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UPCOMING PUBLICATIONS:

Pie, H.V., Mitchelmore, C.L. 2014. Determining the acute toxicity of current and alternative oil spill chemical dispersants to early life-stage blue crabs (*Callinectes sapidus*). (In Preparation)

Pie, H.V., Schott, E.J., Mitchelmore, C.L. 2014. Investigating molecular and physiological effects in juvenile blue crab, *Callinectes sapidus*, exposed to sediments contaminated by oil from the Deepwater Horizon Incident. (In Preparation)

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ABSTRACT

Title of Dissertation: Assessing the Exposure to and Impacts of Oil Constituents and Chemical Dispersants on Marine Invertebrates

Hannah Victoria Pié, Doctor of Philosophy, 2014

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While the impacts of oil spill events on marine ecosystems have been well studied over the past half-century, continued large scale oil leaks/spills like the Deepwater Horizon Incident serve to highlight how much we still do not fully understand regarding the impacts of oil exposure and the data gaps that exist in current damage assessments and remediation strategies. These events also emphasize how critical it is to have a thorough understanding of the native ecosystem where the oil spills occurs when deciding the best response options during the event, understanding the damages to organisms, and determining what strategies are needed to achieve and assess recovery. Therefore, this dissertation aimed to address observed data gaps in response options, damages assessments, and remediation strategies relevant to the Northern Gulf of Mexico. Firstly, it addresses the lack baseline data necessary to assess exposure to and impacts of oil during and recovery following an oil spill event for offshore pelagic and benthic zones by describing a novel resource for future offshore biomonitoring using oil rig fouling invertebrates. The low baseline accumulation of polycyclic aromatic hydrocarbons (PAHs) and the quick accumulation response following a contamination event observed in this study demonstrates the benefits of using oil rig fouling invertebrates as offshore biomonitoring resources. Secondly, it addresses the lack of data on the acute or sublethal

toxicity of oil exposures on an important ecological and economic species in the Gulf of Mexico, the blue crab, *Callinectes sapidus*. The enclosed studies provide a crucial foundation for understanding the sensitivity of blue crabs at multiple early life stages to oil, chemically-dispersed oil, and chemical dispersants. The blue crab exposure studies highlight the benefit of fully characterizing exposure solutions beyond total petroleum hydrocarbons by using a suit of PAHs and distinguishing between dissolved and particulate fractions of exposure solutions to better understand observed toxicities and potential routes of uptake by organisms. Overall, the data gaps addressed by this research can aid future managers and responders as they decide on response and remediation options following an oil spill in the Northern Gulf of Mexico.

Assessing the Exposures to and Impacts of Oil Constituents and Chemical Dispersants on
Marine Invertebrates

by
Hannah Victoria Pié

Dissertation submitted to the Faculty of the Graduate School of the
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To my parents, thank you for all you have sacrificed to provide me the opportunity to
achieve such a prestigious degree

To my husband, thank you for all your unconditional love and support

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research. I am also grateful to the CBL Graduate Education Committee for travel support to be able to present my research at scientific meetings.

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List of Abbreviations

AK	Arginine kinase
AN	Anthracene
ANOVA	Analysis of variance
ARC	Aquatic Research Center
ASW	Artificial seawater
BaP	Benzo[a]pyrene
BAS	Bioanalytical Service
BDL	Below detection limit
BLAST	Basic local alignment search tool
BP	British Petroleum
CBL	Chesapeake Biological Laboratory
CEWAF	Chemically enhanced water accommodated fraction
CRRC	Coastal Response Research Center
CT	Cycle threshold
CuMT	Copper metallothionein
CYP	Cytochrome p450
DCM	Dichloromethane
DMN	Dimethylnaphthalene
DOSS	Diethyl sulfosuccinate
DWH	Deepwater Horizon
EPA	Environmental Protection Agency
ERL	Effects range low
ERM	Effects range median
EROD	Ethoxyresorufin O-deethylase
EST	Expressed sequence tag
FID	Flame ionization detection
FLA	Fluoranthene
GC	Gas chromatograph(y)
GOOMEX	Gulf of Mexico Offshore Operations Monitoring Experiment
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione S-transferase
H ₂ O ₂	Hydrogen peroxide
HBSS	Hank's buffered saline solution
HMW	High molecular weight
hr	Hour
HSD	Honest significance difference
IMET	Institute of Marine and Environmental Technology
K _{ow}	Octanol-water partitioning coefficient
LC ₅₀	Median lethal concentration
LMPA	Low melting point agarose

LMW	Low molecular weight
min	Minute
MnSOD	Manganese superoxide dismutase
MS	Mass spectrometer/spectrometry
NCBI	National Center for Biotechnology Information
NCP	National Contingency Plan
NMPA	Normal melting point agarose
NOAA	National Oceanographic and Atmospheric Administration
NOEC	No observed effect concentration
NRC	National Research Council
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerized chain reaction
PHE	Phenanthrene
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per thousand
PY	Pyrene
qPCR	Quantitative polymerized chain reaction
ROS	Reactive oxygen species
RT	Reverse transcribed
SCAT	Shoreline Clean-up Assessment Team
SEM	Standard error of the mean
SIM	Selective ionization mode
SLC	Sweet Louisiana Crude
TEH	Total extractable hydrocarbons
TOC	Total organic carbon
TPAH	Total polycyclic aromatic hydrocarbons
TPH	Total petroleum hydrocarbons
UV	Ultraviolet
WAF	Water accommodated fraction
WMA	Wildlife Management Area
ZI-ZVII	Zoea stage 1 to 7

Chapter 1. Introduction

The rising demand for and the use of petroleum based products over the last few decades has resulted in an increase in oil pollution within marine environments through multiple sources. These include oil spills/leaks from transportation and extraction processes (e.g. Exxon Valdez and the Deepwater Horizon Incident) or from urban runoff and discharge. Anthropogenic releases of petroleum due to oil and gas exploration and production only account for an average of 3,000 tonnes per year in North America (NRC, 2003). However, these inputs are not trivial as they can occur as large, acute spills or slow, chronic releases that are concentrated around production fields and can pose significant risks to sensitive environments. It is also important to note that oil pollution in the ocean can come from natural sources such seepage from geological formations below the seafloor or from large scale weather events like hurricanes or flooding. In North America, natural seepage of crude oil from beneath the seafloor is estimated to be 1,123,000 barrels of crude oil per year and is responsible for over 60% of the total petroleum entering North American waters (NRC, 2003; Schmidt, 2009). These natural seeps tend to occur sporadically and at low enough flow rates that allow for an ecosystem to adapt so there is little if any net harm to organisms or ecosystem services. The extent of natural seepage is not homogenous throughout all waters in North America. Regions like the Northern Gulf of Mexico and Southern California are rich in oil deposits and are known to have the highest levels of continuous natural seeps, with best estimates of 980,000 and 140,000 barrels of crude oil per year, respectively. Other regions like the northern and southern coastline of Alaska have been shown to have orders of magnitude

lower natural levels with best estimate of only 2,800 barrels of crude oil per year (NRC, 2003; Schmidt, 2009). In the Northern Gulf of Mexico, this natural release of oil is potentially enhanced by the presence of close to 4,000 active petroleum and gas platforms resulting in an ecosystem exposed to potentially higher levels of oil components, including petroleum polycyclic aromatic hydrocarbons (PAHs).

The Northern Gulf of Mexico's continental shelf is among the most ecologically productive and economically important regions in North America. The Gulf of Mexico's coastal wetlands span about five million acres (approximate half of the U.S. total) and serve as essential habitat for numerous fish and wildlife species (Turner and Streever, 2002; Felder and Camp, 2009). Additionally, the fisheries of the Gulf of Mexico are among some of the most productive in the world. The 2012 commercial fish and shellfish harvest from the five U.S. Gulf states was estimated to be 1.6 billion pounds valued at over \$754 million (NMFS, 2013). Louisiana alone had the second highest landing volume at 1.2 billion pounds and the fourth highest landing value at \$328 million for all U.S. States in 2012 (NMFS, 2013). Beyond commercial fishing, the recreational fishing industry is incredibly important to the economy of the Gulf Coast states with almost 3.1 million residents participating in marine recreational fishing in 2012 (NMFS, 2013). Therefore, given the ecological and economical importance of the Northern Gulf of Mexico it is imperative to develop a thorough understanding of the potential impacts of oil and PAH exposures on its ecosystems and organisms residing within.

1.1. Crude Oil and Polycyclic Aromatic Hydrocarbons

Crude oil is composed of hundreds of types of hydrocarbons (predominately aliphatic, alicyclic and aromatic hydrocarbons) as well as trace metals like nickel, vanadium, and chromium and chemicals containing sulfur, nitrogen, and oxygen. A broad average shows that crude oils contain 84% carbon, 14% hydrogen, 1-3% sulfur, and approximately 1.0% nitrogen, 1.0% oxygen, and 0.1% minerals and salts (US EPA, 2011). However, the specific chemical composition and proportions of different chemicals can vary drastically between different producing regions and even from within a specific formation. Crude oils are identified by various factors that define their composition including viscosity, specific gravity or density (light or heavy), type of predominant hydrocarbons (paraffinic or naphthenic), sulfur content (sweet or sour) and API gravity indicating gasoline potential (US EPA, 2011).

Furthermore, the chemical composition of crude oil in an oil spill is substantially altered over time due to chemical and physical changes (i.e. weathering) caused by processes like evaporation, photooxidation, biodegradation, and water-in-oil emulsification (NRC, 2005). The extent of weathering can differ depending on environmental conditions, especially temperature, and crude oil type. Evaporation, in particular, is considered to be the most important and rapid of all weathering processes, especially as it results in the extensive loss of lower molecular weight hydrocarbons from the oil (McAuliffe, 1989).

The wide variety in chemical composition and physical properties of different crude oils can produce variable profiles of toxic effects in marine organisms. Therefore, to more fully understand the toxic effects observed, it is crucial to fully characterize oil

exposures in the field and laboratory by measuring as many chemical components as possible and expanding beyond just measuring the total petroleum hydrocarbon (TPH) concentration. In addition to TPH, other recommended components for chemical analysis include volatile organic compounds (e.g. benzene, toluene, ethylbenzene, and xylenes), individual alkanes, and a suite of parent and alkylated homolog PAHs (NRC, 2005).

Although they comprise only a small fraction (0.2-7%) of the total composition of crude oil, PAHs represent one of the major contributors to the toxicity of oil in the environment; and therefore, are important to examine in detail for oil exposure studies (US EPA, 2011). PAHs are natural, ubiquitous substances in the marine environment that can range in size and complexity from 2-ring naphthalene up to complex 10 ring structures. The main sources of PAHs into the ecosystem are pyrogenic, combustion of fossil fuels, and petrogenic, fossil fuel (e.g. oil) derived (Hylland et al., 2006). The majority of PAHs found in the aquatic environment originate from pyrogenic sources, as pyrogenic PAHs tend to distribute on a broader geographic scale due to long-range atmospheric transport. Petrogenic PAHs, however, appear to be more associated with local or point sources (e.g. oil refineries, spills, and transport). While pyrogenic and petrogenic PAHs are the two primary sources in the environment, diagenic sources can also play a major role in PAH inputs within terrestrial and marine sediments (Venkatesan, 1988). Diagenic PAHs are formed during early diagenesis of biogenic precursors like plant terpenes within anaerobic sediments (Venkatesan, 1988; US EPA, 2003).

PAHs are divided into two main categories by size: low molecular weight (LMW) that are composed of less than 4 aromatic rings and high molecular weight (HMW) that

are composed of 4 or greater aromatic rings. Petrogenic sources are primarily composed of LMW 2- and 3-ringed PAHs, while pyrogenic sources are dominated by HMW 4-, 5-, and 6-ringed PAHs (Neff et al., 2005). Additionally, PAHs containing straight carbon side chain(s) are referred to as substituted or alkylated PAHs, while their unsubstituted homologs are called parent PAHs. The relative concentrations of individual PAHs and the extent of alkylation can vary depending upon the source(s). Alkyl substitutions tend to be eliminated by the high temperatures (greater than several hundred °C). Therefore, processes like pyrogenic fossil fuel combustion that occur at high temperatures will produce predominately unsubstituted, parent PAHs. Petrogenic sources, however, are formed at lower temperatures over a longer time period (Steinhauser and Boehm, 1992). This generally results in a higher relative abundance of alkyl-substituted PAHs with a bell-shaped distribution of parent and alkylated homologs and highest concentrations in single and double substituted homologs (Neff et al., 2005; Kobilinsky, 2011).

The overall fate and partitioning of individual PAHs between environmental compartments varies by size (molecular weight) and by water-solubility (octanol-water partitioning coefficient: K_{ow}). Higher molecular weight PAHs are generally considered less bioavailable as they are less water-soluble, less volatile, and tend to associate more with particles and dissolved organic carbons (POC and DOC) leading to higher rates of sedimentation (Meador et al., 1995). However, lower molecular weight PAHs are more water-soluble and less strongly sorbed by particles and sediments; and therefore, more bioavailable for uptake by marine organisms. Exposure routes and uptake of PAHs in marine organisms can vary by many factors including life history, life stage, location in aquatic system (i.e. benthic vs. pelagic), and feeding methods. In general, PAHs can be

taken up by marine organisms directly from the water (dermal uptake or via respiratory surfaces) or through direct (or indirect) ingestion pathways (Hylland et al., 2006).

Generally, the higher the water solubility of a PAH (lower K_{ow}), the more bioavailable it is to marine organisms as dissolved hydrocarbons are more likely to diffuse across gills, skin, or other exposed membranes (Meador et al., 1995). Whereas, the PAHs bound to particulates (e.g. oil droplets or colloidal organic matter) tends to impact marine species mostly through physical coating of gills and other bodily surfaces or by incorporation into their diet. Incorporation into their diet can be an important route of exposure for marine invertebrates like filter-feeding bivalves or larval stages of species whose typical prey size can fall within the common size range of oil particles. Additionally, species that feed within sediments might indirectly ingest PAHs bound to suspended particulate matter.

1.2. Effects of Oil exposure on Marine Organisms

Exposure to oil and its constituents can be highly detrimental to marine organisms and can result in acute toxicity and/or sublethal effects. Sensitivity to oil and its toxic components (e.g. PAHs) can vary within and between species due to many factors: specific life stage, habitat, route of uptake and extent of bioaccumulation, metabolism and other physiological/biochemical pathways, and elimination capacity. During the past few decades, an extensive body of research has been published on the biological effects of petroleum PAHs in the marine environment (see reviews by NRC, 2003; NRC, 2005; Hylland et al., 2006). Acute impacts of oil are generally related to its constituents, most notably LMW PAHs, acting as non-polar (Type I) narcotics (DiToro et al., 2000). Non-

polar narcotics generally result in depression of biological activity, which is thought to occur due to alterations of cell membranes (Rand et al., 1995). Although the exact mechanism remains unclear, the two predominant theories, critical-volume and protein-binding, suggest that narcosis is caused by either changes in the lipid component of cell membranes due to increase in volume or by binding of toxicants to receptor sites located in the hydrophobic regions of proteins in cell membranes, respectively (Abernethy et al., 1988; Rand et al., 1995). While this process can be completely reversible, if concentrations are high enough or the organism cannot escape exposure it can also eventually lead to paralysis and even death (Rand et al., 1995). As Type 1 narcotics, the toxicological effects of PAHs are considered additive, which can make aid in predicting acute toxicity (Di Torro et al., 2000). The use of toxic unit models based on additive effects has been suggested to estimate acute toxicity of PAHs and other oil components (DiTorro et al., 2000). The extent of acute toxicity of PAHs varies by molecular weight. In general, more water-soluble lower molecular weight PAHs have significantly higher acute toxicities than less water-soluble higher molecular weight PAHs (Donkin et al., 1990).

While narcosis models have the utility in predicting acute mortality, they may underestimate toxicity in cases where sublethal and delayed effects are manifested, particularly for chronic exposures (Barron et al., 2004). Other non-narcotic modes of action have been considered to explain sublethal and delayed effects in marine organisms including receptor mediated toxicity through interaction with the aryl hydrocarbon receptor (AhR), edemas, cardiovascular dysfunction, genotoxicity, and endocrine disruption (Brinkworth et al., 2003; Barron et al., 2004). These non-narcotic models for

sublethal effects of PAHs have been linked to disruptions in energetic functions and normal homeostasis, enzyme inductions (e.g. cytochrome p450)/inhibitions, and DNA damage (NRC, 2003; Hylland et al., 2006). Multiple biomarker assays have been designed to examine these impacts, particularly for DNA damage and enzyme inductions/inhibitions within metabolic and oxidative stress systems (see Section 1.5.2). Biomarkers of exposure and effects can serve as early warning signs of adverse biological effects from exposure to a contaminant. By examining early effects at a molecular or cellular level (e.g. enzyme activities and DNA damage) it can be possible to predict effects of a contaminant at later levels such as tissue, systemic, individual, and even population (van der Oost et al., 2003). Examples of later sublethal and delayed effects of PAHs include disruption of energetic processes, alterations to immune responses, disruption of ionic and osmotic regulation, alterations in feeding, decreases in growth, and decreases in reproductive success (NRC, 2003; Martínez-Gómez et al., 2010, Luna-Acosta et al., 2011).

Another major concern with oil exposures is the carcinogenic and mutagenic effects of certain PAHs, particularly HMW PAHs. There exists extensive knowledge of how carcinogenic PAHs interact with biological systems (review see Neff, 2002). The carcinogenic effects of PAHs results from biotransformation and metabolism within organisms, which is thought to be more prevalent in vertebrates. Carcinogenic PAHs are metabolized through the phase I and phase II metabolic systems (Morgan et al., 1998). The mutagenic and carcinogenic potential of different PAHs is associated with the propensity of phase I enzymes (mixed function oxidase system coupled to the aryl hydrocarbon hydroxylase system) to generate reactive PAH metabolites (e.g. epoxides).

These metabolic products can be detoxified by phase II enzymes and subsequently excreted or they can bind to cellular components like DNA resulting in DNA adduct formation. If not repaired, the DNA damage can ultimately lead to unregulated cell growth/division and tumor formation (Collier et al., 1992).

1.3. Responses to and the Remediation of Oil Spills

Multiple means of remediation are used to reduce the impacts from oil spills and leaks on marine ecosystems. While the primary means of clean-up remains mechanical containment and recovery of the oil slick, chemical dispersants are used as a secondary mechanism to reduce the overall environmental and economic impact of an oil spill/leak. The decision to use dispersants both on surface plumes and more recently directly at the oil source/leak is based upon minimizing the overall environmental harm of oil coupled with human health considerations. Dispersant use results in environmental tradeoff decisions on what resources are most important to protect in an oil spill event. The aim of using chemical dispersants is to reduce the amount of oil reaching sensitive and highly productive shoreline habitats. However, their use increases the amount of oil entrained in the water column potentially leading to increased impacts on pelagic (water column) and benthic (bottom dwelling) organisms. Chemical dispersant use in oil spill response strategies has always been a controversial issue, particularly given the data gaps and uncertainties regarding their sublethal and delayed impacts as well as long-term implications of large-scale dispersant use in the marine environment (NRC, 1989; NRC, 2005).

Current formulation chemical dispersants generally contain two or more nonionic or anionic surfactants and/or solvents. The exact composition of these mixtures, beyond the hazardous substances listed on an MSDS sheet, is generally proprietary with the exception of Corexit 9500 and Corexit 9527. Following the extensive use of these Corexit products during the Deepwater Horizon (DWH) Incident and subsequent pressure from the Environmental Protection Agency (EPA), Nalco released the detailed chemical composition of both dispersants (Nalco, 2011). The composition details for Corexit 9500 and the other oil spill remediation chemical dispersants and surface washing agents examined in this dissertation are described in Table 1.1.

Chemical dispersants reduce the interfacial tension between oil and water in order to enhance the natural dispersion process by increasing the generation of small droplets of oil. These droplets enter into the water column by wave energy, which serves to dissipate and dilute the oil rapidly in three dimensions. It is often not the inherent toxicity of the dispersant itself but what it does to the oil (i.e. the dispersant:oil mixture) that is of toxic concern. These small oil droplets tend to remain suspended in the water column, especially within the top 10m, before diluting in three dimensions over time post-spill. This increases the bioavailability of oil to organisms within those systems as the oil is more likely to remain in the water column rather than return to the surface to reform oil slicks. It is important to note that use of chemical dispersants in remediation does not actually remove the oil from the ecosystem, but instead serves to alter the overall fate of the oil, minimizing net harm. Depending on the weather and environmental conditions, surface plumes sprayed with chemical dispersants can be rapidly diluted, although significant concentrations of petroleum hydrocarbons can still exist in the top 10m of the

Table 1.1 Chemical composition details for chemical dispersants and surface washing agents used in the dissertation.

Chemical Name	Manufacture	pH	Specific Gravity	CAS Number	Composition Ingredient
Corexit 9500	Nalco Environmental Solutions LLC	6.2	0.95	1338-43-8	Sorbitan, mono-(9Z)-9-octadecenoate
				9005-65-6	Sorbitan, mono-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs
				9005-70-3	Sorbitan, tri-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs
				577-11-7	Butanedioic-acid, 2-sulfo-,1,4-bis(2-ethylhexyl) ester, sodium salt (1:1)
				29911-28-2	Propanol,1-(2-butoxy-1-methylethoxy)
				64742-47-8	Distillates (petroleum), hydrotreated light
Sea Brat #4	Alabaster Corp.	7.0-8.0	1.0	NA	All ingredients proprietary
Petroclean	Alabaster Corp.	7.0-8.0	1.02	NA	All ingredients proprietary
Orca	Alabaster Corp.	7.0-8.0	1.02	NA	All ingredients proprietary
Dispersit SPC 1000	U.S. Polychemical Corp.	10.0	0.995	34590-94-8	Dipropylene glycol ether

water column where pelagic organisms, particularly the early life stages of many species, are concentrated (NRC, 2005).

To date, Corexit 9500 and its older formulation Corexit 9527 are the most commonly used chemical dispersants within U.S. waters and their toxicity has been extensively studied in multiple species and life stages (see reviews in NRC, 1989; NRC, 2005). Both dispersants were utilized in the DWH incident, although Corexit 9500 was used to a larger extent once supplies of the older (and not currently manufactured) formulation Corexit 9527 were exhausted. During the near three months prior to the capping of the Macondo 252 wellhead, approximately 1.8 million gallons of dispersants were applied to both the surface (58%) and at the wellhead (42%) (Barron, 2012). Given the extensive use of dispersants during the DWH incident and the EPA's call for British Petroleum (BP) to use a less toxic alternative than Corexit 9500 (US EPA, 2010), there has been an increased emphasis on the potential use of less toxic, but equally or more effective dispersant alternatives. While Corexit 9500 and Corexit 9527 are the most commonly used dispersant, numerous alternatives listed under the EPA's National Contingency Plan (NCP) Product Schedule are also available (US EPA, 2014). Corexit 9500 has a 54.7% effectiveness for dispersing South Louisiana Crude (SLC) oil, which is similar to the Sweet Louisiana Crude from the DWH incident (US EPA, 2013). The many alternative dispersants in the list have higher percent effectiveness and similar or less acute toxicity with dispersed oil (No. 2 fuel oil) than Corexit 9500 for SLC (US EPA, 2013). A higher effectiveness means less dispersant would be needed to achieve the same amount of dispersion, resulting in potentially fewer toxicological impacts. However, very few acute toxicity studies have been conducted on these alternatives beyond the data directly

supplied to the EPA from the manufacturers (US EPA, 2013) and the EPA's recent toxicity tests by Hemmer et al. (2010). While these alternatives might have similar or less acute toxicity, the sublethal or delay effects (e.g. endocrine disruption) of most of the alternatives have not been assessed, which would be important to understand before large scale use. For example, the original formulation of Sea Brat #4 had similar acute toxicity to Corexit 9500 (US EPA, 2013), but contained nonylphenol ethoxylates, which can undergo biodegradation to nonylphenol, a known endocrine disrupter. A recent study by Judson et al. (2010) conducted *in vitro* tests to examine the endocrine disruption of 8 chemical dispersants focusing on the estrogen (ER) and androgen (AR) receptors. Only Nokomis 3-F4 and ZI-400 showed weak ER activity with EC₅₀ of 16 and 25 parts per million (ppm), respectively (Judson et al., 2010).

Other factors to consider with dispersant use are the potential persistence in the marine environment. One recent study by Campo et al. (2013) examined the biodegradation of Corexit 9500 alone and in combination with SLC oil using microbial cultures collected from near the Macondo wellhead (cryo cultures) and surface waters above wellhead. Dioctyl sulfosuccinate (DOSS) in Corexit 9500 with and without SLC was almost completely reduced by surface dwelling cultures (25°C) within 8 and 14 days, respectively, while there was very limited reduction in many of the cryo cultures (5°C) with Corexit 9500 alone and a 28 day lag in reduction of Corexit 9500 in the presence of SLC (Campo et al., 2013). This suggests that dispersants sprayed near the wellhead (~0.76 million barrels total) might have persisted in the environment for a much longer period of time than surface applied dispersants. Additionally, little information is known

about what sort of breakdown or degradation by-products are produced from chemical dispersants and what effect those might have on toxicity.

1.4. Assessing the Exposure to and Impacts of Oil During and After a Spill Event

Multiple risk and impact assessment regimes have been suggested for monitoring the environmental effects of offshore oil and gas drilling activities (OSPAR, 2004; Thain et al., 2008; Martinez-Gomez et al., 2010). The consensus for these assessments is that it is essential to use a set of biological endpoints, including biomarkers known to be responsive to PAHs, as well as other general stress or health biomarkers. These biological assessments should be carried out in conjunction with chemical analyses (e.g. for PAHs) of water, sediment, and/or biota samples (Martinez-Gomez et al., 2010). Additionally, the use of long-term biomonitoring with sessile marine invertebrates, particularly bivalves, has been considered a useful method in determining the toxicological impacts from the activity of offshore oil and gas platforms (Hylland et al., 2008; Gomiero et al., 2011).

