', *Chemical research in toxicology*, 11: 1-11.

Tansel, Berrin, Ariadna Arreaza, Derya Z. Tansel, and Mengshan Lee. 2015. 'Decrease in osmotically driven water flux and transport through mangrove roots after oil spills in the presence and absence of dispersants', *Marine Pollution Bulletin*, 98: 34-39.

Teal, John M, and Robert W Howarth. 1984. 'Oil spill studies: a review of ecological effects', *Environmental Management*, 8: 27-43.

Tjeerdema, Ronald, Adriana C Bejarano, and Sara Edge. 2013. "Biological Effects of Dispersants and Dispersed Oil on Surface and Deep Ocean Species." In *From the Oil Spill Dispersant-Related Research Workshop, hosted by the Center for Spills in the Environment*.

Turner, R Eugene, Giovanna McClenachan, and Andrew W Tweel. 2016. 'Islands in the oil: Quantifying salt marsh shoreline erosion after the Deepwater Horizon oiling', *Marine Pollution Bulletin*.

Valentine, David L., John D. Kessler, Molly C. Redmond, Stephanie D. Mendes, Monica B. Heintz, Christopher Farwell, Lei Hu, Franklin S. Kinnaman, Shari Yvon-Lewis, and Mengran Du. 2010. 'Propane respiration jump-starts microbial response to a deep oil spill', *Science*, 330: 208-11.

Vaughan, Douglas S, Kyle W Shertzer, and Joseph W Smith. 2007. 'Gulf menhaden (*Brevoortia patronus*) in the US Gulf of Mexico: fishery characteristics and biological reference points for management', *Fisheries Research*, 83: 263-75.

Vignier, J, L Donaghy, P Soudant, FLE Chu, JM Morris, MW Carney, C Lay, M Krasnec, R Robert, and AK Volety. 2015. 'Impacts of Deepwater Horizon oil and associated dispersant on early development of the Eastern oyster *Crassostrea virginica*', *Marine Pollution Bulletin*, 100: 426-37.

Vogelbein, Wolfgang K, John W Fournie, Peter A Van Veld, and Robert J Huggett. 1990. 'Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site', *Cancer Research*, 50: 5978-86.

Wade, Terry L, José L Sericano, Stephen T Sweet, Anthony H Knap, and Norman L Guinasso. 2016. 'Spatial and temporal distribution of water column total polycyclic aromatic hydrocarbons (PAH) and total petroleum hydrocarbons (TPH) from the Deepwater Horizon (Macondo) incident', *Marine Pollution Bulletin*.

Walker, William J., Richard P. McNutt, and CarolAnn K. Maslanka. 1999. 'The potential contribution of urban runoff to surface sediments of the Passaic River: Sources and chemical characteristics', *Chemosphere*, 38: 363-77.

Wang, Zhendi, BP Hollebone, M Fingas, B Fieldhouse, L Sigouin, M Landriault, P Smith, J Noonan, G Thouin, and James W Weaver. 2003. 'Characteristics of spilled oils, fuels, and petroleum products: 1. Composition and properties of selected oils', *United States Enviromental Protection Agency*.

Weng, KC, MJW Stokesbury, AM Boustany, AC Seitz, SLH Teo, SK Miller, and BA Block. 2009. 'Habitat and behaviour of yellowfin tuna *Thunnus albacares* in the Gulf of Mexico determined using pop-up satellite archival tags', *Journal of Fish Biology*, 74: 1434-49.

White, Helen K., Pen-Yuan Hsing, Walter Cho, Timothy M. Shank, Erik E. Cordes, Andrea M. Quattrini, Robert K. Nelson, Richard Camilli, Amanda W. J. Demopoulos, and Christopher R. German. 2012. 'Impact of the Deepwater Horizon oil spill on a deep-water coral community in the Gulf of Mexico', *Proceedings of the National Academy of Sciences*, 109: 20303-08.

Whitehead, Andrew, Benjamin Dubansky, Charlotte Bodinier, Tzintzuni I Garcia, Scott Miles, Chet Pilley, Vandana Raghunathan, Jennifer L Roach, Nan Walker, and Ronald B Walter. 2012. 'Genomic and physiological footprint of the Deepwater Horizon oil spill on resident marsh fishes', *Proceedings of the National Academy of Sciences*, 109: 20298-302.

Williams, Rob, Shane Gero, Lars Bejder, John Calambokidis, Scott D. Kraus, David Lusseau, Andrew J. Read, and Jooke Robbins. 2011. 'Underestimating the damage: interpreting cetacean carcass recoveries in the context of the Deepwater Horizon/BP incident', *Conservation Letters*, 4: 228-33.

Wolf, Jeffrey C, Wes A Baumgartner, Vicki S Blazer, Alvin C Camus, Jeffrey A Engelhardt, John W Fournie, Salvatore Frasca Jr, David B Groman, Michael L Kent, and Lester H Khoo. 2015. 'Nonlesions, misdiagnoses, missed diagnoses, and other interpretive challenges in fish histopathology studies: a guide for investigators, authors, reviewers, and readers', *Toxicologic pathology*, 43: 297-325.

Woodhead, AD. 1981. 'Penetration and distribution of carbon particles in a teleost fish, *Poecilia formosa* (Girard), the Amazon molly', *Journal of Fish Biology*, 19: 237-42.

Wurl, Oliver, and Jeffrey Phillip Obbard. 2004. 'A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms', *Marine Pollution Bulletin*, 48: 1016-30.

Ylitalo, Gina M, Margaret M Krahn, Walton W Dickhoff, John E Stein, Calvin C Walker, Cheryl L Lassitter, E Spencer Garrett, Lisa L Desfosse, Karen M Mitchell, and Brandi T Noble. 2012. 'Federal seafood safety response to the Deepwater Horizon oil spill', *Proceedings of the National Academy of Sciences*, 109: 20274-79.

Zhang, Youyu, Lixing Huang, Chonggang Wang, Dongxu Gao, and Zhenghong Zuo. 2013. 'Phenanthrene exposure produces cardiac defects during embryo

development of zebrafish (*Danio rerio*) through activation of MMP-9', *Chemosphere*, 93: 1168-75.

Zhang, Youyu, Chonggang Wang, Lixing Huang, Rong Chen, Yixin Chen, and Zhenghong Zuo. 2012. 'Low-level pyrene exposure causes cardiac toxicity in zebrafish *Danio rerio* embryos', *Aquatic Toxicology*, 114: 119-24.