With all such assessments, it is extremely important that areas in oil exploration/transportation zones have baseline data regarding the ecological (species diversity), biological and chemical state of resident organisms. Furthermore, sessile marine invertebrates have routinely been utilized to examine oil exposure and PAH accumulation as they are excellent point source monitors, quickly and readily accumulate oil components (e.g. via filtration in bivalve species) but are often slow to metabolize/depurate them, possibly reflecting reduced contaminant metabolism/detoxification pathways (Seriano et al. 1996). However, as mentioned earlier for such biomonitoring to be successful, the existence of pre-exposure baseline data is

absolutely essential to fully understand the risk and impact when monitoring the effects of oil exposures during a spill events and remediation efforts to allow for a better understanding of recovery and future impacts to the ecosystem (OSPAR, 2004; Martinez-Gomez et al., 2010).

1.4.1. Oil exposure methods and PAH chemical analysis

Careful thought needs to go into the exposure design of laboratory studies examining the impacts of oil and dispersed oil, particularly with regards to what ultimate question(s) the study hopes to answer. A variety of factors need to be considered when designing such a study including the method used to prepare of exposure solutions, the types of chemical analyses conducted to detail the exposures, and the possible limitations that come with conducting a laboratory exposure studies.

Numerous reviews and analyses of petroleum toxicity in the literature highlight the need for standardized exposure solution preparations when examining the impacts of oil, dispersant and dispersed oil in the laboratory (Singer et al., 2000; Barron and Kaehne, 2003). Suggested methods for preparations for the water accommodated fraction (WAF) of oil and chemical enhanced water accommodated fraction (CEWAF) of oil with chemical dispersant added are discussed in length in the National Research Council (NRC) report (NRC, 2005). The preparation method used is important to consider as variations in oil loading and/or mixing energies can greatly affect the relative concentrations and forms of the oil components test organisms are exposed to. Therefore, with all oil exposure studies detailed chemical analysis of exposure media is crucial for understanding any of the biological impacts observed from exposure to oil, its

components (e.g. PAHs) and/or chemical dispersants. For a complete toxicological understanding regarding bioavailability, bioaccumulation, toxicity, and specific modes of action we need to know the specific chemicals involved, their individual concentrations, the timing of exposure, and also their form (i.e. dissolved or particulate) in the exposure medium. This latter point is quite often not considered when assessing toxicity and yet is the basis for exposure routes and ultimately bioavailability, which is organism and life stage specific.

Many studies that examine the toxicities of oil exposure continue to conduct limited chemical analysis focusing on only total petroleum hydrocarbon (TPH) concentrations, which does little to characterize specific toxicants of exposure. Until recently, any detailed PAH chemical analysis was conducted by examining a limited number of PAHs, mostly parent compounds only, such as the EPA16 priority PAHs. Individual alkylated PAHs should be included with parent PAHs in analyses as they contribute substantially to the total PAH composition of oil exposures and can also contribute to overall toxicity of exposures. Therefore, reviews like the NRC (2005) report recommend that chemical analyses in conjunction with toxicity tests should include analysis of a full suite of parent and alkyl-substituted PAH as well as measuring the fractional components of aqueous oil (e.g. TPH and/or total PAH).

Although thoroughly measuring the concentration and composition of PAHs in the exposure media is important to more completely understand the toxicological impacts, it can be equally, if not more, imperative to determine the partitioning or form of the PAHs within exposure solutions such as dissolved (in-solution) or particulate (droplet) forms. Estimating the amount of PAHs in the dissolved versus particulate fractions is

particularly important when taking into account a test organism's uptake or route of exposure (ultimately bioavailability). Studies have shown that chemically-dispersed oil particles typically range in size from $3-80\mu\text{m}$ with the majority of particles being less than $60\ \mu\text{m}$ (Franklin and Lloyd, 1986; Lunel, 1993, 1995). This range encompasses the preferred size range of prey (e.g. phytoplankton) for the larval stages of many species as well as filter feeding bivalves. However, such analysis is rarely conducted or estimated, leading to potentially over or under reported toxicity estimates. The use of filtration techniques like GF/F filters with a gravity feed or vacuum, although not perfect, have been shown useful in providing an estimate of the total dissolved fraction of PAHs, which can subsequently be used to calculate the particulate fraction by mass balance from the unfiltered exposure solution preparations (Payne and Driskell, 2003; Mitchelmore and Baker, 2010).

In addition to detailed chemical analysis of the exposure media, examining bioaccumulation in organism tissues and the extent of metabolism and types of metabolites produced are also important for understanding how chemical exposures relate to biological impacts and the bioavailability of oil components in the environment. Bioaccumulation and metabolic capacity vary widely between species and taxa. Marine invertebrates such as filter-feeding molluscs have a remarkable ability to bioaccumulate hydrocarbons due in part to their high (and indiscriminate) filtration rates as well as their lower enzymatic activity towards these compounds and slower depuration rates compared to that of most vertebrate species. Other organisms, particularly vertebrates like fish, can readily metabolize and excrete oil components like PAHs making it generally inefficient to examine bioaccumulation within tissues except in acute scenarios. Examining the

bioaccumulation of PAHs in molluscs can be a useful indicator to estimate oil spill exposure as bioavailable chemicals like PAHs accumulate in their tissues at concentrations generally much greater than or in proportion to the surrounding water. They also can be used to monitor post-spill recovery of an ecosystem. Furthermore, understanding bioaccumulation in molluscs is important as their consumption can expose higher trophic level organisms to significant quantities of oil components over protracted time periods.

While many alterations to the PAH profiles of an oil spill/leak can occur between the release into the environment and measuring bioaccumulation in organism tissues (e.g. weathering, biodegradation, photooxidation, and metabolism), extensive chemical analysis of individual parent and alkylated PAHs within water and sediment samples can allow for fingerprinting back to a source oil profile. Various diagnostic ratios of PAHs have been utilized to examine the possible source inputs of PAHs (e.g. petrogenic, pyrogenic, and diagenic) in environmental and tissues samples (e.g. Venkatesan , 1988; Budzinski et al., 1997; Qian et al., 2001; Neffs et al., 2005; Saha et al., 2009). The diagnostic ratios utilized for examining PAH source inputs in sediments and biological tissues for this dissertation are described in Table 1.2.

Due to the distinct differences in PAH size distribution and abundance of alkylation homologs between petrogenic and pyrogenic sources of PAH, ratios comparing the sums of parent to alkylation homologs and HMW to LMW PAHs have been utilized to differentiate between those two sources (Saha et al., 2009; Budzinski et al., 1997, Qian et al., 2001). Additionally, ratios between anthracene and phenanthrene as well as fluoranthene and pyrene are often used in source distinction analyses as they have been

Table 1.2 PAH diagnostic ratios used in the dissertation to distinguish between petrogenic and pyrogenic source inputs within sediment and tissue samples.

Ratio	Petrogenic	Pyrogenic	Reference
Alkylated Homologs/ Parent	> 1	< 1	Saha et al. 2009
HMW/ LMW	< 1	> 1	Qian et al. 2001
PHE/AN	> 10	< 10	Saha et al. 2009
AN/(AN+PHE)	< 0.1	> 0.1	Yunker et al 2002
FLA/PY	> 1	<< 1	Neff et al. 2005
FLA/(FLA+PY)	< 0.4	0.4-0.5 fossil fuel > 0.5 grass, wood, and coal	Yunker et al. 2002
MPHE/PHE	> 2	< 2	Neff et al. 2005
FLAPY/(FLAPY+[C24PHE])	< 0.1	> 0.75	Prahl and Carpenter 1983

*HMW (high molecular weight), LMW (low molecular weight), PHE (phenanthrene), AN (anthracene), FLA (fluoranthene), PY (pyrene), MPHE (sum of alkylated phenanthrenes), FLAPY (sum of fluoranthene and pyrene), C24PHE (sum of C₂- to C₄-phenathrenes)

well characterized in numerous petrogenic and pyrogenic sources as summarized in Neff et al. (2005) and Yunker et al. (2002). Anthracene (AN) and fluoranthene (FLA) are considered less thermodynamically stable than their isomers phenanthrene (PHE) and pyrene (PY), respectively (Baumard et al., 1998). They are produced during rapid, high-temperature pyrosynthesis, but are not likely to remain during the slow generation of fossil fuels (Neff et al., 2005). Furthermore, in distinguishing between petrogenic and pyrogenic PAH assemblages in environmental samples, ratios of parent to alkyl substituted PAHs can be very useful such as the ratio of fluoranthene plus pyrene (FLAPY) to FLAPY plus the sum of C₂- to C₄-phenathrenes (C₂₄PHE) or the ratio of the sum of alkylated phenanthrenes (MPHE) to phenanthrene (Prah and Carpenter, 1983; Neff et al., 2005).

Diagenic sources of PAHs in terrestrial and marine sediments can be distinguished by high concentrations of perylene in relation to other PAHs as perylene is considered one of the most prevalent diagenic PAHs encountered in sediments (Venkatesan, 1988). Perylene is derived from terrestrial or marine biogenic precursors via post-deposition during early diagenesis, but can also come from petrogenic and pyrogenic sources. However, only trace or small amounts of perylene are found in fossil fuels and during most fossil fuel combustions, with a few exceptions like coke production and kerosene combustion (Venkatesan, 1988).

Another important factor to consider for laboratory toxicity tests is the limitations that come with controlled environment exposures as predictors of field impacts. Laboratory experiments, in general, keep constant potentially confounding environmental factors that fluctuate in field exposures, such as temperature and mixing energies of oil (and

chemically dispersed oil) preparations. In the environment for example, temperature can influence both evaporation and emulsification of oil, while both wave action and wind speed can influence the extent of oil dispersion both physically and chemically (NRC, 2005). These processes can ultimately alter the chemical composition and subsequent impacts to marine organisms in an oil spill event. Furthermore, most laboratory experiments examining oil exposure do not account for ultraviolet (UV) light attenuation that organisms living near surface waters (photic zone) would be exposed to in the environment. Some components in oil like specific PAHs can be photoactivated upon exposure to sunlight (UV light). This can ultimately result in extensive damage to cellular macromolecules, including cell membranes, lipids, proteins, and DNA (Mitchelmore and Chipman, 1998; Mitchelmore and Hyatt, 2004). This process, known as photoenhanced toxicity or phototoxicity, has been shown to significantly increase the toxicity of PAHs (up to 3 orders of magnitude) as compared to PAH toxicity in low light conditions typical of most laboratory toxicity tests (Barron and Kawaihue, 2001). Therefore, laboratory studies examining the impacts of oil on pelagic organism that dwell in this photic zone, particularly translucent organisms like corals/anemones and the larval stages of many marine species, can severely underestimate toxicity if not using ultraviolet light in exposures.

Additionally, only a limited number of organisms, particularly marine invertebrates, are amenable to be cultured in a laboratory for toxicity tests; and therefore, have been used extensively as standard organisms for toxicity tests. Such standard species are often used as surrogates for other similar species within the environment during risk assessment analysis and hazard level assessment models. However, a study by Driver et

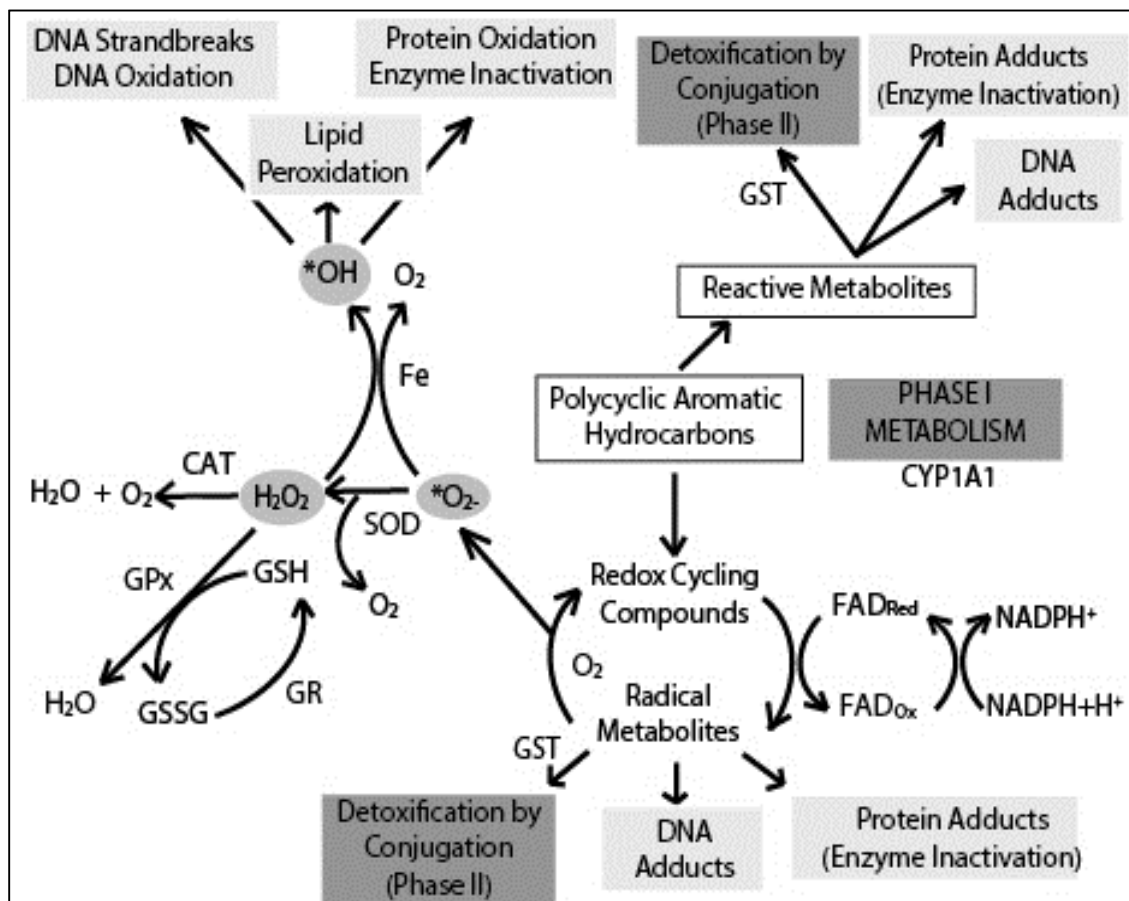


Fig. 1.1 Polycyclic aromatic hydrocarbon (PAH) biotransformation and oxidative stress pathways commonly used for biomarkers of exposure and effects.

al. (2008) suggests that commonly used invertebrate surrogate model species are not always good predictors of toxicity in other invertebrates.

1.4.2. Biomarkers of exposure to and effects of PAHs

As described above (Section 1.2), PAHs in most marine organisms interact with phase I and phase II metabolism systems; and therefore, many biomarkers employed to examine PAH exposure are part of these systems (summary in Figure 1.1). In marine vertebrate (and more complex invertebrate) organisms, the most commonly used biomarker for studying exposure to petroleum PAHs is the hepatic cytochrome P450 system, specifically p4501A1 (CYP1A) in fish species. CYP1A is a phase I metabolism enzyme and part of the mixed function oxidase (MFO) system involved in the biotransformation and excretion of xenobiotic (and endogenous) compounds as well as oxidative stress (Whyte et al., 2000; van der Oost et al., 2003). The ethoxyresorufin *O*-deethylase (EROD) enzyme activity assay, a measurement of CYP1A activity, has been shown to exhibit a time and dose-dependent induction from exposure to PAHs and crude oil in many marine vertebrates (Eggens and Galgi, 1992; Jonsson et al., 2010). Although not as widely applied as the phase I enzyme CYP1A, the phase II metabolism enzyme, glutathione *S*-transferase (GST) has also been utilized as a biomarker for exposure to petroleum PAHs. Certain GST isoforms are involved in both the conjugation of glutathione to xenobiotic metabolites to facilitate excretion and the detoxification of oxidative stress products (Schlenk et al., 2008).

In some species, often invertebrates, metabolism of PAHs by CYP1A and CYP1A-like enzymes can also lead to oxidative stress by the activation of PAHs involved in

redox cycling (Livingstone, 2003). Redox cycling of PAHs results in the production of reactive oxygen species (ROS), which can oxidize most cellular compartments (lipids, protein, DNA) leading to altered cell physiology and even mutations or cell death (Cadenas, 1995). However, cells contain multiple antioxidant enzymes that can neutralize these ROS and prevent oxidative stress such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). However, if these antioxidant systems are overwhelmed, the oxidative stress caused by excess ROS can lead to DNA damage, particularly single-strand DNA breaks. Exposure to PAHs and other pollutants have been shown to result in DNA strand breaks in both the liver and blood cells of marine bivalves and fish as reviewed in Mitchelmore and Chipman (1998) and more recently Frenzilli et al. (2009).

1.4.3. Biomonitoring for the exposure to and impacts of oil in the marine environment

One of the most effective ways to assess the extent of damage and the subsequent recovery from large oil spill events is through the use of a multifaceted biomonitoring program. The collection of organisms, particularly marine invertebrates, to examine their PAH accumulation coupled with general health and exposure/stress biomarkers from areas near an oil spill event can help identify the range of oil contamination and the extent of impacts possible, including projections for other species in the vicinity.

Additionally, the collection of organisms at various time points after a spill event can help determine the extent an ecosystem has recovered and is critical for damage assessments and determining the effectiveness of remediation options. To conduct such a

monitoring program, baseline PAH and health/stress biomarkers need to be examined to provide a clear distinction between normal and exposure levels.

Sessile marine invertebrates, primarily bivalves, have been used for long-term biomonitoring of the exposure to, impacts of and the toxicological effects of the activity of offshore oil and gas platforms in European waters (Hylland et al., 2008; Gomiero et al., 2011) and the ultimate fate of released compounds. Sessile invertebrates have also been used to examine contaminant accumulation like PAHs within the coastal wetlands of the United States, including the Gulf of Mexico, through the National Oceanographic and Atmospheric Administration (NOAA) Mussel Watch program (Wade et al., 1988; Qian et al., 2001). As discussed earlier marine bivalves (e.g. oysters, mussels) are capable of filtering substantial quantities of water over large and highly permeable gills. This filtration, in combination with their low metabolic capacity and slow depuration rates, can result in high bioaccumulation of hydrophobic compounds in their tissues. For these reasons as well as their sessile nature (i.e. good point source indicators), oysters have been utilized as an ideal species for the biomonitoring of pollutants in the marine environment (Kimbrough et al., 2008).

1.5. Assessing the Exposure to and Impacts of Oil in the Northern Gulf of Mexico

Given the recommendations highlighted and issues discussed in Section 1.4, this dissertation serves to address two key data gaps in assessing exposure and impacts of oil within the Northern Gulf of Mexico. The first is the lack of baseline biomonitoring data of offshore organisms in areas with exposure to oil and dispersed oil following an oil spill

event within the Northern Gulf of Mexico oil field. The second is the lack of acute or sublethal effects data with exposure to oil for one of the most important fisheries species within the Gulf of Mexico, the blue crab, *Callinectes sapidus*.

1.5.1. Biomonitoring oil exposures using oil rig structure fouling invertebrates

The use of biomonitoring of exposure to and impacts from oil and gas activities is common in European waters, particularly with caged organisms (Gorbi et al., 2008; Hylland et al., 2008; Sundt et al., 2011, 2012). One study by Gomiero et al. (2011) collected native mussels directly from rig structures in the Adriatic Sea to examine PAH bioaccumulation and biomarker analyses. However, to my knowledge, only one monitoring study, the Gulf of Mexico Offshore Operations Monitoring Experiment (GOOMEX), has been conducted near oil rig locations in the Gulf of Mexico, which is detailed in Kennicutt et al. (1996). GOOMEX was an extensive monitoring study around five oil rigs off the coast of Texas to examine the near-field impacts of oil and gas activities on organisms over a range of distances (30-3000m) from the rigs (Kennicutt et al., 1996). Tissue contamination of hydrocarbons (PAHs included) and metals as well as sublethal detoxification responses were examined in various marine invertebrates and fish (Kennicutt et al., 1994; McDonald et al., 1996). A few other large scale studies have been conducted in the late 1970s examining strictly the ecology of rig fouling communities in offshore Texas or throughout the Central Gulf offshore Louisiana (Fotheringham, 1981; Gallaway et al., 1981; Gallaway and Lewbel, 1982). More recent studies have examined coral communities of offshore oil rigs near Texas (Sammarco et al., 2004), the impacts of oil rigs on the nearby benthic and demersal community structures and food webs (Daigle,

2011), and pelagic fish associated with rigs in the Gulf of Mexico as reviewed in Frank (2000). However, other than the GOOMEX study there has been no monitoring to date of contaminant levels in offshore organisms on or near oil rigs within the Gulf of Mexico. Additionally, no studies to my knowledge have examined ecological changes to oil rig fouling communities following a spill event to determine impacts and subsequent recoveries.

Although their structures represent possible areas of oil exposure, oil and gas platforms provide a habitat for a thriving, abundant, and diverse community of organisms by introducing extensive amounts of hard substrate (>5000km²) into a region of the Gulf of Mexico normally dominated by soft-sediments (Grippo et al., 2010). Such fouling assemblages consist of reef-dependent taxa including algae, sponges, byozoans, corals, molluscs, and many others invertebrates (Gallaway and Lewbel, 1982; Dokken et al., 2000). These vertical artificial reefs also provide habitat and food for numerous fish species making the platforms important components for both recreational and commercial fishing industries (NRC, 1996; Gallaway et al., 2009). Therefore, fouling marine invertebrates living on oil platforms represent a unique resource that can be utilized in biomonitoring to understand the impacts of oil spills in the offshore pelagic and benthic zones of the Gulf of Mexico, particularly large scale events like the DWH incident. This is particularly important as large data gaps remain concerning the impacts of oil and/or chemical dispersants in the offshore regions of the Gulf of Mexico.

In addition to the use of marine bivalves, other marine invertebrates such as soft and hard coral species could also serve as environmental indicators of exposure to and impacts of petroleum exposure in the marine environment. Like bivalves, corals are quite

abundant on the oil rig fouling assemblages within the Northern Gulf of Mexico (Gallaway and Lewbel, 1982; Carney, 2005). Corals are particularly susceptible to contaminants dissolved in seawater due to the very thin layer of tissue covering the coral skeleton, large-surface to area volume ratios, and high lipid content which can facilitate the direct uptake and bioaccumulation of lipophilic chemicals like PAHs (Peters et al., 1981; Peters et al., 1997). Recent studies examining the impacts of petroleum products on corals (Mitchelmore and Baker, 2010; White and Strychar, 2011) show the sensitivity of corals to petroleum products and PAHs as well as the ability of corals to accumulate PAHs. Corals also appear to be much more sensitive than other marine invertebrates to chemical oil spill dispersants and chemically dispersed oil, although it is unclear if this is due to animal or symbiotic algal components in symbiotic coral species (Negir and Heyward, 2000; Shafir et al., 2007; Mitchelmore and Baker, 2010). Some researchers have suggested careful consideration before using dispersants near coral reef systems (Mitchelmore and Baker, 2010), while others recommend ruling out the use of dispersants, in general, in the vicinity of corals (Shafir et al., 2007).

Therefore, the aim of the study in Chapter 2 of this dissertation was to conduct a baseline screen of PAH accumulation in the most abundant bivalve (crested oyster- *Ostrea equestris*) and coral (orange cup coral-*Tubastrea coccinea*) extracted from support structures of various oil rigs within the Northern Gulf of Mexico oil field off the coast of Louisiana. The concentration and distribution of 52 individual (parent and alkylation homology) PAHs were examined in the oysters and corals collected from two sampling trips in April and May 2011 and various diagnostic ratios of PAH were utilized to examine source inputs of exposure to PAHs as described in Section 1.4.2. The ultimate

goal of this study was to determine if invertebrate organisms collected from oil rig structures could serve as offshore biomonitors for future oil spill events within the Northern Gulf of Mexico.

1.5.2. *Examining the impacts of oil and dispersed oil on larval and juvenile blue crabs*

Most toxicity studies conducted in the laboratory use model test organisms that can be easily cultured and examined with standardized toxicity methods that allow for comparison of results between different toxicants. Such standard species are often used as surrogates for other similar species within the environment during risk assessment analysis and hazard level assessment models. However, the range of approved test species, particularly for marine invertebrates, is limited and the sensitivities can often vary between species exposed to the same chemicals, highlighting the importance of multiple species comparisons. One of the most commonly used invertebrate species to examine the impacts of contaminant toxicity on marine crustaceans is the mysid shrimp, *Americamysis bahia*, which is native to the Gulf of Mexico (ASTM, 1999; US EPA, 2000). The mysid shrimp has been examined extensively for the impacts of oil, dispersed oil, and chemical dispersant exposures (NRC, 2005; Hemmer et al., 2010). While information gained from these species can be helpful in risk assessment decisions during an oil spill event, it does not guarantee that other important non-model invertebrate species native to the Gulf of Mexico, like blue crabs *Callinectes sapidus*, will respond similarly to oil exposure. Therefore, it is also important to examine the impacts of oil, dispersant and dispersed oil on native non-model species whenever possible. Blue crabs can be very difficult to culture in mass from larvae to juveniles within an aquaculture

setting. However, the blue crab hatchery in the Aquatic Research Center (ARC) of the Institute of Marine and Environmental Technology (IMET) has developed a successful culturing system for mass production of larvae and juvenile blue crabs (Zmora et al., 2005). This blue crab hatchery provided the means to examine the impacts of oil and dispersed oil exposures on the early life stages of hatchery raised blue crabs (larvae to juveniles) in this dissertation.

The blue crab ranges from Nova Scotia to northern Argentina, including the entire coast of the Gulf of Mexico (Williams, 1974). This range overlaps substantially with oil production regions in both North and South America, including the Northern Gulf of Mexico. Within the Northern Gulf of Mexico, the blue crab is a keystone species both ecologically and economically. Blue crabs contribute ecologically to the estuarine and coastal food webs as they serve as both prey and predators to numerous other species as summarized in Guillory et al. (2001). Blue crabs are economically important as they are one of the top ten key domestic commercial fisheries nationally as well as for many individual states, particularly Louisiana, in weight and monetary value (NMFS, 2013). As of 2012, hard blue crab U.S. landings were valued at \$186.1 million with 25 percent of the total landings coming from Louisiana (NMFS, 2013). In spite of this, however, there is very little information in the literature regarding the acute, let alone sublethal impacts of oil/PAH exposure and chemical dispersants on blue crabs, especially the early larval and juvenile life stages.

The distribution of blue crab in the environment differs with life stage as outlined in Figure 1.2. Adult crabs typically inhabit lower salinity estuaries and coastal rivers with brackish water (Tagatz, 1968a). After mating, adult female blue crabs within the Gulf of

Mexico migrate to higher salinity (euhaline) regions in the lower-estuaries or near-shore coastal locations where spawning occurs on an ebbing tide in the spring, summer, and fall (Perry and Stuck, 1981; Steele and Perry, 1990). Hatching of eggs occurs between 12 and 17 days following spawning (Millikin and Williams, 1984). Newly hatched larvae (stage I zoea) are found in substantial numbers in the mouths of estuaries where spawning occurs and are transported out into the open waters of the continental shelves (Perry and Stuck, 1982; McClintock et al., 1993). They continue to develop in the open ocean where they live a planktonic existence on the surface, particularly the upper 3m including the neuston (Epifanio, 1995; Johnson and Perry, 1999). During this period, the larvae go through seven (or occasionally eight) zoeal molt stages over 31-49 days (Costlow and Bookhout, 1959; Sulkin, 1978) before molting into the megalopa stage, which generally lasts 6-20 days (Costlow, 1967).

Megalopa re-enter the estuarine waters (ingress) upon tidal and salinity cues with wind-driven transport and transition from a planktonic to a benthic stage (Epifanio, 1995). Once settled in the benthos, the megalopa metamorphose into the first instar juvenile crab, which tend to migrate to shallower, lower-salinity waters where they continue to grow to maturity following 18 to 20 post-larval molts (Van Engel, 1958). Typically, juveniles are found in shallow nursery habitats like marsh and seagrass beds (Orth and van Montfrans, 1987; Thomas et al., 1990). The overall time to maturity from hatching ranges from 10 to 12 months in Mississippi (Perry, 1975) and Florida (Tagatz, 1968a).

The difference in distribution of blue crabs throughout their life stage alters their potential exposure routes to oil and PAHs with oil spill events as shown in Figure 1.3. As

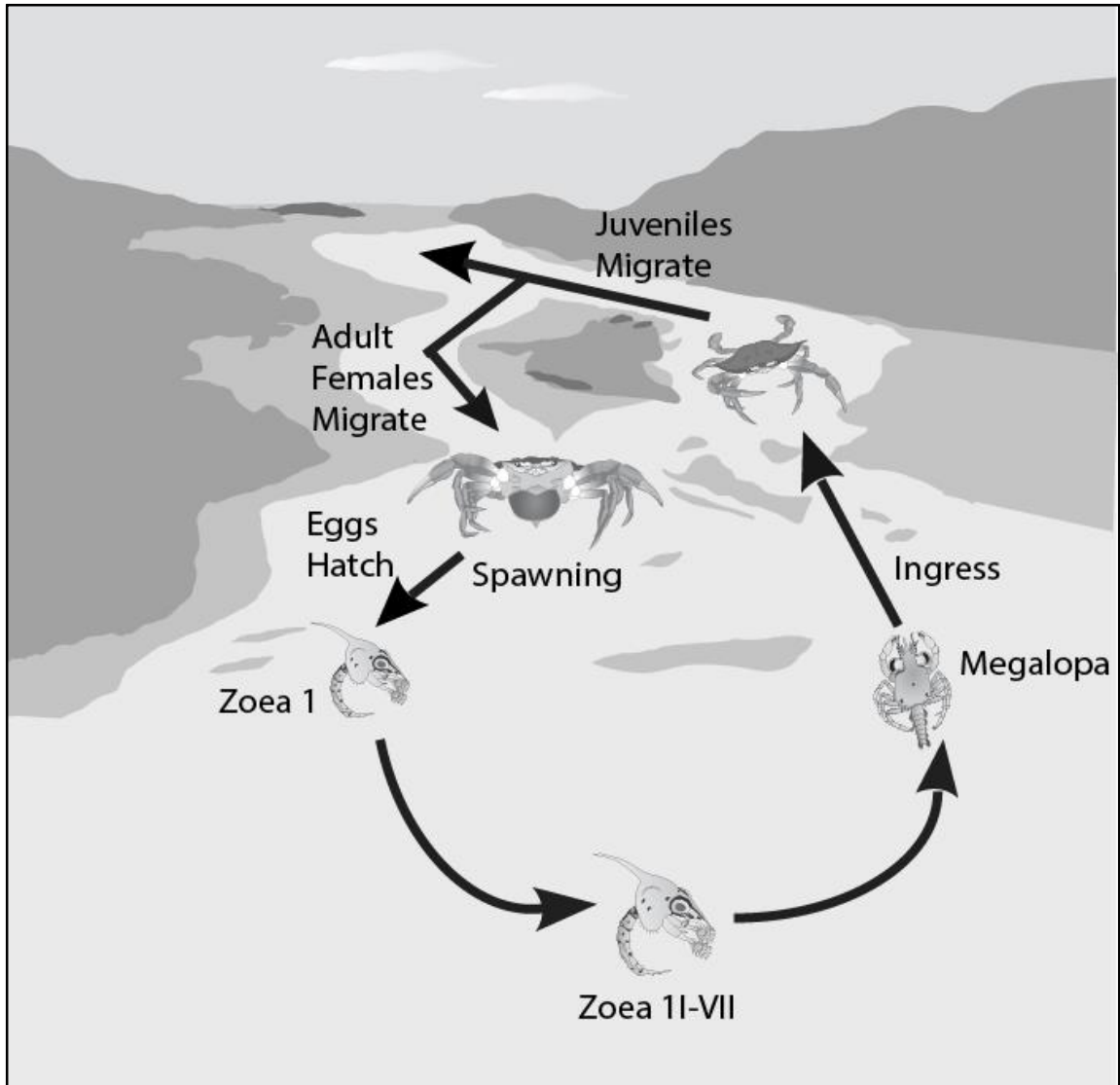


Fig. 1.2 Distribution of blue crabs within the environment varies with life stage.

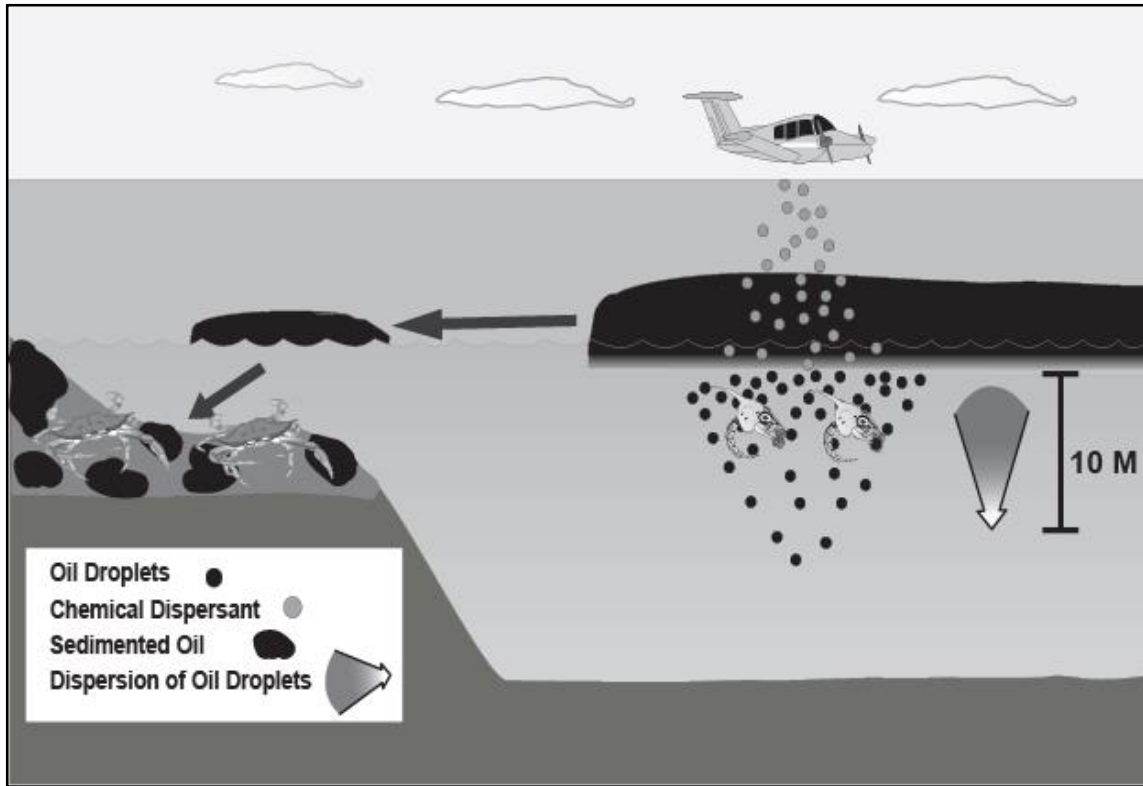


Fig. 1.3 Exposure of blue crabs to oil differs between larval and juvenile stages.

a benthic species, juvenile crabs live and feed within coastal wetlands and estuarine sediments; and therefore, would be more likely exposed to sedimented oil that reached the coastal wetlands and estuaries. During the DWH incident in particular, multiple response options were utilized to minimize the amount of oil reaching the shoreline ecosystem. However, in spite of these efforts, oil reached the shoreline and contaminated many marshlands areas in the northern Gulf of Mexico, particularly the Louisiana coast (NOAA, 2010; National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011). These contaminated coastal areas can then chronically release oil for significant periods of time post-spill event as is evident by persistence of oil from the Exxon Valdez oil spill in subsurface sediments of exposed shores 16 years post spill (Short et al., 2007). More than 650 miles of the Gulf of Mexico's coastal habitats including salt marshes, mangroves, mudflats, and sand beaches were oiled following the DWH incident with more than 130 miles having been designated moderately to heavily oiled (National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011). All these oiled areas are potential habitat for juvenile blue crabs highlighting the importance for understanding the impacts of oil exposure to this species.

Blue crab zoea, however, live in the offshore surface waters of the continental shelf, which is well within the 3-miles offshore pre-approved guidelines for dispersant use. Therefore, blue crab zoea are likely to be exposed to oil and chemically dispersed oil in an offshore oil spill event, especially those events like the DWH incident that coincided with blue crab spawning. Additionally, the larvae live within the top 3m of the water column, which means they will be exposed to the highest concentrations of hydrocarbons

with chemical dispersion before the oil can dilute with dispersion to potentially less harmful concentrations below thresholds of concern for most organisms.

Therefore, given the importance of blue crabs to the Gulf of Mexico environment and economy, the likelihood of exposure to oil during a large oil spill event, and the substantial lack of toxicity data regarding blue crab exposure to oil, the studies in Chapters 3, 4, and 5 of this dissertation serve to address this critical data gap. Chapter 3 examines the impacts of oil contaminated sediments following the DWH incident on juvenile blue crabs, while Chapter 4 examines the acute and sublethal impacts of oil, dispersed oil, and chemical dispersant on blue crab zoea. The sublethal impacts of oil exposure on larvae and juveniles (Chapter 3 and 4) were examined through the use of various biomarkers highlighted in Section 1.4.1 such as the gene expression levels of enzymes involved in detoxification and oxidative stress as well as DNA damage through the Comet assay. As the study in Chapter 3 extended for 31-days, additional sublethal endpoints such as impacts to growth and molting were examined in juvenile blue crabs. Comprehensive chemical analysis was conducted and PAH source diagnostic ratios were examined for both studies as described in Sections 1.4.2. The study in Chapter 5 examines the acute toxicity of the presently used dispersant, Corexit 9500 and four alternative dispersants on blue crab zoea to address the need for substantially more toxicity data on dispersant alternatives that may be less toxic and/or more effective than Corexit 9500; and therefore better options for use in oil spill remediation within the Gulf of Mexico.

Chapter 2. PAH Accumulation in Oil Platform Marine Fouling Invertebrates within the Northern Gulf of Mexico and Their Potential Use as Biomonitors¹

2.1. Abstract

Accumulation of polycyclic aromatic hydrocarbons (PAHs) has been routinely monitored in bivalves throughout the coastal Gulf of Mexico through the NOAA Mussel Watch Program. Oil rig fouling reef communities offer a unique and promising opportunity to expand biomonitoring efforts into the offshore areas of the Northern Gulf of Mexico. Therefore, the aim of this study was to conduct a baseline screen of PAH accumulation in crested oysters, *Ostrea equestris*, and orange cup corals, *Tubastrea coccinea*, collected from various oil rigs within the oil field off the coast of Louisiana. The concentration and distribution of 52 individual parent and alkylated homolog PAHs were examined in oysters and corals collected from two sampling trips in April and May 2011 and various diagnostic ratios of PAH were utilized to estimate source inputs of exposure to PAHs. Overall, the coral and oysters collected from both sampling trips had lower PAH accumulation than most oysters and mussels collected near the shoreline and in wetlands of Louisiana and elsewhere in the Gulf of Mexico. For the April sampling trip, corals had TPAH concentrations ranging from 8.73 to 15.17 ng g⁻¹ and oysters from 2.52 to 22.04 ng g⁻¹. For the May sampling trip, corals had elevated concentrations of TPAH ranging from 24.28 to 79.23 ng g⁻¹ and oysters from 7.18 to 95.55 ng g⁻¹. This increase in TPAH between sampling trips could be due to Mississippi River flooding that occurred in mid-May prior to the second sampling trip. Oysters and corals collected in

¹ Hannah V. Pié, Andrew Heyes, Carys Mitchelmore. In preparation for submission.

May from oil rig MC21B, closest rig to the Mississippi River Delta, had the highest TPAH concentrations observed among all locations and the only rig to have predominantly petrogenic source inputs. All other rig locations appeared to have either predominantly pyrogenic or a mixture of sources. Distribution of accumulated PAHs was similar between species in April, but differed in May samples with oysters accumulating large proportions of 5 ring PAHs, particularly benzo[e]pyrene and perylene, while corals from the same locations primarily accumulated 2 ring PAHs. Overall, given the low baseline of PAHs and the rapid accumulation in response to a possible contamination event, invertebrates residing on oil rig structures could make excellent biomonitoring tools to examine the exposure to and recovery from oil and petroleum PAHs in the offshore Northern Gulf of Mexico. In order to conduct a successful biomonitoring program for offshore oil exposure, a more thorough sampling study both spatially and in sample size that incorporates biological biomarkers of PAH exposure and effects, an ecological survey, and PAH accumulation would need to be conducted to have a more comprehensive understanding of baseline levels.

2.2. Introduction

The Northern Gulf of Mexico continental shelf is among the most ecologically productive regions in North America. It is also an area rich in oil deposits resulting in continuous natural seepage of oil components into the ecosystem. The natural release of oil is enhanced by the presence of approximately 4,000 petroleum platforms in the region. The net result is in an ecosystem exposed to potentially high levels of petroleum polycyclic aromatic hydrocarbons (PAHs). In a region dominated by soft-sediments, oil

and gas platforms provide an extensive amounts of hard substrate (>5000km²) that supports a thriving habitat for an abundant and diverse community of organisms (Grippio et al., 2010). Such fouling assemblages consist of reef-dependent taxa including algae, sponges, byozoans, corals, molluscs, and many others (Gallaway and Lewbel, 1982; Dokken et al., 2000). These vertical artificial reefs also provide habitat and food for numerous fish species making the platforms important components of both recreational and commercial fishing industries (NRC, 1996). Additionally, the quantity and spatial distribution of oil rigs in the Northern Gulf of Mexico provides an ideal lattice for a thorough sampling regime needed for a large scale biomonitoring program that could address natural and anthropogenic distribution of oil derived compounds over a substantial portion of the offshore region of the Northern Gulf of Mexico. Therefore, fouling marine invertebrates living on oil platforms represent a unique resource that can be utilized in understanding the impacts of oil spills in the Gulf of Mexico, particularly large scale ones like the Deepwater Horizon (DWH) Incident.

Sessile marine invertebrates, primarily mussels, have been used for long-term biomonitoring of the impacts the toxicological effects of the activity of offshore oil and gas platforms in European waters (Hylland et al., 2008; Gomiero et al., 2011). While these studies generally use caged organisms to examine exposure, one study by Gomiero et al. (2011) collected native mussels directly from rig structures in the Adriatic Sea to examine PAH bioaccumulation and biomarker analyses. However, to our knowledge, only one oil monitoring study, the Gulf of Mexico Offshore Operations Monitoring Experiment (GOOMEX), has been conducted near oil rig locations in the Gulf of Mexico, which is detailed in Kennicutt et al. (1996). GOOMEX was an extensive

monitoring study around five oil rigs off the coast of Texas to examine the nearfield impacts of oil and gas activities on organisms over a range of distances (30-3000m) from the rigs (Kennicutt et al., 1996). Tissue contamination of hydrocarbons (PAHs included) and metals as well as sublethal detoxification responses were examined in various marine invertebrates and fish (Kennicutt et al., 1994; McDonald et al., 1996). Other than this study there has been no consistent monitoring to date of contaminant levels or biomarkers of health and detoxification responses in offshore organisms on or near oil rigs within the Gulf of Mexico and none near the Louisiana coastline.

Sessile marine bivalves have routinely been utilized to examine oil exposure and PAH accumulation (e.g. NOAA Mussel Watch program) as they quickly and readily accumulate oil components and are slow to depurate them, possibly reflecting reduced contaminant metabolism and/or detoxification pathways (Seriano et al., 1996). However, few studies have examined the extent of PAH bioaccumulation in fouling organisms living in the coastal oil fields of the Northern Gulf of Mexico. Marine bivalves (e.g. oysters, mussels) are capable of filtering substantial quantities of water over large and highly permeable gills. This filtration in combination with their low metabolic capacity and slow depuration rates can result in high bioaccumulation of hydrophobic compounds like PAHs in their tissues.

Other marine invertebrates such as corals could be used alongside bivalves as environmental bioindicators of the impacts of petroleum exposure in the marine environment. Corals are particularly susceptible to contaminants dissolved in seawater due to the very thin layer of tissue covering the coral skeleton, large-surface to area volume ratios, and high lipid content which can facilitate the direct uptake and

bioaccumulation of lipophilic chemicals like PAHs (Peters et al., 1981; Peters et al., 1997). Recent studies examining the impacts of oil on corals (Mitchelmore and Baker, 2010; White and Strychar, 2011) show the sensitivity of corals to petroleum products and PAHs as well as the ability of corals to accumulate PAHs. On the oil-rig structures examined in this study, the most abundant fouling bivalve observed was the crested oyster, *Ostrea equestris*, and the most abundant translucent invertebrate was the orange cup coral, *Tubastrea coccinea*. Other ecological surveys of oil rig fouling communities in the Gulf of Mexico have also observed high abundance and prevalence of *O. equestris* (Fotheringham, 1981; Gallaway et al., 1982) and *T. coccinea* (Sammarco et al., 2004). *O. equestris* is native to the Gulf of Mexico (Abbott, 1974) and is predominately found in high salinity environments from estuarine conditions of 20 parts per thousand (ppt) to oceanic conditions (Galtsoff and Merrill, 1962). *O. equestris* will replace the more common Eastern oyster, *Crassostrea virginica*, on beds when salinity reaches 30 ppt (Parker, 1960). While quite a few studies have examined the impacts of oil and PAH exposure (Fucik et al., 1995; Norena-Barroso et al., 1999; Liu et al., 2006) and bioaccumulation of PAHs (Wade et al., 1988; Serciano et al., 1996; Qian et al., 2001) in *C. virginica*, we are not aware of any studies conducted on *O. equestris*. *T. coccinea*, a non-indigenous species to the Gulf of Mexico, primarily appears on artificial substrates such as submerged steel wrecks and oil rig support structures (Fenner and Banks, 2001; Sammarco et al., 2004; Ferry, 2009). These artificial structures make the impacts of *T. coccinea* invasion a concern for the ecological integrity of native reef communities with the Florida Keys and Gulf of Mexico (ONMS, 2011). Given their abundance on offshore oil rigs surveyed, *O. equestris* and *T. coccinea* could be useful species in examining the

variability in PAH accumulation within fouling communities living in highly exposed environments and for potential future monitoring of the impacts of offshore oil exploitation activities.

Therefore, the aim of this study was to conduct a baseline screen of PAH accumulation in *O. equestris* and *T. coccinea* collected from various oil-rigs within the Northern Gulf of Mexico oil field off the coast of Louisiana. The concentration and distribution of 52 individual (parent and alkylated homologs) PAHs were examined in the oysters and corals collected from two sampling trips in April and May 2011. Additionally, various diagnostic ratios of PAH were utilized to examine source inputs of exposure to PAHs.

2.3. Methods

2.3.1. Collection of organisms

Crested oyster, *Ostrea equestris*, and orange cup coral, *Tubastrea coccinea*, were collected from oil rig structures throughout the Northern Gulf of Mexico oil field during two sampling trips: a preliminary trip in April and a definitive trip in May. *O. equestris* and *T. coccinea* were chosen for analysis based on observed abundance on offshore oil-rig structures. Organisms used for analyses were collected by a diver at a depth of 10m. Oil-rig locations are shown in Figure 2.1 and details described are included in Table 2.1.

Oysters were shucked and whole body tissues of 4-5 oysters were pooled per sample in baked scintillation vials ($N=2-6$ and 4-5 sample vials per rig for April and May, respectively). Oyster sizes ranged from 800 to 5,250 mm² (length x width of shell) with a mean and standard error of 2082±58 mm². The ratio of number of males to females for

all oysters was 1.06. Coral tissues samples were scrapped from the substrate and pooled together to fill scintillation vials (N=2-3 and 3-10 sample vials per rig for April and May, respectively). Samples were stored on dry ice until permanent storage at -80°C.

2.3.2. *Chemical analysis of tissue samples*

The analysis of PAHs in organisms was carried out largely following the methods outlined in Ko and Baker (1995) and Rowe et al. (2009). Briefly, pooled whole oysters or coral samples were homogenized, mixed with anhydrous sodium sulfate, and then ground with mortar and pestle to obtain a dry powder. The wet weight of samples used for extraction was recorded for normalization. The powdered samples were extracted with dichloromethane (DCM) using accelerated solvent extraction, (ASE-300, DIONEX), through deactivated alumina to remove the polar/lipid interferences. Additional anhydrous sodium sulfate was added to the extracts to further remove polar interferences. The DCM extracts were then transferred to round bottom flasks and reduced to 2 mL in hexane by rotary evaporation (Rotovapor R-114, Büchi). The extract volumes were transferred to amber glass vials and further reduced to ~1 ml using a purified nitrogen stream. The vials were sealed and stored at -20°C for GC/MS analysis.

Samples were analyzed using an Agilent 6890 gas chromatograph (GC) with a 5975 mass spectrometer (MS) in selected ion monitoring mode using a J&W Scientific 128-5522 DB-5MS fused silica column. Samples were injected in splitless mode at an initial oven temperature of 45°C and an injector temperature of 250°C with helium as the carrier gas. The oven temperature was ramped at 10°C/min to 280°C and then 5.0°C/min to 310°C before holding at 310°C for 16.5 min. Surrogate PAH standards of perdeuterated

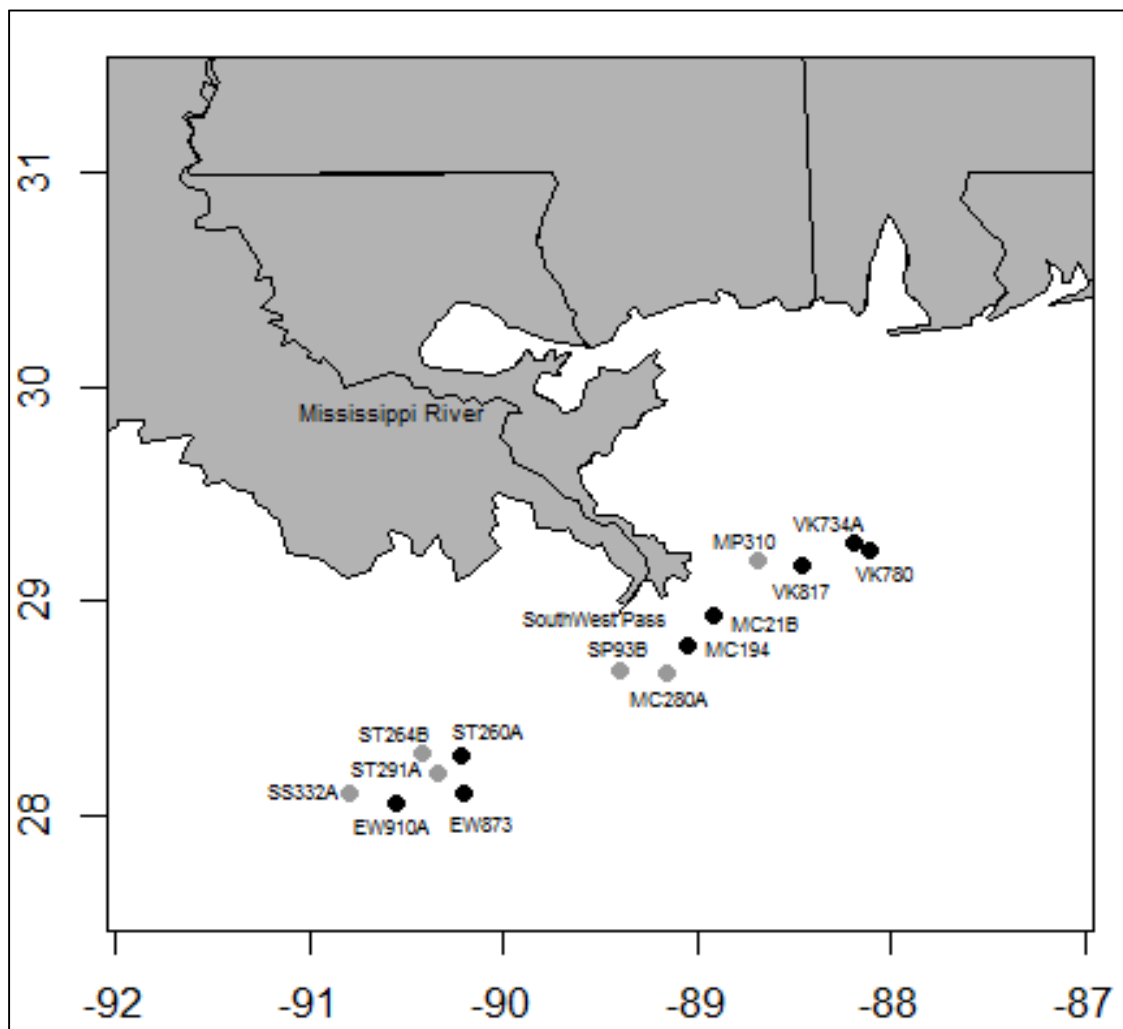


Fig. 2.1 Oil rig sampling locations off the Louisiana coast for April (gray) and May (black).

Table 2.1 Details for oil rigs included in April and May sampling trips.

Rig ID (Area/Block)	Date Sampled	Rig Age	Rig Depth (ft)	Distance to Land (miles)	Active (Y/N)	System Type
SP93B	03/29/2011	01/01/1985	450	20	Y	Fixed
MC280A	03/29/2011	01/01/1984	1,000	23	Y	Compliant Tower
SS332A	04/1/2011	08/31/1985	438	78	Y	Fixed
ST291A	04/1/2011	11/25/1998	394	90	Y	Fixed
ST264B	04/1/2011	2/26/2001	215	51	Y	Fixed
MP310	04/2/2011	11/4/1992	248	41	Y	Fixed
EW873	05/27/2011	07/02/1994	775	69	Y	Fixed
EW910A	05/27/2011	10/16/1998	549	72	Y	Fixed
ST260A	05/28/2011	09/10/1996	303	45	Y	Fixed
VK817	05/28/2011	07/27/1995	671	34	Y	Fixed
VK780	05/28/2011	07/03/1998	722	57	Y	Fixed
VK734A	05/28/2011	08/02/1999	322	32	N	Fixed
MC210	05/29/2011	01/23/2005	667	13	Y	Fixed
MC194	05/30/2011	01/01/1978	1023	15	Y	Fixed

PAHs (d₈-naphthalene, d₁₀-flourene, d₁₀-flouranthene, and d₁₂-perylene) were added prior to analysis on GC/MS to quantify method performance, while internal standards (d₁₀ acenaphthalene, d₁₀-phenanthrene, d₁₂-benzo[a] anthracene, d₁₂-benzo[a]pyrene, d₁₂-benzo[g,h,i]perylene) were added to samples after grinding prior to extraction for quantification. Calibration standards (Supelco Separation Technologies, Bellefonte, PA) for all PAHs were used.

PAHs were identified by retention time relative to that of mixed standards. Identification was further confirmed by the abundance of a secondary mass fragment relative to the molecular ion when possible. In the biological samples, four pairs of individual PAHs (1,4-dimethylnaphthalene and 2,3-dimethylnaphthalene, 1-methylphenanthrene and 1-methylantracene, chrysene and triphenylene, and 4-methylchrysene and 6-methylchrysene) resulted in chromatogram peaks that overlapped each other and individual PAH results could not be resolved. Therefore, the integrated results for each group of two PAHs were summed in the results and the names for each group are detailed in Table 2.2. Integrated responses of each PAH were first normalized to responses of blank extractions (containing perdeuterated-PAH standards only). The blank-normalized integrated area response for each PAH was then adjusted relative to that of the appropriate internal standard (refinement of EPA Method 8270D) and normalized again to wet weight of tissue analyzed. Results are reported as ng PAH per g wet weight tissue. Surrogate recovery averages and standard deviations of all samples for each perdeuterated PAH were 71.7±18.1 (d₈-naphthalene), 98.9±17.0 (d₁₀-flourene), 102.9±16.4 (d₁₀-flouranthene), and 84.9±20.3 (d₁₂-perylene). Instrument detection limits were calculated as sum of the average integrated response and three times the standard

Table 2.2 Specific PAH congeners analyzed including ring size and detection limits (abbreviations in parentheses).

Parent PAH	Ring Size	Detection Limit (ng g ⁻¹)	Alkylated PAH	Ring Size	Detection Limit (ng g ⁻¹)
Naphthalene (NA)	2	0.20-1.82	2-Methylnaphthalene (2-M NA)	2	<0.008-2.03
Acenaphthene (AC)	3	<0.008-0.032	1-Methylnaphthalene (1-M NA)	2	<0.008-0.58
Acenaphthylene (ACL)	3	<0.008-0.16	2,6-dimethylnaphthalene (2,6-M NA)	2	<0.008-0.59
Fluorene (FL)	3	0.13-0.64	1,3-Dimethylnaphthalene (1,3-M NA)	2	<0.008-0.52
Dibenzothiophene (DBT)	3	<0.008-0.23	1,6-Dimethylnaphthalene (1,6-M NA)	2	<0.008-0.38
Phenanthrene (PHE)	3	0.73-1.44	1,4+ 2,3-Dimethylnaphthalene (1,4-/2,3-M NA)	2	<0.008-0.10
Anthracene (AN)	3	<0.008-0.19	1,5-Dimethylnaphthalene (1,5-M NA)	2	<0.008-0.12
Fluoranthene (FA)	4	0.28-0.40	1,2-Dimethylnaphthalene (1,2-M NA)	2	<0.008-0.098
Pyrene (PY)	4	0.15-0.74	1,8-Dimethylnaphthalene (1,8-M NA)	2	<0.008
Benzo[a]fluorene (BaFL)	4	<0.008	2,3,5-trimethylnaphthalene (2,3,5-M NA)	2	<0.008-0.64
Benzo[b]fluorene (BbFL)	4	<0.008	1-Methylfluorene (1-M FL)	3	<0.008-0.011
Benzo[a]anthracene (BaAN)	4	<0.008	2-Methyldibenzothiophene (2-M DBT)	3	<0.008-0.16
Chrysene-triphenylene (CHR-TRI)	4	<0.008-0.27	4-Methyldibenzothiophene (4-M DBT)	3	<0.008
Naphthacene (NAC)	4	<0.008	2-Methylphenanthrene (2-M PHE)	3	<0.008-0.39
Benzo(b)fluoranthene (BbFLA)	5	<0.008-0.20	2-Methylanthracene (2-M AN)	3	<0.008
Benzo(k)fluoranthene (BkFLA)	5	<0.008-0.87	4,5-Methylenepheneanthrene (4,5-M PHE)	3	<0.008-0.65
Benzo(e)pyrene (BeP)	5	<0.008-2.81	1-Methylanthracene + 1-Methylphenanthrene (1-M AN/PHE)	3	0.037-0.094
Benzo(a)pyrene (BaP)	5	<0.008	9-Methylanthracene (9-M AN)	3	<0.008
Perylene (PE)	5	<0.008	3,6 dimethylphenanthrene (3,6-M PHE)	3	<0.008-0.97
Indeno(1,2,3-cd)pyrene (IP)	6	<0.008	2,3 dimethylanthracene (2,3-M AN)	3	<0.008-2.92
Dibenz(a,c+g,h)anthracene (DBAN)	6	<0.008	9,10 dimethylanthracene (9,10-M AN)	3	<0.008-1.26
Benzo(g,h,i)perylene (BghiP)	6	<0.008	1-methylpyrene (1-M PY)	4	<0.008-0.70
Anthanthrene (ANT)	6	<0.008	2 methylfluoranthene (2-M FLA)	4	<0.008
Coronene (COR)	6	<0.008	4- + 6- methylchrysene (4-/6-M CHR)	4	<0.008-0.017
			3-Methylcholanthrene (3-M CA)	5	<0.008

deviation of the mean for the integrated responses for an individual PAH or pairing for blank samples (Table 2.2). Any PAH values below the detection limits or not detected with analysis were recorded as below the detection limit (BDL) in Tables 2.4 and 2. 5.

2.3.3. PAH source diagnostic ratio

Various diagnostic ratios of PAHs have been utilized to examine the source inputs of PAHs (e.g. petrogenic, pyrogenic, and diagenic) in environmental and tissues samples. The ratios used to estimate between petrogenic and pyrogenic PAH source inputs in this study were alkylated to parent PAHs, high molecular weight (HMW) to low molecular weight (LMW) PAHs, phenanthrene (PHE) to anthracene (AN), fluoranthene (FLA) to pyrene (PY), and sum of alkylated phenanthrenes (MPHE) to parent phenanthrene (PHE) as described in Table 2.3. Alkylated to Parent PAH is the ratio of the sum of all parent PAHs to the sum of all alkylated PAHs. HMW to LMW ratio is the sum 4 and 5 ring parent PAHs to the sum of 2 and 3 ring parent PAHs.

Diagenic sources of PAHs were estimated by measuring the concentration of perylene. While perylene, can come from petrogenic and pyrogenic sources, only trace or small amounts of perylene are found in fossil fuels and during most fossil fuel combustions, with a few exceptions like coke production and kerosene combustion (Venkatesan, 1988). High concentrations of perylene in relation to other PAHs indicate a diagenic source input (Venkatesan, 1988).

2.3.4. Statistical analysis

All statistical analyses were conducted using R statistical software 2.15.2. Total PAH

Table 2.3 PAH diagnostic ratios used to distinguish between petrogenic and pyrogenic source inputs

Ratio	Petrogenic	Pyrogenic	Reference
Alkylated Homologs/ Parent	> 1	< 1	Saha et al. 2009
HMW/ LMW	< 1	> 1	Qian et al. 2001
PHE/AN	> 10	< 10	Saha et al. 2009
FLA/PY	> 1	<< 1	Neff et al. 2005
MPHE/PHE	> 2	< 2	Neff et al. 2005

*HMW (high molecular weight), LMW (low molecular weight), PHE (phenanthrene), AN (anthracene), FLA (fluoranthene), PY (pyrene), and MPHE (sum of alkylated phenanthrenes)

(TPAH) is a sum of all individual PAHs examined, except perylene. The mean and standard error of the mean values of TPAH were compared for each species from the two different sampling trips, separately. TPAH values were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test). If one or both tests failed, a log transformation was conducted and the tests redone. If both tests passed with (or without) the transformation, an Analysis of Variance (ANOVA) was conducted. All TPAH values passed both tests with (or without) a log transformation. If a significant difference was found in the ANOVA test (p value <0.05), then a multiple pairwise comparisons was conducted using Tukey's Honest Significant Difference (HSD) test. Any comparison with a p value < 0.05 was considered a significant difference between treatments.

All the TPAH values for both species were binned and scaled together to allow for direct comparison between species, rig locations, and sampling trips. The scaled values were used to create bubble plots of TPAH for each separate species for both sampling trips (Figs. 2.2 and 2.4). The size of each bubble is proportional to the TPAH concentration at that location. The percentage of each different PAH ring size from the total PAH was calculated and plotted to examine accumulation based on PAH size.

2.4. Results

2.4.1. Accumulation of PAHs in oysters

TPAH (PAHs) concentrations in oysters were 2.52-22.04 ng g⁻¹ from the April sampling trip and 7.18-95.55 ng g⁻¹ from the May sampling trip. Distribution of TPAH by rig location for oysters from both sampling trips is shown in Figure 2.2. Average

individual PAH concentrations and total PAH concentrations are shown in Table 2.4. No oysters contained 6 ring PAHs (IP, DBAN, BghiP, ANT, or COR) or 5-ring 3-methylcholanthrene above the detection limits. There were no significant differences in TPAH between rigs for oysters collected in April. However, oysters collected in May from MC21B had significantly higher TPAH than oysters from all other rig locations from that sampling trip ($p < 0.05$). Additionally, May oysters from VK817 had significantly high TPAH than oysters from VK734A, ST260A, EW873, and EW910A ($p < 0.05$). Overall, oysters collected in May had higher TPAH concentrations than oysters from April with the exception of oysters from SP93B.

Oysters from the May collection contained high concentrations of perylene (3.92-41.49 ng g⁻¹). If perylene was included in the TPAH, the total PAHs would range from 11.10-137.04 ng g⁻¹ and perylene would account for 21.3-66.2% of TPAH. Oysters from April, however, did not have perylene concentrations above the detection limit. Besides perylene, the most predominant PAH measured in oysters from May was benzo[e]pyrene (BeP), which accounted for 4.9-55.2% of TPAH, not including perylene. Alkylated naphthalenes also accounted for a large percentage of TPAH in oysters from May with C₁-naphthalenes and C₂-naphthalenes ranging from 3.3-44.4% and 3.3-34.4% of TPAH, respectively. However, C₁-naphthalenes were below the detection limit for oysters from EW910A. For oysters collected in April, naphthalene, C₁-naphthalenes, and C₂-naphthalenes were the predominant PAHs and accounted for 4.6-32.2%, 8.2-28.4%, and 12.1-34.1% of TPAH, respectively. Other PAHs that accounted for a large portion of TPAH were phenanthrene and C₁-phenanthrene which ranged from BDL-21.4% and 2.8-20.7% of TPAH, respectively.

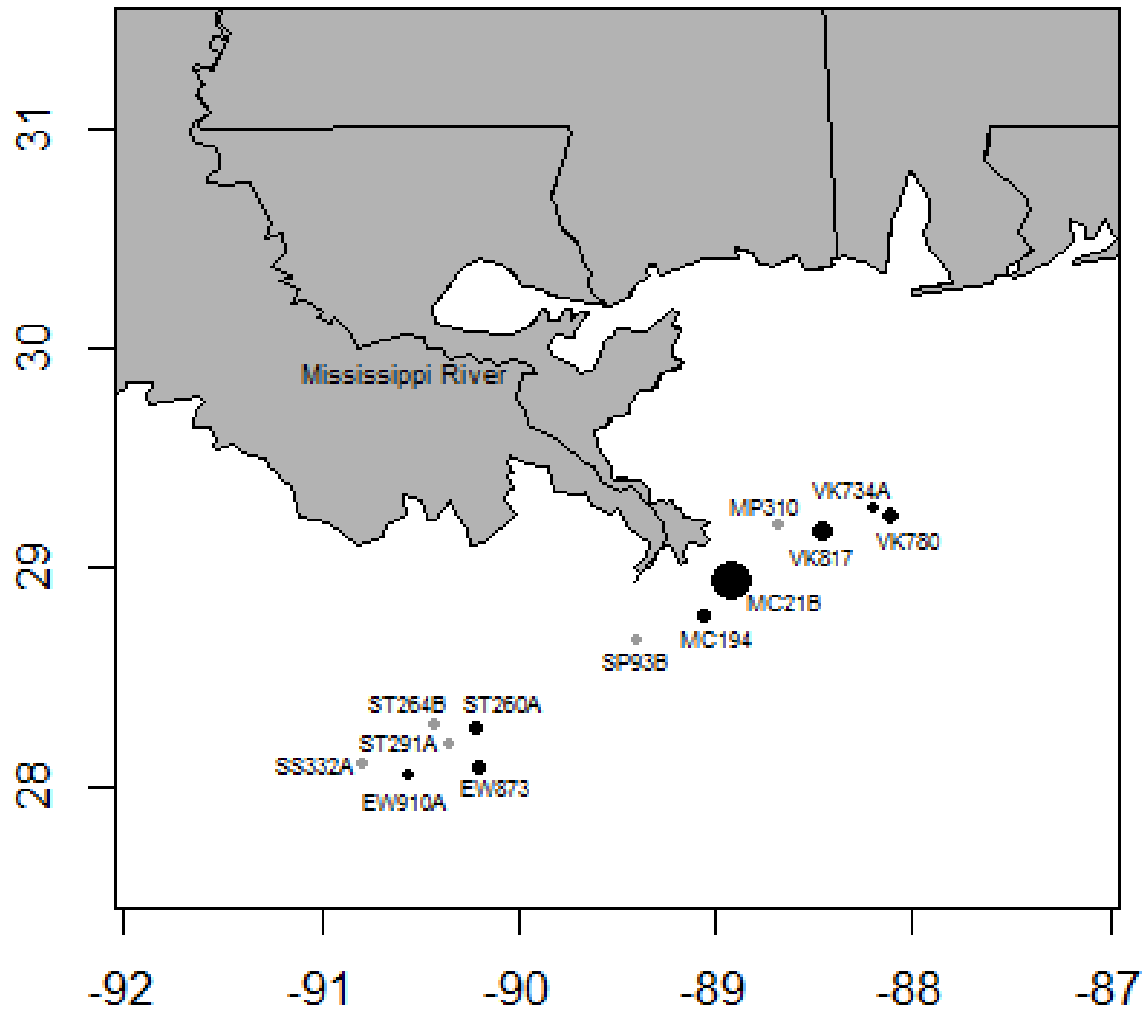


Fig. 2.2 Total PAH accumulation by rig location for oysters collected in April (light gray) and May (black). Size of circles is proportional to TPAH concentration. Exact concentrations detailed in Table 2.4.

Table 2.4 Individual and total PAH concentrations (ng g^{-1} wet weight) in oysters collected in April and May 2011. Perylene (PE) concentrations included separately.

PAHs	Units	April Sampling					May Sampling								
		SS 332A	ST 291A	ST 264B	SP 93B	MP 310	EW 910A	EW 873	ST 260A	MC 194	MC 21B	VK 817	VK 734A	VK 780	
NA	ng g^{-1}	0.67	0.69	0.66	0.84	0.58	BDL	BDL	0.90	1.12	1.41	1.19	1.48	1.46	
2-M NA	ng g^{-1}	0.42	0.55	0.54	0.71	0.43	BDL	3.72	1.14	1.40	3.74	0.81	0.68	1.34	
1-M NA	ng g^{-1}	0.54	0.28	0.30	0.49	0.22	BDL	0.81	0.37	0.57	2.08	0.33	1.04	0.74	
2,6-M NA	ng g^{-1}	0.22	0.28	0.34	0.62	0.73	1.85	1.58	0.75	1.99	7.44	0.65	0.82	1.16	
1,3- M NA	ng g^{-1}	0.24	0.27	0.24	0.38	0.24	0.80	0.88	0.39	0.82	8.20	0.29	0.30	0.95	
1,6- M NA	ng g^{-1}	0.11	0.13	0.12	0.18	0.37	BDL	BDL	0.16	0.29	2.52	0.19	0.17	0.61	
1,4/2,3 M NA	ng g^{-1}	0.03	0.04	0.03	0.05	0.04	0.20	0.16	0.05	0.42	1.29	0.12	0.10	0.18	
1,5- M NA	ng g^{-1}	0.06	0.05	0.10	0.05	0.06	BDL	BDL	0.04	0.29	1.80	0.04	0.08	0.14	
1,2- M NA	ng g^{-1}	0.09	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.98	BDL	BDL	BDL	BDL	
1,8- M NA	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
2,3,5-M NA	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	1.28	BDL	BDL	BDL	3.79	BDL	BDL	BDL	
AC	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.28	BDL	BDL	BDL	
ACL	ng g^{-1}	0.20	0.27	0.28	0.22	0.20	0.40	0.34	0.38	0.38	0.88	0.26	0.23	0.24	
FL	ng g^{-1}	0.39	0.69	0.50	0.56	0.54	BDL	BDL	1.96	1.47	2.26	0.98	0.92	1.12	
1-M FL	ng g^{-1}	BDL	0.33	BDL	0.24	BDL	0.02	0.02	0.02	0.03	0.17	0.02	0.01	0.03	
DBT	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
2-M DBT	ng g^{-1}	BDL	BDL	BDL	0.38	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
4-M DBT	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
PHE	ng g^{-1}	0.57	1.12	0.92	1.11	1.04	BDL	BDL	1.22	1.41	5.58	1.68	1.04	1.71	
AN	ng g^{-1}	0.04	0.07	0.08	0.06	0.04	0.35	0.39	0.21	0.25	0.23	0.14	0.13	0.18	
1-M AN/PHE	ng g^{-1}	0.10	0.34	0.30	0.78	0.36	0.26	0.19	0.17	0.21	2.44	0.20	0.14	0.21	
2-M PHE	ng g^{-1}	0.20	0.31	0.20	0.49	0.23	0.73	0.60	0.39	0.53	8.41	0.56	0.32	0.67	
2-M AN	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
9-M AN	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.39	BDL	BDL	BDL	BDL	
4,5-M PHE	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
3,6-M PHE	ng g^{-1}	BDL	0.11	BDL	0.20	BDL	0.21	0.14	0.11	0.18	3.06	0.26	0.11	0.24	
2,3-M AN	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
9,10-M AN	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
FLA	ng g^{-1}	BDL	0.71	BDL	0.45	0.99	0.41	0.36	0.71	2.77	1.97	2.62	0.30	0.46	
PYR	ng g^{-1}	0.15	0.29	0.20	0.23	0.36	0.50	0.43	0.23	0.49	2.82	0.84	BDL	0.39	
1-M PYR	ng g^{-1}	BDL	0.07	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
2-M FLA	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
BaFL	ng g^{-1}	BDL	0.41	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
BbFL	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
BaAN	ng g^{-1}	BDL	BDL	BDL	0.28	0.46	BDL	BDL	BDL	BDL	2.39	BDL	BDL	BDL	
CHR-TRI	ng g^{-1}	BDL	BDL	BDL	1.21	0.85	0.10	BDL	BDL	0.20	4.60	0.41	0.09	0.13	
NAC	ng g^{-1}	BDL	BDL	BDL	1.17	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
4/6 M CHR	ng g^{-1}	BDL	BDL	BDL	0.68	BDL	BDL	BDL	BDL	BDL	2.53	BDL	BDL	BDL	
BbFLA	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	3.41	BDL	BDL	BDL	
BkFLA	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
BeP	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	4.52	2.58	2.52	1.96	8.76	14.13	3.17	10.50	
BaP	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	1.19	BDL	BDL	BDL	
ΣPAHs	ng g^{-1}	4.01	7.00	4.79	11.38	7.73	11.63	12.21	11.73	17.16	84.23	25.72	11.11	22.47	
PE	ng g^{-1}	BDL	BDL	BDL	BDL	BDL	13.73	9.30	8.56	14.13	30.68	32.96	6.70	28.02	

The percentage of TPAH for each different ring sizes was examined for oysters collected from both sampling trips (Fig. 2.3). This figure includes 2-5 rings PAHs only as there were no 6-ring PAHs detected in oysters. Perylene was not included in the 5-ring PAH calculation as it would have accounted for such a substantial proportion of the TPAH for oysters collected in May. Distribution of PAH size by ring number for oysters in April and in May varied between rig locations and between sampling trips. Oysters from both sampling trips accumulated various proportions of 2-4 ring PAHs, but only oysters from May's sampling trip contained 5 ring PAHs above the detection limits. Oysters from April predominately accumulated 2 and 3 ring PAHs with lower proportions of 4 ring PAHs, except for oysters from MP310 which had equivalent distributions between 2, 3, and 4 ring PAHs with mean and standard error values of $39.5 \pm 5.4\%$, $26.1 \pm 4.0\%$, and $34.5 \pm 2.6\%$ of TPAH, respectively. The primary ring size accumulated by oysters from SS332A was 2 ring PAHs with a mean and standard error of $64.4 \pm 0.04\%$ of TPAH. However, oysters from rigs ST291A, ST264B, and SP93B accumulated equivalent proportions of predominantly 2 and 3 ring PAHs ranging from 31.6-58.9% and 34.3-51.1% of TPAH, respectively.

Unlike oysters collected in April, 5 ring PAHs, predominately benzo[e]pyrene, accounted for a large percentage of TPAH for most oysters collected in May. The ring size distribution was similar for EW873, VK817 and VK780 with the highest percentage being PAHs of 5 rings with a range of 39.0-57.2% of TPAH for oysters from these locations. Oysters from MC194 and MC21B had similar distributions with the lowest proportions of 5 ring PAHs (4.95-18.4% of TPAH) and the highest proportion of 4 ring PAHs with mean and standard error values of $16.8 \pm 2.2\%$ and $19.3 \pm 6.8\%$ of TPAH,

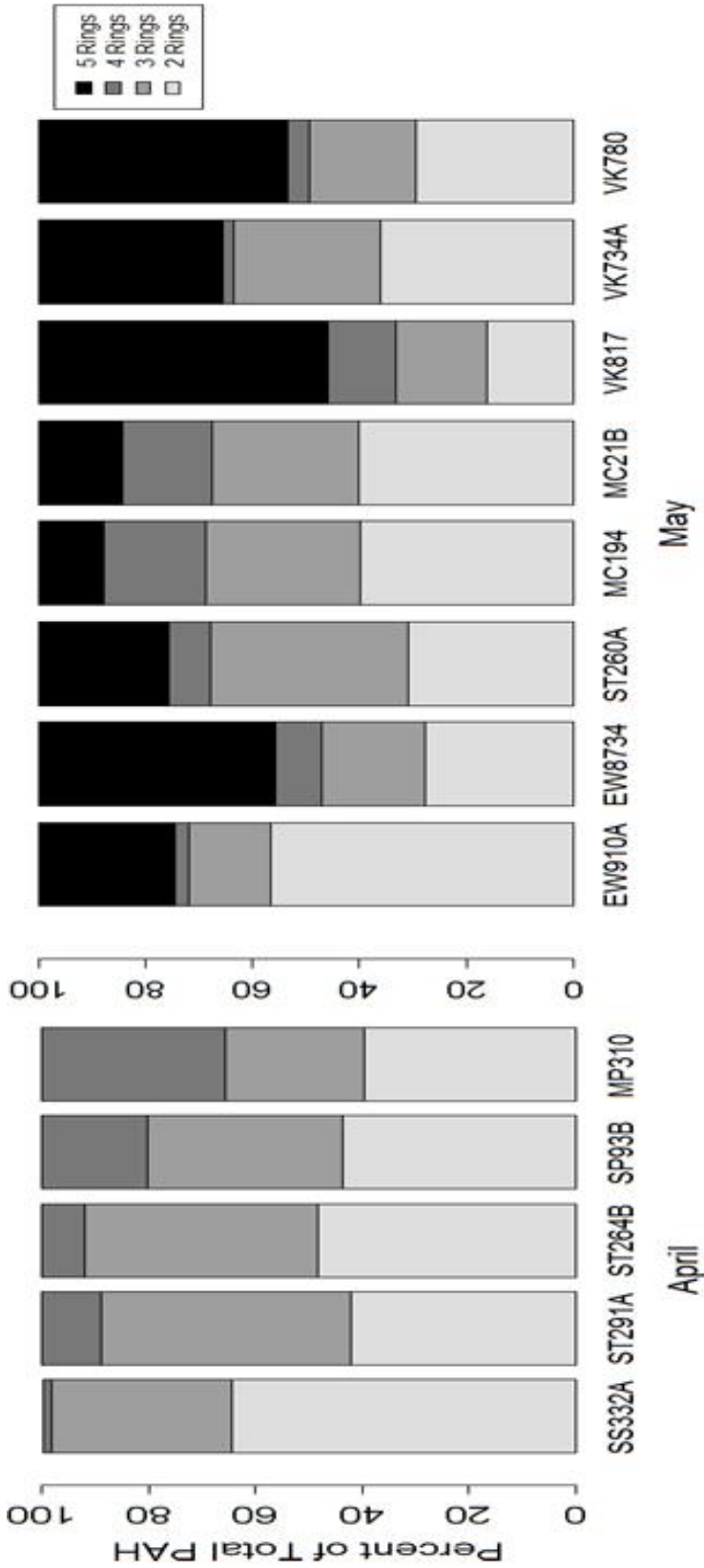


Fig. 2.3 Percentage of TPAH distribution by PAH ring size (2-5 ring PAHs) in oysters for each rig location collected in April (left) and May (right).

respectively. VK734A, ST260A, and EW910A had similar distributions for 4 and 5 ring PAHs, but varied for 2 and 3 ring PAHs. Oysters from VK734A had predominantly 2 ring PAHs ($56.6 \pm 7.9\%$ of TPAH), while oysters from ST260 and EW910A had similar proportions of 2 and 3 ring PAHs ranging from 21.8-58.0% and 23.0-47.2% of TPAH for the respective ring sizes.

2.4.2. *Accumulation of PAHs in corals*

TPAH (PAHs) concentrations in corals from the April collection ranged from 8.73-15.17 ng g⁻¹ and corals from the May collection ranged from 24.28-79.23 ng g⁻¹. Distribution of TPAH by rig location for oysters from both sampling trips is shown in Figure 2.3. Average individual PAH concentrations and total PAH concentrations are shown in Table 2.5. No corals contained 6 ring PAHs (IP, DBAN, BghiP, ANT, or COR) or 5-ring 3-methylcholanthrene above the detection limits. There were no significant differences in average TPAH between rig locations in corals from either April or May collections. However, as with the oysters, corals from MC21B had the highest overall TPAH concentrations ranging from 28.93-79.23 ng g⁻¹. Corals collected in both April and May sampling trips had higher TPAH concentrations than oysters collected from the same rig locations, with the exception of MC21B.

The predominant individual PAH in corals collected in April was 2,6-dimethylnaphthalene which ranged from 15.6-57.3% of TPAH for all samples. Other individual PAHs that account for a large percent of TPAH for April corals are phenanthrenes ranging from 6.9-19.2% of TPAH, 2-methylnaphthalene ranging from 5.1-14.0%, and acenaphthylene ranging from 7.5-15.1% of TPAH, except one sample from

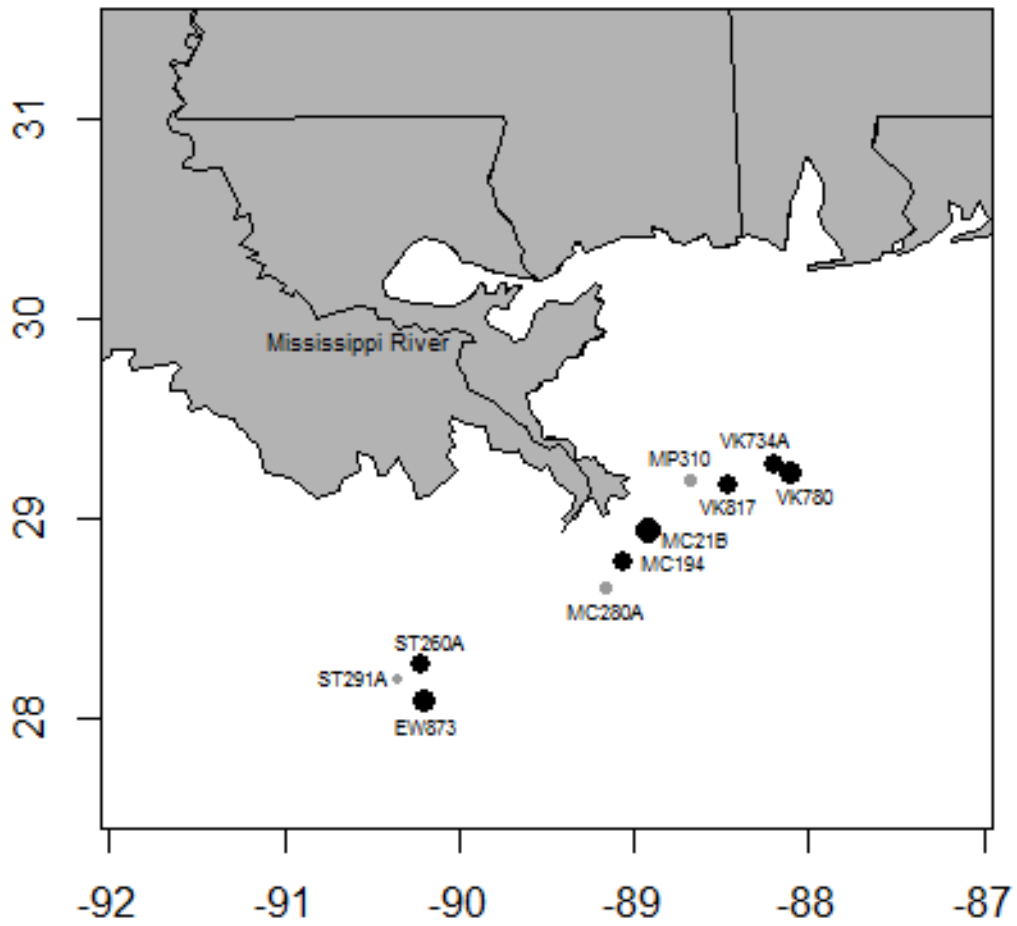


Fig. 2.4 Total PAH accumulation by rig location for corals collected in April (light gray) and May (black). Size of circles is proportional to TPAH concentration. Exact concentrations detailed in Table 2.5.

Table 2.5 Individual and total PAH concentrations (ng g⁻¹ wet weight) in corals collected in April and May 2011. Perylene (PE) concentrations included separately.

PAHs	Units	April Sampling			May Sampling						
		SP	MC	MP	EW	ST	MC	MC	VK	VK	VK
		291A	280A	310	873	260A	194	21B	817	734A	780
NA	ng g ⁻¹	1.07	0.79	0.54	18.74	8.01	5.53	7.67	14.53	3.77	5.92
2-M NA	ng g ⁻¹	1.13	0.95	0.74	1.05	0.89	1.25	3.00	0.83	1.72	1.06
1-M NA	ng g ⁻¹	0.29	0.25	0.24	0.38	0.35	0.51	1.35	0.31	0.58	0.53
2,6-M NA	ng g ⁻¹	1.44	6.63	3.75	3.30	13.52	16.90	11.27	6.38	13.30	19.12
1,3- M NA	ng g ⁻¹	0.35	0.35	0.50	8.18	3.38	3.19	6.12	2.91	1.67	3.20
1,6- M NA	ng g ⁻¹	0.39	0.51	0.30	1.31	0.36	0.76	2.10	0.35	0.72	1.27
1,4-/2,3- M NA	ng g ⁻¹	0.10	0.21	0.12	0.06	BDL	0.44	0.59	0.07	2.08	0.18
1,5- M NA	ng g ⁻¹	0.21	BDL	0.34	0.08	0.10	0.29	1.20	0.13	0.31	0.37
1,2- M NA	ng g ⁻¹	BDL	BDL	BDL	0.31	0.11	0.48	0.80	0.36	0.52	0.82
1,8- M NA	ng g ⁻¹	BDL	BDL	BDL	BDL	0.08	BDL	BDL	BDL	BDL	BDL
2,3,5-M NA	ng g ⁻¹	BDL	BDL	BDL	0.59	BDL	0.62	1.35	0.39	0.42	2.09
AC	ng g ⁻¹	1.01	1.01	1.64	0.06	0.09	0.66	1.51	0.41	0.52	0.22
ACL	ng g ⁻¹	BDL	BDL	BDL	0.50	0.25	0.40	0.54	0.32	0.52	0.34
FL	ng g ⁻¹	0.57	0.33	0.32	1.31	0.57	1.27	1.78	0.77	1.50	1.05
1-M FL	ng g ⁻¹	BDL	BDL	BDL	0.03	0.01	0.03	0.08	0.02	0.03	0.09
DBT	ng g ⁻¹	0.26	BDL	BDL	0.35	0.26	0.37	0.63	0.29	0.40	0.30
2-M DBT	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
4-M DBT	ng g ⁻¹	BDL	BDL	BDL	0.16	BDL	BDL	BDL	BDL	BDL	BDL
PHE	ng g ⁻¹	1.51	1.11	1.31	2.10	1.99	2.13	4.85	1.85	2.98	2.16
AN	ng g ⁻¹	BDL	BDL	BDL	0.23	0.21	0.35	0.27	0.29	0.25	0.37
1-M AN/PHE	ng g ⁻¹	BDL	0.08	0.22	0.74	0.34	0.40	0.63	0.47	0.28	0.53
2-M PHE	ng g ⁻¹	0.25	0.19	0.25	BDL	BDL	0.41	1.40	0.51	0.52	1.05
2-M AN	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.61	BDL	BDL
9-M AN	ng g ⁻¹	BDL	BDL	BDL	0.08	0.05	BDL	0.08	0.08	BDL	0.06
4,5-M PHE	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
3,6-M PHE	ng g ⁻¹	BDL	BDL	BDL	0.07	0.07	0.10	0.29	0.09	0.10	0.29
2,3-M AN	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.47	BDL	BDL
9,10-M AN	ng g ⁻¹	0.14	0.13	0.24	BDL	BDL	BDL	BDL	BDL	BDL	BDL
FLA	ng g ⁻¹	BDL	0.28	0.80	0.39	BDL	0.48	0.76	0.39	0.64	0.40
PYR	ng g ⁻¹	0.26	0.26	0.53	0.38	BDL	0.50	1.04	0.42	0.45	0.63
1-M PYR	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
2-M FLA	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
BaFL	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
BbFL	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.12	BDL	BDL
BaAN	ng g ⁻¹	BDL	BDL	BDL	0.37	BDL	BDL	BDL	BDL	BDL	0.74
CHR-TRI	ng g ⁻¹	0.27	0.39	1.30	0.17	BDL	0.36	1.44	0.42	0.42	0.42
NAC	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	0.80	BDL	2.69	0.56
4-/6- M CHR	ng g ⁻¹	0.12	0.16	0.24	1.22	1.86	0.61	2.21	1.14	1.58	1.79
BbFLA	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
BkFLA	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
BeP	ng g ⁻¹	BDL	BDL	BDL	3.22	2.87	BDL	2.36	3.45	BDL	2.15
BaP	ng g ⁻¹	BDL	BDL	BDL	BDL	BDL	0.89	1.79	2.63	1.14	1.31
PAHs	ng g ⁻¹	9.37	13.64	13.36	45.40	35.38	38.92	57.90	41.01	39.09	49.00
PE	ng g ⁻¹	BDL	BDL	BDL	BDL	1.29	BDL	BDL	BDL	BDL	BDL

MC280A which was below the detection limit.

Unlike the oysters collected in May, only one coral sample (from rig ST260A) had a perylene concentration (1.29 ng g^{-1}) above the detection limit, which would have only accounted for 4.0% of TPAH if included in the TPAH calculation. Naphthalene was the dominant individual PAH for corals from EW873 and VK817 with mean and standard error values of $43.6 \pm 2.3\%$ and $39.3 \pm 4.8\%$ of TPAH, respectively. However, 2,6-dimethylnaphthalene was the dominant individual PAH for coral from the other five rig locations with percent of TPAH ranging from 12.7-61.1%. Other individual PAHs that account for a large percent of TPAH for May corals were 1,3-dimethylnaphthalene ranging from 1.6-21.6% of TPAH and phenanthrene ranging from 2.9-11.5% of TPAH, when above the detection limit. Additionally, benzo[a]pyrene and benzo[e]pyrene were prominent in ST260A (6.3-12.1% of TPAH) and VK817 (2.4-13.5% of TPAH, when above detection limit), respectively.

The percentage of the TPAH concentration for each different ring size was examined for corals collected from both sampling trips (Fig. 2.5). This figure includes 2-5 rings PAHs only as there were no 6-ring PAHs detected in corals. For consistency with oysters, perylene was not included in the 5 ring PAH calculation. The primary ring size accumulated for all corals collected in April and May was 2 ring PAHs, which ranged from 45.0-81.2% and 56.6-91.1% of TPAH, respectively. As with oysters, only corals from the May sampling trip had 5 ring PAHs above the detection limit, although they did not account for a large percent of TPAH.

Corals from April varied slightly in ring size distribution between locations. Corals from MC280A had the highest proportion of 2 ring PAHs ($76.5 \pm 4.7\%$ of TPAH), and

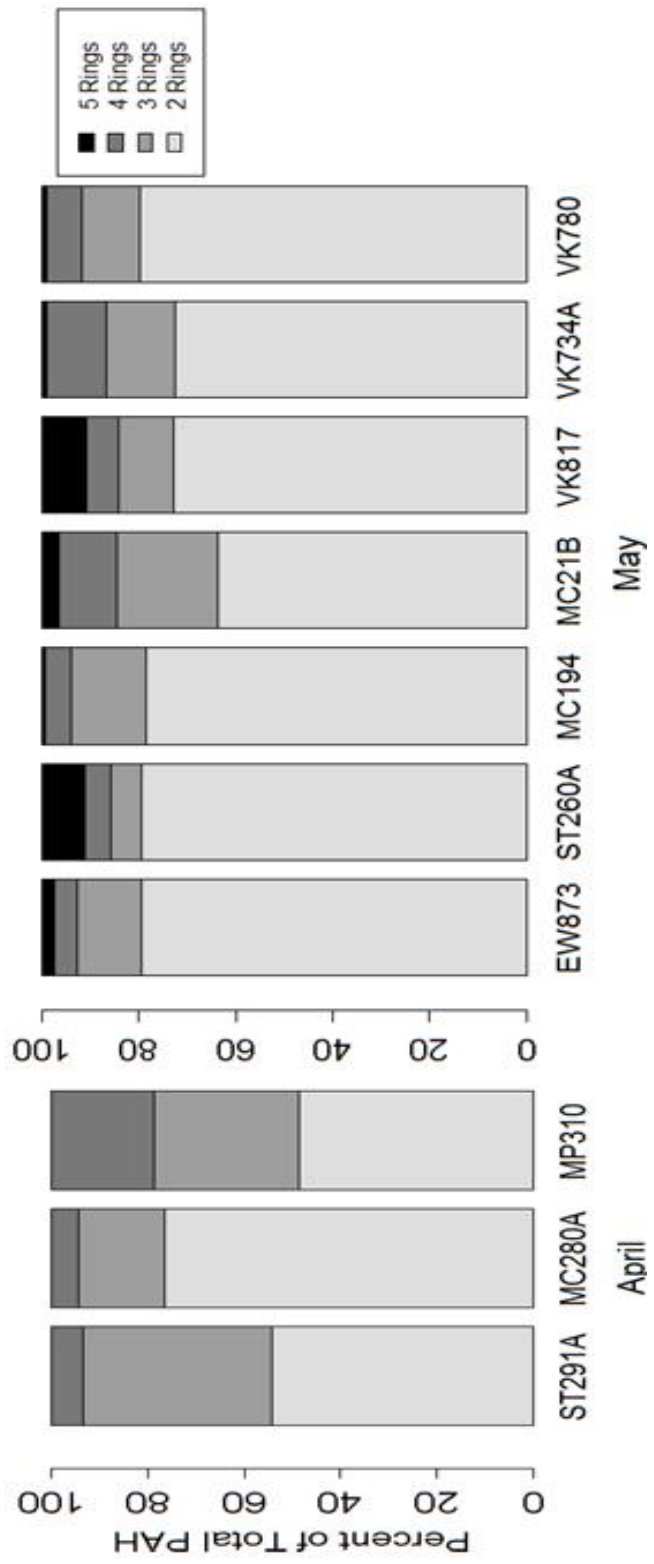


Fig. 2.5 Percentage of TPAH distribution by PAH ring size (2-5 ring PAHs) in corals for each rig location collected in April (left) and May (right).

lowest proportions of 3 and 4 ring PAHs with mean and standard errors of $17.9 \pm 0.3\%$ and $6.6 \pm 4.4\%$ of TPAH, respectively. Corals from ST29A had higher proportions of 2 and 3 ring PAHs ($54.2 \pm 3.2\%$ and $39.2 \pm 1.6\%$ of TPAH resp.) than corals from MP310 ($48.5 \pm 1.6\%$ and $30.3 \pm 2.4\%$ of TPAH, resp.), while corals from MP310 had higher proportions of 4 ring PAHs ($21.2 \pm 3.1\%$ of TPAH) than those from ST291A ($6.6 \pm 1.6\%$ of TPAH). PAH ring size distribution of corals collected in May did not vary appreciably between rig locations. All corals from May had low percentages of 3, 4 and 5 ring PAHs ranging from 3.3-28.1%, 3.19.9%, and below detection limit to 26.1% of TPAH, respectively.

2.4.3. Diagnostic ratios

Multiple diagnostic ratios were examined to differentiate between petrogenic and pyrogenic sources of PAHs in oysters and corals (Table 2.6). The majority of oysters from both sampling trips contained lower concentrations of alkylated homologs than parent PAHs (Alkyl/Parent < 1.0), while the majority of corals had higher concentrations (Alkyl/Parent > 1.0). All corals and the majority of oysters from April contained lower concentrations of high molecular weight PAH than low molecular weight PAHs (HMW/LMW < 1.0), while most oysters in May contained higher concentrations of high molecular weight PAHs (HMW/LMW > 1.0). The ratio of alkylated phenanthrenes to phenanthrene was below 2.0 for all samples except oysters from MC21B. In general for both sampling trips, oysters and corals accumulated more phenanthrene than anthracene as all PHE/AN ratios (from samples with PHE or AN above the detection limit) were > 5. While a PHE/AN ratio of 10 is considered the conservative differentiating point between

Table 2.6 PAH diagnostic ratios for corals and oysters collected in April and May (bold/italic indicates petrogenic)

	Rig ID	Alkyl/Parent	HMW/LMW	ΣMPHE/PHE	FLA/PY	PHE/AN
Oysters	SP93B	0.90 ± 0.18	0.54 ± 0.11	0.92 ± 0.36	2.0 ± 0.41	15.15 ± 1.96
	SS332A	1.36 ± 0.23	0.05[^]	0.47 †	NA	11.60 †
	ST291A	0.91 ± 0.18	0.27 ± 0.09	0.78 ± 0.11	2.10 ± 0.35	NA
	ST264B	0.86 ± 0.25	0.17 ± 0.09	0.57 ± 0.10	1.59*	8.74
	MP310	0.79 ± 0.16	1.35 ± 0.39	0.64 ± 0.04	2.73 ± 0.21	24.39 †
	EW910A	0.68 ± 0.11	7.18 ± 0.44	NA	0.80 ± 0.13	NA
	EW873	2.11 ± 0.36	4.82 ± 1.01	NA	0.96*	NA
	ST260A	0.51 ± 0.04	1.02 ± 0.24	0.60 ± 0.11	3.51 ± 1.13	5.81 ± 0.83
	VK817	0.18 ± 0.03	0.18 ± 0.03	0.59 ± 0.07	2.80 ± 0.32	11.81 ± 3.60
	VK734A	0.58 ± 0.11	1.54 ± 0.49	0.54 ± 0.01	NA	8.28 ± 0.86
VK780	0.40 ± 0.08	2.42 ± 0.23	0.65 ± 0.03	1.17 ± 0.18	9.47 ± 0.81	
	MC21B	1.40 ± 0.12	0.45 ± 0.05	2.51 ± 0.29	0.70 ± 0.04	25.66 ± 3.70
Corals	MC194	0.72 ± 0.13	1.17 ± 0.24	0.94 ± 0.14	5.50 ± 2.72	5.68 ± 0.42
	ST291A	0.94 ± 0.20	0.10 ± 0.06	0.18 ± 0.08	NA	NA
	MC280A	2.59 ± 0.51	0.27 ± 0.22	0.23 †	1.08*	NA
	MP310	1.09 ± 0.10	0.68 ± 0.12	0.36 ± 0.06	1.38 ± 0.33	NA
	EW873	0.75 ± 0.17	0.07 ± 0.03	0.44 ± 0.06	1.19 ± 0.06	9.29 ± 1.22
	ST260A	1.64 ± 0.16	0.30 ± 0.63	0.15 †	NA	10.6 †
	MC194	2.24 ± 0.14	0.16 ± 0.03	0.47 ± 0.07	0.95 ± 0.04	6.05 ± 0.79
	MC21B	1.52 ± 0.33	0.35 ± 0.06	0.51 ± 0.05	0.73 ± 0.03	18.22 ± 4.77
	VK817	0.72 ± 0.10	0.35 ± 0.13	0.41 ± 0.05	0.94 ± 0.08	6.83 ± 1.21
	VK734A	2.56 ± 0.60	0.37 ± 0.09	0.32 ± 0.04	1.62 ± 0.70	16.17 ± 2.97
VK780	3.38 ± 0.58	0.25 ± 0.10	0.76 ± 0.28	0.78 ± 0.08	6.16 ± 0.93	

[^]Only one sample had high molecular weight PAHs above the detection limit

*Only one sample had anthracene or phenanthrene above the detection limit

†Only one sample had fluoranthene or pyrenes above the detection limit

NA: One or both PAHs in ratio were below the detection limit

petrogenic and pyrogenic, most pyrogenic sources have PHE/AN ratios <5 (Neff et al., 2005). Additionally, both species collected in April and oysters collected in May accumulated more pyrene than fluoranthene, while corals collected in May contained more fluoranthene than pyrene. However, the oysters and corals that contained more pyrene than fluoranthene have ratios that are still close to 1.0 (0.7-0.95). Most petrogenic sources have FLA/PY ratios well below 1.0 as summarized in Neff et al. (2005), with a FLA/PYR ratio of 0.25 for a mean of 60 crude oils from around the world (Kerr et al. 1999).

Overall, oysters and corals collected in April appeared to have accumulated PAHs from a mix of both petrogenic and pyrogenic sources for all sampling locations. However, not all of the ratios could be examined for every location/organism due to one or more PAHs in the ratios being below the detection limit for all or most of the samples. Both corals and oysters collected from MC21B in May appear to have accumulated PAHs from predominately petrogenic sources. All other oysters collected in May and corals from EW873 appear to have accumulated predominately pyrogenic PAHs, while the remaining corals from the same locations in May show a mix of both sources.

2.5. Discussion

In this study, the baseline PAH accumulation of crested oysters, *O. equestris*, and orange-cup corals, *T. coccinea*, collected from oil rig structures off the coast of Louisiana was measured to determine if these organisms can be used as biomonitors for offshore oil exposure in the Northern Gulf of Mexico. While PAH concentrations varied by species and sampling trip, the total PAH accumulation was generally low for both species from

the two trips (2.51-95.55 ng g⁻¹). The PAH concentrations within these offshore fouling species are substantially lower than most oysters and mussels collected near the shoreline and in wetlands of the Louisiana and the Gulf of Mexico (Wade et al., 1988; NCCOS, 2011) and corals collected near the Texas coast (Sabourin et al., 2013). Through the NOAA Mussel Watch Program, Wade et al. (1988) examined the accumulation of 18 PAHs (only 4 alkylation homologs) in oysters (predominantly the Eastern oyster, *Crassostrea virginica*) collected from 51 sites along the coastal Gulf of Mexico from Texas to Florida. Total PAH concentrations ranged from below detection limit of 20 ng g⁻¹ to 18,620 ng g⁻¹ with a mean of 507 ng g⁻¹ (Wade et al., 1988). It is important to note that only 18 PAHs (14 parent and 4 alkylated homologs) were examined for these surveys, which likely underestimated TPAH. While that study accounted for sites throughout the Gulf of Mexico, other collections have been conducted specifically within the Mississippi River Delta (Tigers Pass and Pass a Loutre) in January through the NOAA Mussel Watch Program from 1990-2000. Oysters collected from these locations ranged from 321-4,775 ng g⁻¹ TPAH for 39 individual parent and alkylation group PAHs (NCCOS, 2011). While the PAH accumulation in corals, has not been well characterized, one study by Sabourin et al. (2013) showed much higher levels bioaccumulation than observed by this study in sea whips, *Leptogoria setacea*, collected from along the Port Aransas Jetty off the coast of Texas with total PAH (16 individual PAHs) concentrations ranging from 243-2,121 µg g⁻¹.

Furthermore, oysters and corals from this study appear to be on the low end of PAH bioaccumulation observed in invertebrates collected from sediments 30-3000m away from three oil rigs in Texas for the GOOMEX study (Kennicutt et al., 1994). The TPAH

concentrations (39 parent and alkylated, including perylene) within the soft tissue of a variety of different invertebrate species collected near the three oil rigs screened ranged from 17.73 ng g⁻¹-10,424.87 ng g⁻¹ for their first cruise and from 34.82-349.19 ng g⁻¹ for their second cruise (Kennicutt et al., 1994). The authors did not address the distribution of individual PAHs or if TPAH varied by type of invertebrate species (i.e. crustaceans or bivalves) as they included all invertebrate species collected into one category for analysis. Additionally, while perylene was measured among the PAHs, the authors did not specifically indicate its concentration in sediments or tissues of invertebrates.

Oysters and corals collected along coastal or wetland areas of the Gulf of Mexico (Wade et al., 1988; NCCOS, 2011; Sabourin et al., 2013) and invertebrates living in sediments near oil rigs in Texas (Kennicutt et al., 1994) could have a more persistent source of PAH exposure due to living within contaminated sediments. Conversely, organisms living essentially in the water column on the vertical reef communities of offshore oil rigs might have a more transient exposure to PAHs due to water currents around the rig and hence movement and dilution (in three dimensions) of contamination. This could explain why the PAH accumulation observed in organism collected from oil rigs were much lower than similar species collected from nearby coastal areas or slightly lower than invertebrates in sediments nearby rigs. The only other study, to our knowledge, that examined PAH contamination on native oil rig fouling species also showed much lower levels of bioaccumulation of the PAHs in blue mussels, *Mytilus galloprovincialis*, collected from oil rigs in the Adriatic Sea (Gomiero et al., 2011) compared to mussels collected from coastal sites along and near the Mediterranean Sea (Claisse, 1989; Villeneuve et al., 1999; Valavanidis et al., 2008).

Oysters and corals collected in May, especially corals, had higher TPAH concentrations than organisms collected in April from similar rig locations. In particular, oysters and corals collected in May from oil rig MC21B had the highest TPAH concentrations observed among all locations. This increase in PAH accumulation between sampling trips could be due to the flooding of the Mississippi River in mid- to late-May 2011. This flooding was one of the largest on record with the Mississippi River delivering extensive suspended sediment loads to the Gulf of Mexico (Falcini et al., 2012). The plumes that emanated from the mouth of the Mississippi River (<http://earthobservatory.nasa.gov/NaturalHazards/view.php?id=51179>) exhibited a narrow and focused jet of sediment-laden water that penetrated the coastal current, particularly from the Southwest Pass, delivering sediments far offshore into the northern Gulf of Mexico (Falcini et al., 2012). The jet from the Southwest pass was easily recognized as a persistent core of high current velocity and suspended solids even 50 km offshore (Falcini et al., 2012). This increased sediment load offshore was visible during this study's sample collections as rig locations which had blue water in April, were green-brown in May. The flood plume could have resulted in an influx of anthropogenic and diagenic PAHs into the offshore ecosystem, which might explain the increases in accumulation of PAHs in the organisms collected from oil rigs in May as the oil rigs would be near enough the outflow and within observed the flood plume flow (Androulidakis and Kourafalou, 2013). However, there were no correlations between the TPAH concentrations in either species and distance from nearest pass of Mississippi River outflow. Although, the oysters and corals with the highest TPAH concentrations were from rig MC21B, which was the closest rig to the shoreline and the South Pass of

the Mississippi River. The substantially higher PAHs observed in organisms from this particular station could also be due to a localized source of exposure, especially as the source ratios indicate predominantly petrogenic inputs.

Beyond the increase in total PAHs accumulated in oysters and corals collected in May, the increased concentrations in high molecular weight PAHs, particularly benzo[e]pyrenes and perylene in oysters, could be indicative of exposure to the sediment-laden plume from the Mississippi River. Due to their low water solubility, higher molecular weight PAHs, particularly 5- and 6-ring PAHs, are found in low proportions both within the water column (as compared to sediments containing anthropogenic contamination) and within petrogenic PAH sources (Meador et al., 1995). However, a large flooding event that results in resuspension of sediment particles into the water column could make the HMW PAHs bound to particulate matter from the Mississippi River plume more accessible for filtering feeding organisms like oysters on the oil rigs. Studies examining the simulated resuspension of sediment bound PAHs suggest that resuspension does result in substantial increases in particle-bound PAHs into the water-column as well as increases of PAHs into the dissolved phase through desorption (Latimer et al., 1999; Yang et al., 2008). However, PAH concentrations within water samples from each rig location would have needed to be examined to determine what PAHs were available for uptake and if the sediment laden plume did indeed play a factor in the concentrations of PAHs observed in organism collected in May.

Coastal and wetland sediments near the Mississippi River Delta do have high proportions of 5- and 6-ring sediments. For sediment samples collected in the Tiger Pass and Pass a Loutre sampling sites (1996 and 2005), 5- and 6-ring PAHs accounted for 24-

31% of TPAH and perylene, in particular, accounted for 11-19% of TPAH (NCCOS, 2011). Further up the Mississippi River at the Head of Passes sampling site (~10km south from Venice, LA), 5- and 6-ring PAHs constituted 46% of TPAH, with perylene alone accounting for 18% of TPAH (NCCOS, 2011). Additionally, perylene has been observed in appreciable levels in the particulate phase ($>0.7\mu\text{m}$) in surface and bottom waters (12 ± 3.5 and $14.8\pm 1.2\text{ ng L}^{-1}$, respectively) of the lower Mississippi River in April (Mitra and Bianchi, 2003). Within deep ocean surface sediments collected throughout the northern Gulf of Mexico region, perylene was the major PAH detected ranging from below detection limit to 110 ng g^{-1} with some of the highest concentrations in two of the sampling locations closest to the Mississippi River Delta near the west-most oil rigs surveyed in this study (Wade et al., 2008). This could indicate that terrestrial PAH inputs from the Mississippi River are indeed reaching the offshore shelf.

In addition to the differences in PAH accumulation observed between sampling trips, the bioaccumulation in response to the plume differed between the two species. The ring size distributions of PAH for oysters and corals were not appreciably different in April, but there was a noticeable difference between oysters and corals from the same rig locations collected in May. Oysters accumulated large proportions of 5 ring PAHs, particularly benzo[e]pyrene (and perylene), while corals exposed to the same environmental conditions primarily accumulated 2 ring PAHs. This difference in bioaccumulation could be due to different primary routes of exposure between the two species. Oysters are filter feeding organisms, which makes particle associated PAHs (e.g. higher molecular weight) a primary source for PAH accumulation (Baumard et al., 1998; Baumard et al., 1999). Prior studies examining bioaccumulation of PAHs in primarily *C.*

virginica, in the coastal Gulf of Mexico have shown the highest accumulation of 4- and 5-ring PAHs (Serciano et al., 1996) or 3- and 4-ring PAHs (Wade et al., 1988).

Additionally, Qian et al. (2001) showed that oysters accumulated higher concentrations of HMW parent PAHs than what was measured in sediments from the same location, further indicating the preferential bioaccumulation and possibly retention of HMW PAHs.

Unlike oysters, non-symbiotic stony corals like *Tubastrea coccinea* are not filter-feeders, but are heterotrophic feeders using their tentacles to bring prey, particularly zooplankton, directly to their mouths (Goreau et al., 1971). Besides, consuming contaminated prey, the main route of exposure to PAHs for stony corals is likely direct uptake (Knap et al., 1982; Kennedy et al., 1992; Peters et al., 1997). Corals can be susceptible to organic contaminants because the layer of tissue covering the coral skeleton is thin (~100µm) and lipid rich, which can facilitate the direct uptake of lipophilic compounds like PAHs (Peters et al., 1997). Furthermore, the detoxification and depuration of lipophilic contaminants in corals can be slow (Knap et al., 1982; Kennedy et al., 1992). Lower molecular weight PAHs would be more bioavailable in the water column due to their lower K_{ow} values (i.e. higher water solubility). Additionally, their smaller size could facilitate easier direct uptake, which might explain the high accumulation of 2-ring PAHs in the corals from this study. While PAH accumulation has not been well studied in corals, PAH accumulation in sea whip, *Leptogorgia setacea*, collected at Port Aransas Jetty off the coast of Texas only had a mean of 27.5% of TPAH for 2-ring PAHs in coral body tissue, but the study was limited in the number of PAHs measured and corals were collected from a coastal region with high anthropogenic

contamination (Sabourin et al., 2013).

The difference in bioaccumulation between the two species from the same locations during an event resulting in environmental contamination of PAHs highlights the need to be cautious while using ratios of PAHs to examine source inputs of contamination within organisms. This is especially evident in ratios like HMW:LMW that can be highly biased based on preferential accumulation by each species. The HMW:LMW ratios for oysters in May indicated predominantly pyrogenic inputs, while all corals from the same locations indicated strong petrogenic inputs. This emphasizes the need to examine multiple ratios to attempt to understand source inputs, particularly the use of ratios that examine PAHs in the same ring size class like PHE/AN or FLA/PYR. Also, while ratios can be useful for identifying PAH sources in environmental samples, it can be hard to account for the potential metabolism or depuration of PAHs within an organism, even in organisms that are thought to have more limited detoxification pathways.

Furthermore, while this study suggests the quick response to an environmental contamination event like the Mississippi River flooding, it also shows the potential recovery. Most, if not all, organisms collected during this study were old enough to be alive during the 3 month leak of oil into the Gulf of Mexico from the Deepwater Horizon (DWH) incident (April 20th-July 15th, 2010). Organisms on all but the western most set of oil rigs in this study, particularly the four east most rigs (VK780, VK734A, VK817, and MP310), would have been exposed to the surface oil plume from the DWH incident (Optical Oceanography Laboratory, 2010). However, evidence of the potential exposure to petroleum PAHs from the oil was not observed in oysters or corals collected approximately 9 months later.

2.6. Conclusions

Overall, oysters and corals collected from oil rigs show a low baseline level of PAH contamination compared to similar species collected from nearby coasts and wetlands. Additionally, both organisms showed a quick response to a potential contamination event highlighting the benefits of using these sessile invertebrate species as indicators of exposure to environmental contamination in the offshore region such as oil spill events. Due to this study's limited sampling regime, a more thorough sampling of fouling species on offshore oil rigs would need to be conducted to have a more comprehensive understanding of baseline levels of PAH accumulation in the offshore Gulf of Mexico. Additionally, future studies should incorporate biological biomarkers of PAH exposure and effects including physiological stress, DNA damage, oxidative stress, and detoxification pathways to understand the baseline health of fouling organisms as understanding both exposure and effects of contaminants are crucial to using these species and indicators of environmental contamination. Future studies should also conduct baseline ecological surveys (e.g. species diversity) of oil rig fouling communities to determine ecological impacts from oil exposure such as population shifts or alterations in recruitment. Furthermore, this study highlights the necessity of examining multiple species for biomonitoring as there were differences in accumulation and distribution of PAHs between the oysters and corals. This could be particularly important when attempting to assess diagnostic ratios for PAH sources as the differences in accumulation by ring size for a given species might bias the diagnostic analysis, especially when examining ratios that span more than one ring size like alkylation homolog to parent PAH

or high molecular weight to low molecular weight ratios.

2.7. Acknowledgements

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Chapter 3. Investigating molecular and physiological effects in juvenile blue crab, *Callinectes sapidus*, exposed to sediments contaminated by oil from the Deepwater Horizon Incident.²

3.1. Abstract

Hatchery reared juvenile blue crabs, *Callinectes sapidus*, were exposed for 31 days to six different sediments collected from the Pass a Loutre State Wildlife Management Area after the Deepwater Horizon (DWH) Incident that differed in their levels of oil contamination. The concentration of polycyclic aromatic hydrocarbons (PAHs), saturated hydrocarbons, and total extractable hydrocarbons were determined in each sediment, as were biologically relevant metals (As, Ag, Cd, Cr, Cu, Fe, Hg, Ni, Pb, and Zn), grain size distribution, percent total organic carbon, and percent total solids. Water samples collected during the exposure were also analyzed to determine PAH concentrations. Multiple biological endpoints were measured including mortality, growth, and molting responses. Additionally, various biomarkers of early biological stress were examined in the hemolymph and hepatopancrease of crabs, including DNA damage (Comet assay) and gene expression levels of Cu-metallothionein (CuMT), glutathione-S-transferase (GST), and mitochondrial manganese superoxide dismutase (MnSOD). Over the 31 day exposure, there were no treatment related mortalities in juvenile blue crabs. The overall growth and molting of the crabs were not substantially different between the various sediment exposures. Additionally, none of the biomarkers of early biological stress were correlated with PAH concentrations. However, GST gene expression levels were positively correlated with metal concentrations in the sediments ($p < 0.05$). Overall,

² Hannah V. Pié, Eric J. Schott, Carys L. Mitchelmore. In preparation for submission.

juvenile blue crabs did not appear to be negatively impacted by sediment PAH contamination during the 31 day exposure.

3.2. Introduction

For three months, the Macondo well (MC252) blowout of April 20th, 2010 leaked an estimated 4.1 million barrels of Louisiana Sweet crude oil into the Northern Gulf of Mexico (Camilli et al., 2010). Multiple oil recovery and response options such as chemical dispersants (applied at the water surface and directly at the well-head), burning, boomers, and skimmers were utilized in attempt to minimize the amount of oil reaching sensitive shoreline ecosystems. Despite these various oil recovery and response options, oil made it ashore and contaminated many marshland areas in the northern Gulf of Mexico, particularly the Louisiana coast (NOAA, 2010; National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011). More than 650 miles of the Gulf of Mexico's coastal habitats including salt marshes, mangroves, mudflats, and sand beaches were oiled following the DWH incident with more than 130 miles having been designated moderately to heavily oiled (National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011).

Large-scale oil contamination of sediments in such an ecologically productive region of the Gulf of Mexico could impact numerous benthic species, including important fishery species like blue crabs, *Callinectes sapidus*. Blue crabs are a keystone species for the Atlantic coast and Gulf of Mexico both ecologically and economically. They contribute ecologically to the estuarine and coastal food webs as both prey and predators to numerous other species (Guillory et al., 2001). Blue crabs are economically important as they are one of the top ten key domestic commercial fisheries nationally as well as for

many individual states, particularly Louisiana, in weight and monetary value (NMFS, 2013). As of 2012, hard blue crab U.S. landings were valued at \$186.1 million with 25 percent of the total landings coming from Louisiana (NMFS, 2013). As a benthic species, they live and feed within sediments; and therefore, could be exposed to oil that reaches the coastal wetlands and estuaries. Exposure to oil and its constituent polycyclic aromatic hydrocarbons (PAHs) can result in acute mortality but can also lead to delayed and sublethal effects that would ultimately impact the population. In marine organisms, these effects can range from mutagenesis, impacts on energetic processes, alterations to immune responses, disruption of ionic and osmotic regulation, alterations in feeding, decreases in growth, and decreases in reproductive success (NRC, 2003; Martínez-Gómez et al., 2010; Luna-Acosta et al., 2011). For example, water-borne exposures of juvenile blue crabs to aromatic hydrocarbons like benzene and dimethylnaphthalene as well as the water-accommodated (or soluble) fraction (WAF) of No #2 fuel oil and South Louisiana crude oil have been shown to inhibit growth and molting (Karinen and Rice, 1974; Cantelmo et al., 1981, 1982; Wang and Stickle, 1987; and Hale, 1988). Other studies have shown that exposure to petroleum products and PAHs in water and sediments causes physiological stress, oxidative stress, and increases in detoxification biomarkers in crabs and other marine invertebrates (Lee, 1988; Morales-Caselles et al., 2008; Gomiero et al., 2011; Zanette et al., 2011).

The overall environment impacts of these DWH contaminated sediments have yet to be determined. Many data gaps and uncertainties remain in regards to the lethal and sublethal toxicological impacts of oil contaminated sediments on invertebrate organisms. Therefore, to examine the lethal and sublethal impacts of exposure to DWH contaminated

sediments on benthic invertebrates, juvenile blue crabs were exposed for 31 days to sediments collected from various contaminated and reference sites in the Pass a Loutre Wildlife Management Area (WMA), the closest wetland to the Macondo 252 (MC252) well-head. The sediments collected contained a wide range of PAH contamination (186 - 2,196,658 $\mu\text{g kg}^{-1}$ total PAH). The organisms were exposed to six sediment types in total, including one reference sediment. These sediments had been previously fingerprinted for the presence of MC252 oil. Various diagnostic ratios to differentiate source inputs of PAHs were also examined in this study to further explore other possible inputs within the sediments.

In addition to mortality, we investigated various sublethal impacts of oil / PAH exposure and effects including, alterations in growth, changes in molting timing and frequency, behavior (including lack of feeding), genotoxicity (DNA damage) and multiple molecular biomarkers. The Comet assay, which measures single-strand DNA damage, was used to determine genotoxicity in blue crab hemocytes. Changes in gene expression were examined in hepatopancrease tissue using several genes involved in the detoxification of xenobiotics, metals, and reactive oxygen species.

The genes of the two most common biomarkers for PAH exposure involved in the metabolism of PAHs, cytochrome P450 and glutathione-*S*-transferase (GST), have not yet been fully characterized in blue crabs. However, a putative GST gene (Accession # KJ925004) was used to examine GST gene expression for this study. Within invertebrate species, the metabolism of PAHs can lead to oxidative stress through redox cycling, which is a common metabolic route in marine invertebrates, including crabs (Livingstone, 2003; Vijayavel et al., 2004). To examine if exposure to oil and PAHs

results in oxidative stress impacts, the gene expression levels of the mitochondrial manganese superoxide dismutase (MnSOD) and the GST genes were examined. Furthermore, the gene expression levels of Cu-II metallothionein (CuMT) were measured. Metallothionein is involved in heavy metal detoxification and also potentially oxidative stress protection (Cavalento et al., 2002).

3.3. Materials

3.3.1. Organisms used for study

Juvenile blue crabs, *Callinectes sapidus*, used in this study were hatched and raised in the Institute of Marine Biotechnology (IMET)ø Aquatic Research Center (ARC). All of the 144 crabs used in the study were 3 months old, from the same brood stock, and raised under standard hatchery conditions. At the start of the experiment, the average crab width was $32.1 \pm 0.3\text{mm}$ (SEM; range 24.2 - 43.1mm). Crabs of all sizes were randomly distributed to the different treatments. While crabs in this study had a 20mm range in sizes at the start of this experiment, other studies and field surveys have not shown a significant difference in growth or molting in juvenile blue crabs ranging from 20-49mm or with sex of crabs in this range (Tagatz, 1968b), so there should not be significantly different growth rates between crabs examined based on initial size or sex.

3.3.2. Collection of sediments

Sediments were collected from various locations within the Pass a Loutre WMA located in southern Plaquemines Parish at the mouth of the Mississippi River (Fig. 3.1) and were provided by Stratus Consulting for DWH National Resource Damage

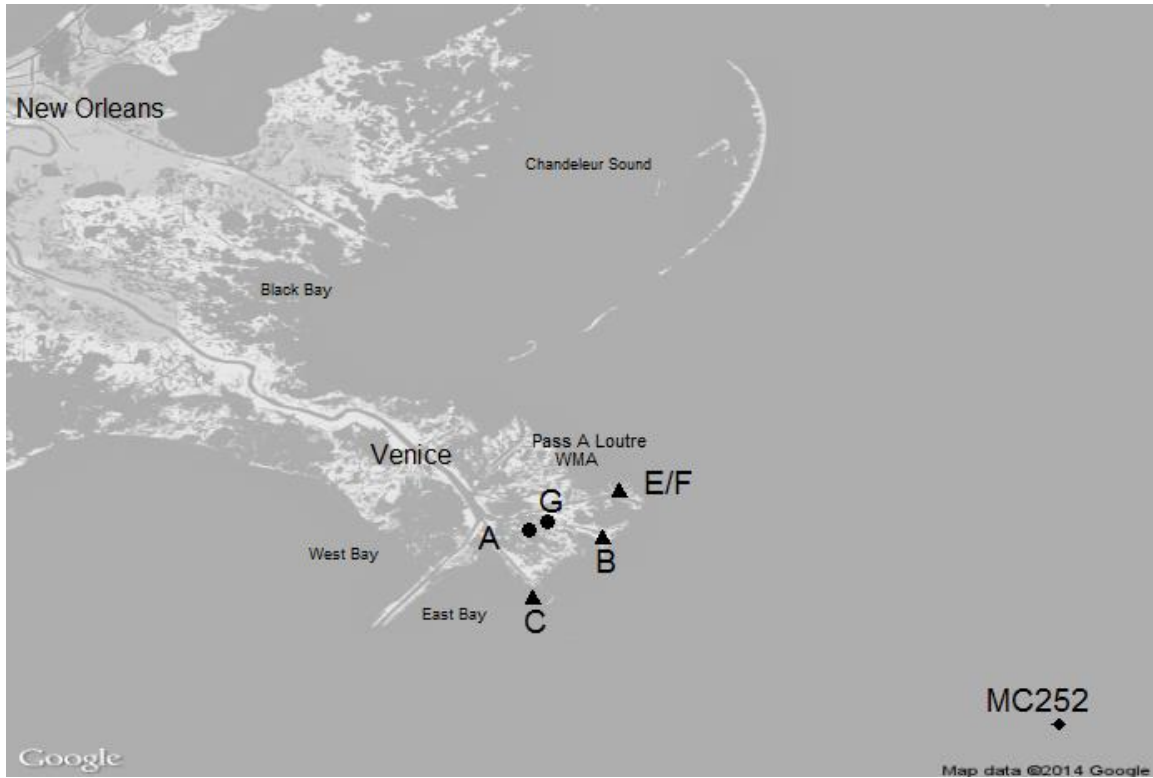


Fig. 3.1 Sampling locations for sediments collected within the Pass-a-Loutre State WMA in southern Louisiana including sites contaminated by the DWH incident (triangles), reference sites with little to no observed contamination by the DWH incident (circles), and the location of the Macondo 252 wellhead (diamond).

Assessment toxicity testing activities. Sediments A and G were collected from a Section of the WMA with little to no observed surface oil contamination from the DWH incident and served as reference sediments, while sediments B, C, and F were collected in areas of moderate to heavy oil contamination (NOAA, 2010). Sediment E was collected a little over a year after the first collection trip from the same location as sediment F.

Forensic analysis to fingerprint for DWH (MC252) oil was conducted for all sediments except sediment E. Sediments B, C, and F had positive identification for MC252 oil, while sediments A and G had indeterminate identifications, which indicated that some hydrocarbons were present, but MC252 oil was not recognized (data not shown; Scott Stout, NewFields Companies, LLC, Rockland, MA, per. communication, April 2014). For each sediment type, detailed chemical analyses were conducted which included analysis of 50 individual PAHs/alkylation groups, total PAHs (TPAH₅₀), total saturated hydrocarbons (sum of 37 individuals), total extractable hydrocarbons (TEH), total organic carbon (TOC), total solids, grain size distribution, and concentration of a variety of metals for all sediments by ALS Environmental (Kelso, WA) or Alpha Analytical (Westborough, MA). Description of the sediment collection dates, TOC, total solids, and grain size percentages are described in Table 3.1.

3.4. Methods

3.4.1. Exposure methods

Eight different 8 L glass tanks were used for each sediment type with three crabs added per tank ($N=24$ crabs per sediment type) and separated by pre-conditioned plastic inserts attached to the glass tanks using Aquarium Grade 100% silicon. Water-flow was

Table 3.1 Composition of sediment samples collected from Pass a Loutré WMA for the exposure study.

Sediment ID	Collection Date	TOC (%)	PSEP Grain Size (%)								
			Total Solids (%)	Gravel >2.0mm	Sand, Very Coarse 1.0-2.0 mm	Sand, Coarse 0.5-1.0 mm	Sand, Medium 0.25-0.5 mm	Sand, Fine 0.125-0.25 mm	Sand, Very Fine 0.0625-0.125 mm	Silt 0.0039-0.0625 mm	Clay <0.0039 mm
A	1/26/2011	1.14	48.9	0.00	0.01	0.04	0.16	5.04	31.08	39.55	18.12
B	1/26/2011	2.37	57.2	0.10	0.06	0.17	0.70	36.04	52.02	2.88	3.81
C	1/25/2011	0.94	80.3	0.00	0.00	0.06	0.63	77.96	19.49	0.63	0.40
D ^a	NA	12.8	31.5	0.04	0.12	0.31	0.49	0.8	1.91	32.03	36.47
E	4/9/2012	31.9	11.7	0.06	0.15	0.44	0.66	1.07	2.28	24.67	32.21
F ^b	1/25/2011	58.8	15.6	0.00	0.00	0.00	0.00	0.00	0.00	13.55	40.28
G ^c	1/25/2011	1.14	48.9	0.00	0.07	0.09	0.20	0.33	1.30	44.58	43.74

^aSediment D was a 1:1.7 mixture of Sediment G and Sediment E, respectively

^bThe PSEP Grain Sizes for Sediment F are from sediment collected at the end of the experiment

^cSediment G was not used as exposure treatment, only to make the Sediment D mixture

set-up ahead of time and tanks flushed in artificial seawater (ASW) before addition of the sediments or organisms. Sediments were added at a 1:1 ratio to salinity adjusted ASW at 25 ppt and mixed thoroughly to form a homogenous sediment slurry. Sediment D was a 1:1.7 mixture of sediments G and E, respectively. Overall, there were 6 sediment types for a total of 48 individual tanks and 144 crabs. To each tank, 200 g of the appropriate sediment mixture was added. The tanks were then filled just below the standpipe (6 L) with 25 ppt ASW. The sediments were allowed to settle for at least 24hr before the addition of test organisms.

A partial flow-through system was utilized with a fully sealed standpipe placed in the middle of the tank to allow for water exchange. The tops of the standpipes were covered with pre-conditioned fine mesh to prevent the loss of large, floating sediment particles or any organic matter entrained in the sediment. To achieve minimal sediment disturbance but at the same time provide an adequate water exchange to maintain water quality, water exchanges were conducted daily for 40 minutes using a flow rate of 200 ml min^{-1} (1.3x volume of tank exchange). Furthermore, an air hose was placed into the middle of each tank to allow for aeration and placed high enough in the tank as not to disturb the sediment.

Tanks were initially covered with translucent, plastic coverings, which were eventually determined to be insufficient in preventing movement of crabs between tank sections. Subsequently translucent sealed bags of water were used to weigh down the plastic coverings to minimize crab movement while maintaining light attenuation. All tanks and equipment used in tanks were preconditioned with 25 ppt seawater for a minimum of 24 hr before use. Water quality parameters (e.g. dissolved oxygen,

ammonia, pH, temperature, and salinity) were measured daily. Light cycle was held at 14 hr light/ 10 hr dark. Crabs were daily fed pieces of squid tissue and food consumption was recorded. Any excess food was removed after a few hours to minimize potential impacts on water quality.

3.4.2. *Chemical endpoints*

Water samples were collected from each treatment by pooling equal volume subsamples from each of the eight tanks and thoroughly mixing to homogenize the samples. Water samples were collected every other day for the first week and then once a week until the end of the experiment. Sediment samples were collected at the start and end of the experiment. All water and sediment samples were shipped to and processed by ALS Environmental (Kelso, WA). Water samples were analyzed for PAHs and sediment samples were analyzed for PAHs, summation of saturated hydrocarbons (37 total), TEH, TOC, total solids, and particle grain size as briefly described below.

PAHs including alkyl homologues groups were analyzed by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM). The analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the target analytes. Alkyl PAH homologues are quantified using a response factor assigned from the parent PAH compound. Saturated hydrocarbons were analyzed by gas chromatography with flame ionization detection (GC/FID) based on EPA Method 8015. Total extractable hydrocarbons (TEH) representing the total aromatic and aliphatic hydrocarbon content of sample extracts were analyzed by GC/FID after silica gel clean-up. The result is reported

based on integration of the FID signal over the entire hydrocarbon range from *n*-C₉ to *n*-C₄₄ and calibrated against the average alkane hydrocarbon response factor. Total metals in sediments were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS). Total mercury in sediments was analyzed by cold vapor atomic absorption (CVAA) or cold vapor atomic fluorescence spectrometry (CVAFS). The analytical procedures are based on EPA SW-846 Methods 6010C, 6020A, 7470A, 7471A, 7471B, 7474, and 7742. Total organic carbon (TOC) in sediments was measured by a modified ASTM D 4129-82 method and total solids in sediments were measured using a modified EPA 160.3 method. Particle grain size distribution of sediments was measured using the Puget Sound Estuary Program (PSEP) method with particles binned into eight subcategories based on percent from dry weight (as seen in Table 3.1). Results in Table 3.4 that were below the detection limits were given values of 0.

3.4.3. PAH source diagnostic ratios

Various diagnostic ratios of PAHs have been utilized to examine the source inputs of PAHs (e.g. pyrogenic, petrogenic) in environmental samples (Budzinski et al., 1997; Yunker et al., 2001; Neff et al., 2005; Saha et al., 2009). The ratios used to estimate between petrogenic and pyrogenic PAH source inputs in this study were alkylated to parent PAHs, anthracene (AN) to AN plus phenanthrene (PHE), fluoranthene (FLA) to FLA plus pyrene (PY), and FLA plus PY (FLAPY) to FLAPY plus the sum of C₂- to C₄-phenathrenes (C₂₄PHE) as described in Table 3.2. Alkylated to parent PAH is the ratio of the sum of all parent PAHs to the sum of all alkylated homologue PAHs.

Table 3.2 PAH diagnostic ratios used to distinguish between petrogenic and pyrogenic source inputs within initial sediments.

Ratio	Petrogenic	Pyrogenic	Reference
Alkylated Homologs/ Parent	> 1	< 1	Saha et al. 2009
AN/(AN+PHE)	< 0.1	> 0.1	Yunker et al 2002
FLA/(FLA+PY)	< 0.4	0.4-0.5 fossil fuel > 0.5 grass, wood, and coal	Yunker et al. 2002
FLAPY/(FLAPY+[C24PHE])	< 0.1	> 0.75	Prahl and Carpenter 1983

*PHE (phenanthrene), AN (anthracene), FLA (fluoranthene), PY (pyrene), FLAPY (sum of fluoranthene and pyrene), C24PHE (sum of C₂- to C₄-phenathrenes)

3.4.4. *Mortality, growth and molting responses*

Crabs were monitored daily for mortality and molting. The growth in carapace size and wet weight were examined at the start and end of the experiment and each day after a crab molted. For each crab, a photograph was taken on a standardized grid and Image J software was used to measure carapace size to the nearest 0.01 mm. Carapace width and length were measured as described in Millikin and Williams (1984) with the width being from the tip of one lateral spine to the other. After blotting the crab dry to remove excess water, the wet weight of each crab was measured to the nearest 0.01 g in a pre-tarred small beaker of fresh seawater. Molt shells were removed as soon as possible from tanks to minimize impacts on water quality.

3.4.5. *Dissection and collection of tissues*

The hemolymph was withdrawn first to minimize the impacts of stress on the DNA of the hemocytes. Immediately after the crab was taken out of the water, approximately 50 μ L of hemolymph was extracted directly into a syringe with a 23 gauge needle that had been coated with and contained approximately 50 μ L of crustacean anti-coagulant (1:1 hemolymph). The hemolymph was withdrawn from the crab's hypobranchial sinus through the arthroal membrane between the chelae and the first walking leg. Crustacean anti-coagulant contained 0.3 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 25 mM citric acid, and 3 mM EDTA at a pH of 6. The hemolymph/anti-coagulant solution was transferred into eppendorf tubes and placed on ice. The tubes were centrifuged at 800 x g for 10 minutes at 4°C to concentrate the hemocytes. The supernatant of each tube was

discarded and the pellet resuspended in 50 μ L anti-coagulant, which was kept on ice until processing for the Comet assay. Exposure of tubes to light was minimized.

The crabs were placed on ice for at least 5 minutes until torpid before dissecting. The subsamples of hepatopancrease tissue for pre-exposure (Time 0) crabs were flash frozen in liquid nitrogen then stored at -80°C . The hepatopancrease subsamples collected at the end of the experiment were placed into *RNAlater* (at least 10X volume). After allowing the *RNAlater* (QIAGEN, Venlo, Limburg) to permeate the tissues overnight, the *RNAlater* was removed and the cryovials stored at -80°C .

A subsample of hemolymph from one crab per treatment at the end of the experiment was exposed to hydrogen peroxide to be used as a positive control for the Comet assay. From these selected crabs, 10 μ L of the resuspended hemolymph was placed into Eppendorf tubes containing a solution of 50 μ M hydrogen peroxide in cold Hank's buffered saline solution (HBSS)-Hepes and left on ice for 30 min before analysis with the other samples. These tubes were centrifuged at 800 x g for 10 minutes at 4°C to concentrate the hemocytes. The supernatant of each tube was discarded and the pellet resuspended in 50 μ L anti-coagulant for hemolymph and kept on ice covered until processing for Comet assay. The positive controls were processed as quickly as possible to minimize potential impacts from residual hydrogen peroxide in the resuspension.

3.4.6. *Gene expression*

The hepatopancrease tissue samples from all Time 0 and treatment crabs were homogenized with 425-600 μ m acid-washed glass beads (Sigma-Aldrich, Saint Louis, MO) using a bead beater (Savant FastPrep[®] ; Bio101) and the mRNA extracted as using

an RNAqueous kit (Life Technologies, Grand Island, NY). The extracted mRNA for each crab was DNase treated using a TURBO DNase free kit (Life Technologies) to remove any genomic DNA contamination and then reverse transcribed (RT) to cDNA using a high capacity RNA-cDNA kit (Life Technologies) for absolute quantitative polymerase chain reaction (qPCR) analysis. For each sample, a no reverse transcription (No RT) control was also conducted in parallel to the RT reaction to examine genomic DNA contamination in the cDNA sample remaining after DNase treating.

The primers for MnSOD and CuMT were designed using NCBI Primer BLAST (basic local alignment search tool) from existing blue crab mRNA sequences in GenBank (Table 3.3). The primers for a putative blue crab GST (Table 3.3) were designed from an Expressed Sequence Tag (EST) hit observed by running a basic local alignment search with the mRNA sequence for delta-GST from the Chinese mitten crab, *Eriocheir sinensis*, against a non-public EST library (Place; IMET, Baltimore). Details for the characterization and validation of this gene are described in Appendix 2 and 3.

PCR products for each gene of interest were run on a 1.5% agarose gel, extracted using a QIAQuick gel extraction kit (QIAGEN, Valencia, CA), and ligated into a TOPO pcR-II plasmid. This plasmid was transformed into chemically competent E. Coli (TOP10). The plasmids were then purified from the E. coli using a ZR plasmid classic mini-prep kit (Zymo Research, Irvine, CA) and the plasmid DNA quantified on a NanoDrop. The purified products and plasmids were sequenced by the IMET Bioanalytical Services (BAS) laboratory and the orientation of the sense strand of product insert in relation to T7 promoter was verified. The plasmid DNA for each gene was then linearized using a restriction digest (*Hind iii*) and completion of digestion was verified by

Table 3.3 Real-time qPCR primer pairs for blue crab, *Callinectes sapidus*.

Gene Name	Abbreviation	Accession Number	Primer Sequence (5'-3')	Product Size (bp)
Glutathione <i>S</i> -Transferase	<i>GST</i>	KJ925004	GGACTTCAGCAAGCATACCAAC GGTGCTGAGGATTGGGAAAG	103
Mitochondrial Manganese Superoxide Dismutase	<i>MnSOD</i>	AF264029.1	CAGACCTCCCTACGACTATG GAAGCCAAAGAGAAAGGTGATGT	148
Cu-Metallothionein	<i>MT</i>	AF200420.1	ATGCAAAGTGTGGATCCGGCAAGTGCT TCCTGTTGCTGGGGCGGGACTGTA	146

gel electrophoresis. The linearized plasmids were then appropriately diluted (30-3,000,000 copies) and qPCR run to examine qPCR efficiency for all genes (qPCR efficiencies were 90-108%, with R^2 values >0.995).

To conduct absolute quantification, the plasmid inserted products were transcribed into RNA using a T7 transcription kit (Fermentas, Vilnius, Lithuania). The RNA concentrations for each gene were measured on a NanoDrop and six-point RNA serial dilutions were prepared to give a known copy number for the RT reaction. Each RNA dilution was reverse transcribed to cDNA (5,000-500,000 copies for CuMT, 500-50,000,000 copies for GST and MnSOD) with standard curve R^2 values >0.993 . Absolute quantitative-PCR was conducted using the POWER SYBRGreen kit on an Applied Biosystems 7500 Fast Real-Time PCR System. For each gene, all the crab cDNA samples were run on qPCR in triplicate and in conjunction with the six-point cDNA standards and no template controls, also in triplicate. The No RT crab cDNA samples were run in duplicate to examine genomic DNA contamination. A dissociation curve was conducted for all samples and standards. A linear regression equation was produced for the cDNA standards of each gene and used to determine the copy number of each crab sample. The copy numbers were then normalized to the total RNA concentration (ng) and reported as copy number ng⁻¹ total RNA.

3.4.7. *Comet assay*

Microscope slides were coated with 1% normal melting point agarose (NMPA) in phosphate buffered saline (PBS) and allowed to dry at 37°C in the dark. These frosted slides were stored in slide boxes within plastic bags containing desiccant. From the

hemolymph resuspensions or resuspended H₂O₂ positive controls, 10 µL was added to 100 µL of 0.6% low melting point agarose (LMPA, 37°C) in HBSS-Hepes pH 7.6 and layered over the NMPA layer on the frosted slides. Two replicate slides were made for each crab sample. Coverslips were placed onto slides and agarose allowed to polymerize for at least 5 min on metal tray over ice.

Following solidification, the coverslips were removed and slides placed into lysing solution (10% DMSO, 1% Triton X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris Base, 1% sodium sarconsinate; pH 10) for at least 1 hr, but less than 24 hr at 4°C in the dark. Once removed from the lysing solution, slides were rinsed with distilled water, placed on a horizontal gel electrophoresis tray, and covered with electrophoresis buffer (0.20 M NaOH, 1 mM EDTA; pH>12) for 10 min to allow DNA to unwind. Electrophoresis was then conducted at 25 V, 300 mA for 10 min. Slides were removed and placed in neutralization solution (0.4 M Tris, pH 7.5) for 5 min (repeated for three washes with a total of 15 min). Slides were then drained and placed in 100% ethanol for 5 min, allowed to dry in dark container overnight, and then placed in a desiccated slide box until processing. All solutions kept at 4° C prior to use.

For analyses slides were reconstituted with 2 µM mL⁻¹ ethidium bromide in HBSS-Hepes and examined using an epifluorescent microscope (Olympus BX50) with a green filter at 40x magnification (Q Imaging Retiga 1300 camera). The Komet 5.5 (Kinetic Imaging, Liverpool, UK) software's image analysis package was used to score the cell images. From each duplicate slide, 50 non-overlapping cells were randomly selected for quantification. Results expressed as mean ± standard error of the mean in terms of the percentage of DNA in the tail region (Tail % DNA).

3.4.8. *Statistical analysis*

All statistical analyses were conducted using R statistical software 2.15.2. The mean and standard error of the mean values for all treatment groups and the pre-exposure Time 0 group (when applicable) were compared for the following endpoints: day to first molt, tail percent DNA of hemocytes, and hepatopancrease gene expression levels (copy number per ng total RNA) for all genes of interest. Each endpoint was tested for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test) first. If one or both tests failed, a log transformation was conducted and the tests redone. If both tests passed with (or without) the transformation, an Analysis of Variance (ANOVA) was conducted. If a significant difference was found in the ANOVA test ($p < 0.05$), then a multiple pairwise comparisons was conducted using Tukey's HSD test. Any comparisons with a p value < 0.05 were considered a significant difference between treatments. If the homogeneity of variance test was passed, but not the normality test, then a non-parametric Wilcoxon Rank Sums test was performed to compare treatment means. The chemical concentrations in sediments and biological responses were correlated with parametric Pearson's analysis, ($p < 0.05$), in order to explore relationships between the variables.

3.5. **Results**

3.5.1. *Chemical analysis*

Initial sediment TPAH₅₀ concentrations ranged from 186 $\mu\text{g kg}^{-1}$ to 2,196,658 $\mu\text{g kg}^{-1}$ (Table 3.3). The lowest TPAH₅₀ was from the reference sediment A treatment, while

Table 3.4 Initial and final sediment concentrations of hydrocarbons for each treatment sediment, including individual/alkylation homologue group PAHs, TPAH₅₀, total extractable hydrocarbons (TEH), and summation of saturated hydrocarbons.

	Units	Initial Sediments						Final Sediments					
		A	B	C	D	E	F	A	B	C	D	E	F
Naphthalene	µg kg ⁻¹	5.1	50.7	0.78	5.18	10.4	10.2	10.5	49.9	1.53	11.3	7.07	0
C1-Naphthalenes	µg kg ⁻¹	9.34	65.2	4.37	9.21	22.2	84.9	15.3	58.5	1.69	14.6	7.74	0
C2-Naphthalenes	µg kg ⁻¹	12.3	111	309	17.1	43.6	7890	17.6	77.4	4.05	20.3	12.2	1510
C3-Naphthalenes	µg kg ⁻¹	8.51	339	2040	19.1	156	60400	10.6	112	39.4	18.1	16.3	26500
C4-Naphthalenes	µg kg ⁻¹	4.69	847	3250	44.2	192	92400	8.73	129	272	30.7	87.5	56900
Biphenyl	µg kg ⁻¹	1.69	23.2	2.69	3.03	10.5	65.1	2.49	15.8	0.915	3.67	2.77	49.1
Dibenzofuran	µg kg ⁻¹	1.51	23.8	63.8	2.76	5.13	2200	3.77	19.9	0.529	4.99	2.26	0
Acenaphthylene	µg kg ⁻¹	1.69	32.2	0	4.71	24.7	0	2.58	16.4	1.3	22.7	10.7	0
Acenaphthene	µg kg ⁻¹	0	12.4	4.81	1.88	5.42	0	1.34	8.34	0	3.27	2.01	190
Fluorene	µg kg ⁻¹	0	37.6	186	2.94	0	3690	1.22	19.6	4.02	5.22	4.08	1570
C1 - Fluorenes	µg kg ⁻¹	0	301	1680	0	0	41600	1.44	40.7	0	0	0	19500
C2 - Fluorenes	µg kg ⁻¹	0	1340	4720	0	0	114000	2.23	165	374	0	0	76300
C3 - Fluorenes	µg kg ⁻¹	0	1990	5520	0	507	138000	29.5	342	924	112	236	112000
Anthracene	µg kg ⁻¹	0.54	60.1	48.7	6.04	23.9	1900	2.18	34.7	0	21.1	15.1	3530
Phenanthrene	µg kg ⁻¹	6.67	210	1190	22.8	56.6	18900	12.3	81.9	12.1	26.6	12.7	12100
C1-Phenanthrenes/ Anthracenes	µg kg ⁻¹	7.61	1220	6120	41.2	209	157000	12.2	248	333	50.9	79.7	86000
C2-Phenanthrenes/ Anthracenes	µg kg ⁻¹	6.53	3240	8710	145	1050	280000	11	539	1260	120	247	525000
C3-Phenanthrenes/ Anthracenes	µg kg ⁻¹	0	2890	5860	362	2590	202000	7.04	684	1790	344	833	451000
C4-Phenanthrenes/ Anthracenes	µg kg ⁻¹	0	2520	4120	576	3040	148000	3.74	791	1730	546	1200	335000
Dibenzothiophene	µg kg ⁻¹	0	24.1	160	2.42	0	4410	0.65	10.2	3.76	3.15	0	4860
C1 - Dibenzothiophenes	µg kg ⁻¹	0	278	1060	4.01	0	41900	1.41	44.6	69.7	0	0	59800
C2 - Dibenzothiophenes	µg kg ⁻¹	0	904	2320	35.7	395	103000	1.72	173	385	35.6	111	200000
C3 - Dibenzothiophenes	µg kg ⁻¹	0	1430	1990	204	990	95600	1.32	309	629	98.7	320	199000
C4 - Dibenzothiophenes	µg kg ⁻¹	0	699	1250	132	1070	54600	0	275	558	129	388	126000
Benzo(b)fluorene	µg kg ⁻¹	1.46	176	260	11.9	56.6	8080	2.98	81.2	30.1	26.8	0	16300
Fluoranthene	µg kg ⁻¹	13.2	246	65.2	45.9	76.4	2310	17.7	163	17.3	47.9	23.7	2050
Pyrene	µg kg ⁻¹	14.2	394	272	83.6	254	8240	18.7	188	42.7	65.5	70.9	13100
C1 - Fluoranthenes/Pyrenes	µg kg ⁻¹	10.4	956	1190	172	895	37800	9.37	337	399	185	391	87900
C2 - Fluoranthenes/Pyrenes	µg kg ⁻¹	0	1490	1640	291	1490	58700	7.28	468	781	289	758	148000
C3 - Fluoranthenes/Pyrenes	µg kg ⁻¹	0	1690	1820	396	1940	66800	5.16	517	932	352	947	173000
C4-Fluoranthenes/Pyrenes	µg kg ⁻¹	0	1470	1640	469	2340	53500	0	364	696	261	761	108000
Naphthobenzothiophene	µg kg ⁻¹	0	170	328	17	115	10100	2.04	56.7	80.2	25.4	27.3	11100
C1-Naphthobenzothiophenes	µg kg ⁻¹	0	761	1300	115	1020	39900	2.36	275	557	110	348	49300
C2-Naphthobenzothiophenes	µg kg ⁻¹	0	1060	1510	240	1860	51100	0	505	984	253	859	65100
C3-Naphthobenzothiophenes	µg kg ⁻¹	0	913	1140	252	2280	37200	0	464	797	328	1100	49400
C4-Naphthobenzothiophenes	µg kg ⁻¹	0	554	645	237	1980	23600	0	288	448	267	865	23900
Benz(a)anthracene	µg kg ⁻¹	8.04	186	50	19.4	75.8	1550	10.1	139	31.4	65.6	32.3	2480
Chrysene+Triphenylene	µg kg ⁻¹	11.6	690	1020	324	1600	27800	14.7	380	485	505	956	37800
C1 - Chrysenes	µg kg ⁻¹	4.83	1330	2140	492	2840	62500	11.1	606	1120	594	1550	72700
C2 - Chrysenes	µg kg ⁻¹	0	1810	2360	581	3300	62200	4.64	785	1460	700	1840	78500
C3 - Chrysenes	µg kg ⁻¹	0	1390	1460	444	2790	38900	1.46	562	901	537	1460	45100
C4 - Chrysenes	µg kg ⁻¹	0	922	1130	399	2250	27200	34.2	445	672	485	1160	32800
Benzo(b)fluoranthene	µg kg ⁻¹	13.2	267	101	62.8	275	2940	17.1	190	85.2	175	120	5110
Benzo(j+k)fluoranthene	µg kg ⁻¹	5.81	70.8	0	12.8	47	0	7.73	47.9	0	50.4	0	0
Benzo(a)fluoranthene	µg kg ⁻¹	2.73	56	0	8.5	0	0	3.84	33.8	0	26.9	0	0
Benzo(e)pyrene	µg kg ⁻¹	8.21	279	234	146	662	6460	11.5	159	144	214	355	8060
Benzo(a)pyrene	µg kg ⁻¹	8.5	209	21.2	34.7	121	617	13.8	129	12.4	96	41.6	879
Indeno(1,2,3-cd)pyrene	µg kg ⁻¹	8.75	108	0	28.3	106	0	10.2	83.8	0	94.2	41.2	0
Dibenz(a,h)anthracene	µg kg ⁻¹	0	52	23.6	18.6	64.8	628	2.32	35.4	23.7	34.3	44.5	1170
Benzo(g,h,i)perylene	µg kg ⁻¹	8.7	143	29.7	45.5	163	883	11.7	91.9	24.5	102	93.4	947
TPAH ₅₀	µg kg ⁻¹	186	36041	70990	6588	39003	2196658	381	11640	19117	7512	17440	3329505
Perylene	µg kg ⁻¹	7.1	249	0	101	410	1900	9.7	200	8.0	139	170	1860
TEH	mg kg ⁻¹	0	3430	7080	1230	6720	375000	10.8	1100	1960	1710	370	316000
Saturated Hydrocarbons	mg kg ⁻¹	1.3	49.2	299	14.1	58.8	34611	1.8	19.4	18.2	14.5	41.8	9444

sediment F treatment had the highest TPAH₅₀ concentrations. The sediment F treatment had a visible oil sheen on the surface of exposure water and a distinctly petroleum smell to the exposure sediment/solution. Sediment TPAH₅₀ concentrations did decrease or stay relatively consistent after the 31 days of exposure, with the exception of sediment F. The substantial increase in TPAH₅₀ from sediment F could be a result of variations in sampling as there were larger vegetative particles within that sediment such as leaves, twigs, and other organic matter. The main differences between the initial and final sediment F samples are in the alkylation groups for phenanthrenes/anthracenes, dibenzothiophenes, and fluoranthenes/pyrenes. The summation of saturated hydrocarbons and total extractable hydrocarbons (TEH) in initial sediments followed the same concentration patterns as TPAH₅₀ (Table 3.4). TPAH₅₀ concentrations in the water of exposure (data not shown) were less than 1 µg L⁻¹ for all sediment treatments, except for treatment C (3.01 µg L⁻¹) and treatment F (65.30 µg L⁻¹). By the end of the 31 days, the water TPAH₅₀ concentrations had decreased for all treatments with a 57.8% and 92.5% decrease for treatments C and F, respectively.

Perylene, a PAH of primarily diagenic origins formed from bacterial degradation of biogenic precursor like plant terpenes (US EPA, 2003) and to a lesser extent from combustion/processing of fossil fuels, was not included in the total PAH concentration. Perylene is found in the MC252 source oil, but only accounts for a small fraction (<1%) of the whole oil. Perylene was reported separately due to its elevated levels within sediments of the Mississippi River Delta and Gulf of Mexico (Wade et al., 2008; NCCOS, 2010) compared to the low concentrations within MC252 oil. However, perylene would have only been 0-3.7% of the TPAH, if included for these sediments.

Table 3.5 PAH ratios within initial sediments to distinguish between petrogenic and pyrogenic source inputs (bold indicates petrogenic source).

PAH Ratios	Sediment Treatments							G
	A	B	C	D	E	F		
Alkylated Homologs/ Parent	0.54	9.3	16.49	6.25	9.42	20.81		0.79
FLAPYR/(FLA/PYR+[C24PHE])	0.81	0.07	0.02	0.11	0.05	0.02		0.56
FLA/(FLA + PYR)	0.48	0.38	0.19	0.35	0.23	0.22		0.49
ANT/(ANT + PHE)	0.07	0.22	0.04	0.21	0.3	0.09		0.23

*PHE (phenanthrene), AN (anthracene), FLA (fluoranthene), PY (pyrene), FLAPY (sum of fluoranthene and pyrene), C24PHE (sum of C₂- to C₄-phenathrenes)

Table 3.6 Individual metal concentrations (mg kg⁻¹ dry weight) and the summation of biological relevant metals in sediments for each treatment.

Metals	Sediment Treatments					
	A	B	C	D	E	F
Ag	0.09	0.07	0.02	0.25	0.30	0.01
As	4.1	7.0	1.1	20.5	27.0	1.0
Cd	0.37	0.24	0.03	1.53	2.08	0.04
Cr	11.5	7.6	4.1	19.8	21.7	2.0
Cu	9.9	5.3	1.1	40.3	51.8	1.8
Fe	12000	9780	4490	22292	25525	1360
Hg	0.03	0.04	BDL ^a	0.12	0.16	0.002
Ni	15.8	17.1	9.8	31.0	35.1	3.1
Pb	10.4	9.5	3.3	25.7	30.4	1.0
V	14.2	9.8	5.5	38.8	45.0	3.1
Zn	50.6	37.3	18.2	90.4	99.9	4.9
Sum	12117	9874	4533	22560	25838	1377

^aBDL=below detection limit of 0.00745 mg kg⁻¹ dry weight

PAH ratios (Table 3.5) indicate that sediments B, C, E and F contain PAHs that are primarily from petrogenic sources, which was also confirmed by the MC252 oil fingerprint. However, sediment D, as it is a mix of sediments E and G, appears to be more of a mixture between pyrogenic and petrogenic sources. Reference, in relation to the DWH incident, sediments A and G have PAHs that appear to be mostly from pyrogenic sources.

Individual metal concentrations varied by at least an order of magnitude between sediments treatments (Table 3.6). Sediments D and E had the highest individual and sum metal concentrations for all treatments with sum of metal concentrations of 22,560 and 25,838 mg kg⁻¹ dry weight, respectively. Sediment F had the lowest individual and sum metal concentrations with a sum metal concentration of only 1,377 mg kg⁻¹ dry weight.

3.5.2. *Impacts on mortality, growth and molting*

There were no treatment mortalities in any of the sediment exposure treatments, but there were five non-treatment mortalities, mainly due to cannibalism. Of the remaining 22-24 crabs per treatment used for molting and growth endpoints, there were no significant differences in carapace size (width or length) of crabs between exposures from the start to the end of experiment (Table 3.7). Crabs from treatment A had a significantly lower percent change in weight from the start to end of the experiment than crabs from treatments with sediments C, E and F ($p < 0.05$). Crabs from treatment A had the lowest overall percentage changes in growth endpoints, while crabs from treatment F tended to have the highest. The change in carapace width per molt (percent change in width

Table 3.7 Growth and molting responses of blue crabs exposed to each sediment treatment (mean \pm SEM)

Treatment	Days Till First Molt	# Crabs Molting 2x	Overall Percent Change in Crab				Change in Width Per Molt (%)	Intermolt Period (d)
			Carapace Width (%)	Carapace Length (%)	Weight ^a (%)			
A	8.6 \pm 1.2	1	24.2 \pm 1.8	25.4 \pm 1.8	97.0 \pm 6.0 a	24.3 \pm 1.6	20 ^b	
B	7.8 \pm 1.0	6	29.2 \pm 2.5	29.1 \pm 2.3	121.0 \pm 10.2 a,b	23.8 \pm 1.3	20.1 \pm 1.1	
C	11.6 \pm 1.4	7	27.6 \pm 2.5	31.6 \pm 2.8	122.1 \pm 8.2 b	22.9 \pm 1.2	24.4 \pm 0.5	
D	8.8 \pm 1.1	4	26.7 \pm 1.9	27.8 \pm 1.9	112.3 \pm 8.7 a,b	24.0 \pm 1.2	23.0 \pm 2.1	
E	8.7 \pm 0.9	4	25.4 \pm 2.1	27.6 \pm 1.9	118.1 \pm 8.1 b	24.8 \pm 1.1	20.8 \pm 1.7	
F	10.5 \pm 1.1	3	30.6 \pm 2.6	30.3 \pm 2.2	126.7 \pm 10.6 b	27.0 \pm 1.6	16.0 \pm 4.4	

^aDifferent letters signify significant differences (p < 0.05)

^bOnly one crab molted a second time in treatment A

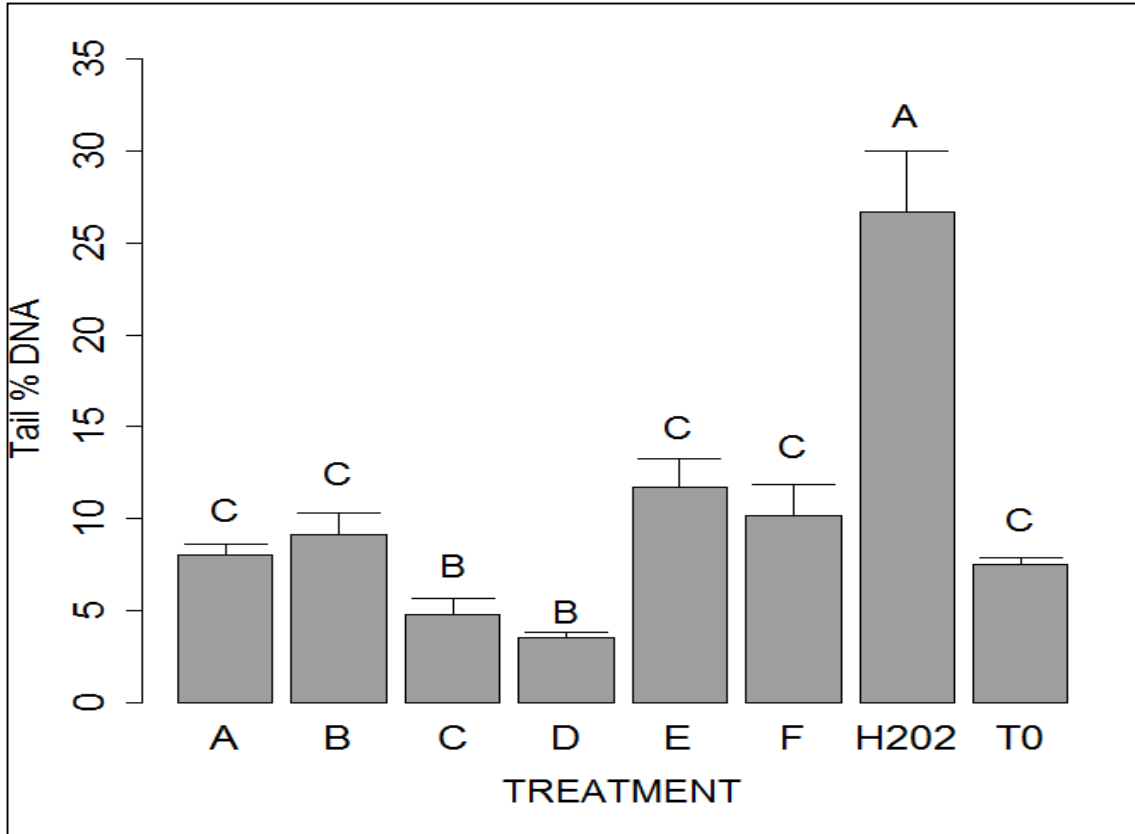


Fig. 3.2 DNA damage in juvenile blue crab hemocytes as Tail % DNA in Comet assay for each sediment treatment, crabs collected prior to exposure (T0), and the *in vitro* H₂O₂ positive control (mean+SEM, N=22-24). Different letters signify significant differences (p<0.05).

between start of experiment and after first molt) is similar between all treatments, with crabs from sediment F having the highest percent change ($27.0 \pm 1.6\%$).

Additionally, there were no significant differences in the average number of days it took for a crab to molt for the first time (days till first molt), but crabs exposed to sediments C and F took the longest. All surviving crabs molted at least once during the 31 day period, but only 17% molted two times with the largest number of second molts occurring in treatments B and C (6 and 7 crabs, respectively). The intermolt period was highest for crabs exposed to sediments B and C and lowest for crabs exposed to sediment F, but the results are limited due to the small sample size of crabs that molted a second time during the experiment.

3.5.3. *Genotoxicity in hemocytes*

The percent of tail DNA (as tail % DNA) in hemocytes for all treatments, pre-exposure (Time 0), and the *in vitro* H₂O₂ positive controls is depicted in Figure 3.2. The hemocytes of all sediment exposed crabs had significantly lower tail percent DNA than those in the H₂O₂ positive controls ($p < 0.01$). Crabs exposed to sediments C and D had significantly lower hemocyte tail percent DNA than those in any of other sediment treatments and the pre-exposure (Time 0) crabs ($p < 0.05$). Only crabs exposed to sediments C and D had hemocyte DNA damage significantly different from the pre-exposure (Time 0) crabs.

3.5.4. *Changes in hepatopancrease gene expression*

Gene expression levels for GST, MnSOD, and CuMT in hepatopancrease tissues for all treatment and the pre-exposure (Time 0) crabs are depicted in Figure 3.3. There were no significant differences in GST gene expression between any of the treatments or pre-exposed crab hepatopancrease tissues. Crab hepatopancrease tissue from treatments C and D had significantly higher MnSOD gene expression than crabs from treatment F (p value<0.05). MnSOD gene expression was not significantly different between crabs exposed to any of other treatment or reference sediments or to pre-exposure (Time 0) crabs. CuMT gene expression in hepatopancrease tissues of crabs from all sediment treatments was significantly higher than pre-exposure (Time 0) crabs (p value<0.05). However, there were no significant differences in CuMT gene between any of the crabs exposed to treatment sediments.

3.5.5. *Correlation analysis*

An exploratory correlation analysis was conducted in order to examine relationships between the chemical constituents and biological endpoints (Table 3.8). In this exploratory analysis, TPAH₅₀ concentrations (logarithmic transformed) in initial sediment and water samples were only correlated with changes in growth (p<0.05). Positive correlations were observed between TPAH₅₀ in sediments and overall changes in carapace size (width and length) and crab weight (p<0.05), while water TPAH₅₀ had a positive correlation with changes in carapace width (p<0.05). There were no correlations between any biological endpoints and TOC or total solids (data not shown). There were positive correlations between all individual and summation of metals and GST gene

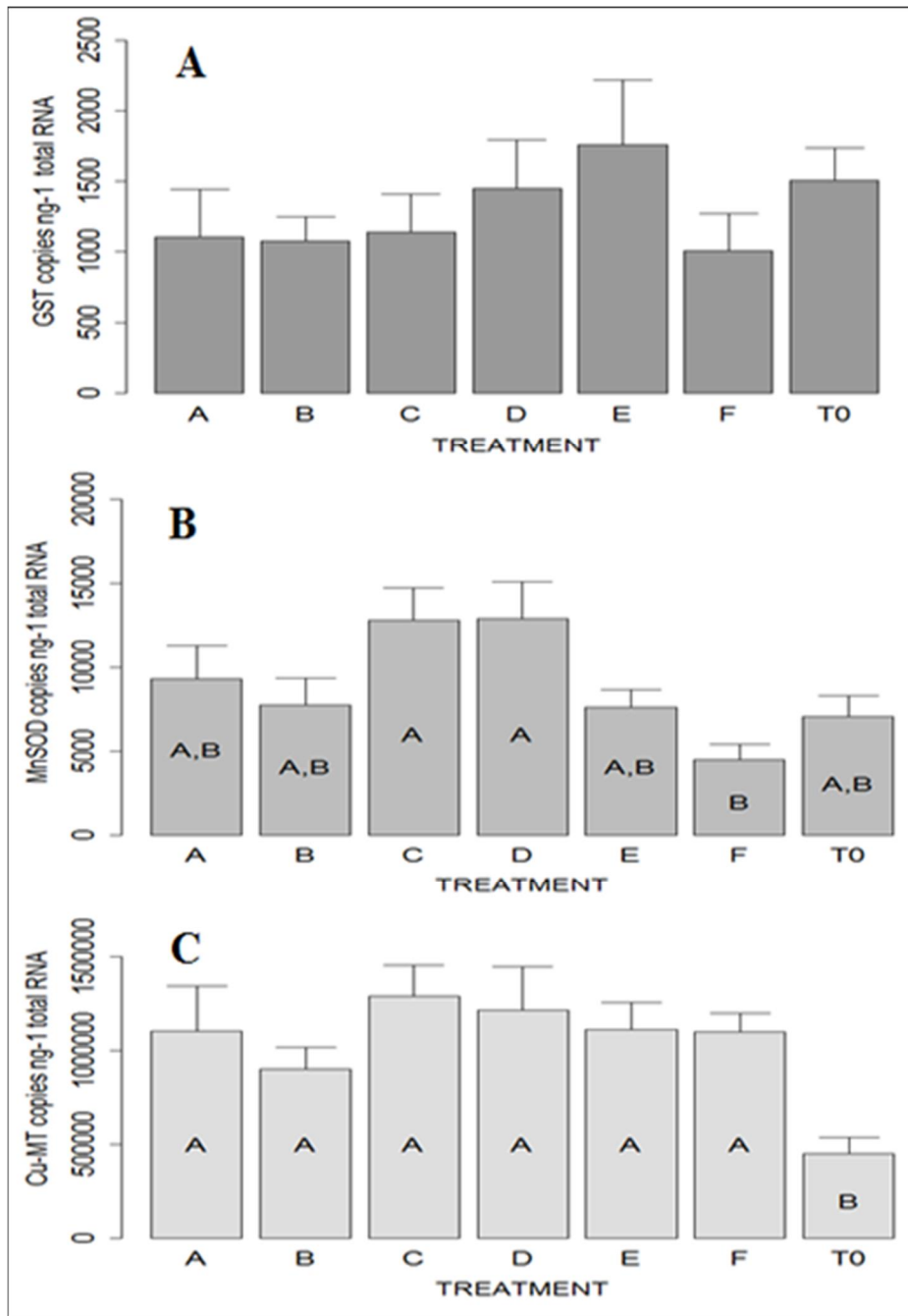


Fig. 3.3 Gene expression levels in hepatopancrease of juvenile blue crabs exposed to the different sediment treatments and crabs (T0) collected prior to exposure (mean+SEM, $N=22-24$): (A) glutathione *S*-transferase (GST), (B) mitochondrial manganese superoxide dismutase (MnSOD), and (C) Cu-metallothionein (CuMT). Different letters signify significant differences ($p < 0.05$).

Table 3.8. Pearson correlations (bolded: *p<0.05, **p<0.01) among chemical compounds (total polycyclic aromatic hydrocarbons [TPAH₅₀] in sediment and water, individual metals in sediments, and sum of individual metals) and biological endpoints: gene expression levels (GST, MnSOD, and MT), tail % DNA (DNA), day to first molt (day to molt), growth endpoints (overall percent changes in carapace width/length and crab weight).

	GST	MT	DNA	Day to Molt	Width	Length	Weight	Ag	As	Cd	Cr	Cu	Fe	Hg	Ni	Pb	V	Zn	Sed. TPAH	Water TPAH	Sum Metals
SOD	0.19	0.73	-0.89 *	0.17	-0.43	0.00	-0.32	0.15	0.22	0.15	0.29	0.17	0.27	0.14	0.31	0.24	0.24	0.30	-0.48	-0.46	0.27
GST	-	0.31	0.13	-0.28	-0.53	-0.32	-0.07	0.96 **	0.94 **	0.97 **	0.90 *	0.97 **	0.91 **	0.95 **	0.91 *	0.94 **	0.95 *	0.90 *	-0.13	-0.51	0.91 *
MT	-	-	-0.61	0.61	-0.45	0.14	-0.17	0.11	0.17	0.20	0.19	0.21	0.15	0.11	0.14	0.15	0.22	0.18	-0.18	-0.12	0.15
DNA	-	-	-	-0.29	0.11	-0.15	0.20	0.12	0.04	0.04	-0.01	0.08	-0.01	0.13	-0.01	0.04	-0.02	-0.01	0.30	0.14	0.01
Day to Molt	-	-	-	-	0.32	0.74	0.44	-0.49	-0.52	-0.43	-0.57	-0.42	-0.59	-0.52	-0.58	-0.54	-0.46	-0.58	0.51	0.67	-0.59
Width	-	-	-	-	-	0.76	0.82 *	-0.46	-0.58	-0.53	-0.68	-0.53	-0.65	-0.51	-0.60	-0.58	-0.58	-0.68	0.84 *	0.85 *	-0.65
Length	-	-	-	-	-	-	0.87 *	-0.41	-0.53	-0.45	-0.64	-0.45	-0.60	-0.48	-0.53	-0.53	-0.50	-0.62	0.81 *	0.76	-0.60
Weight	-	-	-	-	-	-	-	-0.10	-0.26	-0.16	-0.41	-0.17	-0.36	-0.17	-0.29	-0.26	-0.24	-0.39	0.96 **	0.75	-0.36
Ag	-	-	-	-	-	-	-	-	0.98 **	0.99 **	0.95 **	0.99 **	0.96 **	1.00 **	0.96 **	0.98 **	0.98 **	0.95 **	-0.17	-0.55	0.96 **
As	-	-	-	-	-	-	-	-	-	0.99 **	0.99 **	0.99 **	0.99 **	0.99 **	0.98 **	1.00 **	1.00 **	-0.32	-0.64	0.99 **	
Cd	-	-	-	-	-	-	-	-	-	-	0.96 **	1.00 **	0.96 **	0.99 **	0.95 **	0.98 **	0.99 **	-0.20	-0.55	0.96 **	
Cr	-	-	-	-	-	-	-	-	-	-	-	0.96 **	1.00 **	0.97 **	0.98 **	0.99 **	1.00 **	-0.47	-0.75	1.00 **	
Cu	-	-	-	-	-	-	-	-	-	-	-	-	0.96 **	0.99 **	0.95 **	0.98 **	0.99 **	-0.21	-0.54	0.96 **	
Fe	-	-	-	-	-	-	-	-	-	-	-	-	-	0.97 **	0.99 **	0.99 **	0.98 **	-0.43	-0.75	1.00 **	
Hg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.97 **	0.99 **	0.99 **	-0.23	-0.59	0.97 **	
Ni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.99 **	0.97 **	0.99 **	-0.40	-0.74	0.99 **
Pb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.99 **	0.99 **	-0.33	-0.67	0.99 **
V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.99 **	0.98 **	-0.29	-0.61	0.98 **
Ni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.47	-0.76	1.00 **
Sed. TPAH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.87 *
Water TPAH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.87 *
TPAH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.75

expression ($p < 0.05$). Mitochondrial MnSOD gene expression had a negative correlation with DNA damage as percent tail DNA ($p < 0.05$).

3.6. Discussion

Sediments collected in marshland and beaches of Pass a Loutre State WMA contained varying levels of PAHs, with the shoreline areas heavily contaminated with PAHs following the Deepwater Horizon Incident due to their proximity to the MC252 wellhead. Sediments B, C, and F were collected in areas that on the Shoreline Clean-up Assessment Team's (SCAT) ground oil survey from July 12th, 2010 had moderate-heavy ground oil contamination, while sediments A and G were from areas with very light to no observed ground oil contamination (NOAA, 2010). Sediment E was collected in the same location as Sediment F a little over a year later to see how much contamination remained in the sediments and whether it elicited similar impacts to the freshly contaminated sediments from the previous year. The PAH ratios in Table 3.5 were confirmed by the MC252 oil fingerprinting as the three sediments testing positive for MC252 oil (B, C, and F) contained PAHs that appeared to be primarily from petrogenic sources. Furthermore, reference sediments A and G, which did not have recognizable MC252 oil signatures with fingerprinting, appeared to also not have other petrogenic source inputs of PAHs as the ratios indicated they were primarily from pyrogenic sources. Additionally, the changes over time of oil contamination into this region can be observed when comparing sediments E and F. The overall TPAH₅₀ concentrations in this location are greatly reduced after one year and the diagnostic ratios indicate a mixed source input for PAHs between petrogenic and pyrogenic.

Several investigators have developed sediment quality guidelines to aid in assessing potential adverse biological effects from sediment contaminants (Long et al., 1995; MacDonald et al., 1996). The guidelines proposed by Long et al. (1995) sets an effects range low (ERL) and effects range median (ERM) value for each contaminant. The ERL and ERM values are based on field, laboratory, and modeling studies that connect concentrations of contaminants in sediment with adverse biological effects and are defined as the 10th and 50th percentile, respectively, from an ordered list of concentration of substances in sediments that are linked to a biological effect. Contaminant concentrations below the ERL are unlikely to cause a biological effect, while concentrations above the ERM are likely to cause adverse biological effects. While these values are not hard-fast rules for toxicity, they serve as general gauge for concentrations at which exposure to individual and/or mixtures of contaminants in sediments are likely to cause adverse effects.

PAHs are considered a major toxic component of petroleum exposure; and therefore, were the contaminants of focus for this study. The total PAH concentrations (only accounting for the 13 PAHs included in the Long et al., 1995 database) for sediments F were above the ERM of 44,792 $\mu\text{g kg}^{-1}$ at 65,701 $\mu\text{g kg}^{-1}$, while the remaining sediments were below the ERL of 4,022 $\mu\text{g kg}^{-1}$. Individual concentrations of PAHs in Sediment F were mostly above the ERL and ERM as well. Most of the other sediments had individual PAHs below ERL values, except sediments B and C had fluorene concentrations above the ERL value of 19 $\mu\text{g kg}^{-1}$. Given that the total PAH and many individual PAH concentrations for sediment F exceeded the ERM value, it seemed likely to see adverse effects on the juvenile blue crabs exposed to sediment F.

The growth and molting of the juvenile blue crabs, however, did not appear to be negatively impacted by PAH concentrations during the 31 day exposure, even in the heavily contaminated sediment F exposures. In fact, the overall percent changes in growth (carapace size and crab weight) were actually greatest in the treatments with higher PAH sediment concentrations (Table 3.7). However, the only significant difference in growth was that the overall change in crab weight for crabs exposed to the lowest TPAH₅₀ concentration sediment was significantly lower than the crabs exposed to the three highest TPAH₅₀ concentration sediments ($p < 0.05$). Even though there were very few significant differences in growth between treatments, these overall changes in growth results are consistent with other studies from the St. Johns River in Florida examining growth in juvenile blue crabs of a similar size (Tagatz, 1968b). While the direct impacts of aromatic hydrocarbon exposures on growth of juvenile blue crabs has not been well studied, the few studies that have been conducted show decreases in crab growth with aromatic hydrocarbon exposure. For example, a spiked water exposure study by Cantelmon et al. (1982) showed a significant decrease in carapace width and weight in juvenile crabs (1 to 4 cm) exposed to 1ppm benzene and a decreasing trend in growth endpoints in crabs exposed to 0.01ppm dimethylnaphthalene (DMN).

While fewer than 17% of the all crabs molted a second time, there did not appear to be substantial differences in intermolt period between treatments, with crabs exposed to the highest PAH concentrations having the lowest intermolt period (Table 3.7). Prior studies with juvenile blue crabs exposed to aromatic hydrocarbons displayed significantly longer intermolt periods (Cantelmo et al., 1981; 1928). Juvenile blue crabs from 1 to 4 cm in size exposed via spiked water to 1ppm benzene and 0.01 ppm DMN had

significantly longer intermolt periods than control crabs (Cantelmo et al., 1981, 1982). All sediments in this study had combined DMN (C2-naphthalene) concentrations above 0.01 ppm (Table 3.4), while sediments B, C, and F all had individual 2,6-DMN concentrations above that value at 0.036 ppm, 0.099 ppm, and 2.32 ppm, respectively. However, various factors such as availability for uptake and the route of exposure are different between the studies, which could alter the accumulation and eventual impact of those compounds. Other studies have shown the assimilation and depuration of radioactively labeled PAHs in blue crab tissues, particularly accumulating in the hepatopancrease, from water and food exposures (Lee et al., 1976; Hale, 1988) as well as hydrocarbon accumulation in hepatopancrease from exposure to No #2 fuel oil WAF (Melzian and Lake, 1987). However, little is known about the differences in accumulation within blue crabs between water-borne and sediment PAH exposures.

The intermolt periods in this study do seem to be consistent with other studies examining growth of juvenile blue crabs (Tagatz, 1968b; Millikin and Williams, 1984). One laboratory study using a similar temperature and salinity regime showed juvenile blue crabs in the size range used for this study to have average intermolt periods of 22.9-27.7 days (Unpublished results in Millikin and Williams, 1984). Another summer salt-water mesocosm study conducted in the St. Johns River in Florida, showed slightly lower average molt intervals ranging from 10-18 days for crabs of the same size range used in this study (Tagatz, 1968b).

Other than the increase in overall percent change in growth endpoints, there were no observed significant correlations between PAH concentrations and biological responses. In the limited studies that have examined direct impacts of aromatic hydrocarbons and/or

field collected PAH contaminated sediments on crabs, some have shown correlations between PAHs and biological biomarkers, while others have not. A study by Lee (1988) showed increases in hepatopancrease GST activity in field collected shore crab, *Carcinus maenas*, with increases in tissue PAH concentrations. However, studies examining 28 day exposures of *C. maenas* to contaminated sediments collected from Spanish ports in the laboratory or in field cages did not show any correlations between PAH concentrations and various biomarkers of oxidative stress and metabolism of xenobiotic compounds in hepatopancrease, including glutathione *S*-transferase (GST), glutathione reductase (GR), ethoxyresorufin O-deethylase (EROD), and glutathione peroxidase (GPx) activities (Martín-Díaz et al., 2007, 2008). Another 28 day lab exposure of *C. maenas* to contaminated sediments collected from different areas around the Spanish coast did show significant correlations between PAH concentrations and EROD activity, but not GST activity (Morales-Caselles et al., 2008).

While sediments were chosen based on their exposure (or lack of) to DWH oil contamination and PAH content, it is important to remember that these are complex mixtures containing numerous other chemical components that can influence biological responses, particularly heavy metals. Of the nine metals examined in Long et al. (1995), most were below ERL values in exposure sediments, except for sediments D and E. Sediments D and E had metal levels above the ERL for As, Cd (D only), Cu, Hg (E only), and Ni. Overall, metal concentrations in sediments examined from Pass a Loutre are similar to average levels observed for inshore surface sediments of the Northern Gulf of Mexico as summarized in Wade et al. (2008).

Although the metal concentrations were mostly below levels that biological effects are often seen, GST gene expression levels were highest in sediments containing higher metal concentrations. While results examining changes in GST gene expression in crustaceans exposed to xenobiotic compounds is limited, one study in the marine copepod *Tigriopus japonicas*, showed increases in GST gene expression with exposure to trace metals: Ag^{+1} , As^{+3} , Cd^{+2} , and Cu^{+2} (Lee et al., 2008). The most substantial changes in GST gene expression occurred with the sigma isoform of GST, but they did see significant increases in the delta isoform of GST with $2 \text{ mg L}^{-1} \text{ Cu}^{+2}$ and Ag^{+1} ($p < 0.05$). A larger body of research has shown positive relationships between GST activity in marine invertebrates and trace metals in both water-borne and sediment exposures (Canesi et al., 1999; Martín-Díaz et al., 2007, 2008).

Interestingly, CuMT gene expression levels did not show a significant correlation with any metal concentrations in the sediments. However, all crabs exposed to sediments had significantly higher hepatopancrease CuMT gene expression levels than crabs collected just prior to exposure ($p < 0.05$, Fig. 3.3C). Therefore, exposure to higher levels of metals (and other components) in the sediments could be eliciting a threshold response for CuMT gene expression in the more contaminated treatments masking any significant correlations. Changes in metallothionein gene expression levels in response to contaminant heavy metals and xenobiotic compounds in sediments have not been well documented in crabs. A few studies have shown elevated levels of MT or increases in MT activity in crabs with increasing metal contamination in sediments (Pedersen et al., 1997; Martín-Díaz et al., 2007). Pedersen et al. (1997) saw elevated CuMT concentrations in the mid guts of *C. maenas* exposed to metal-contaminated sediments,

while Martín-Díaz et al. (2007) showed a positive correlation between hepatopancrease MT activity of *C. maenas* and metal (As and Hg) concentrations in sediments.

3.7. Conclusion

Overall, juvenile blue crabs do not appear to be highly sensitive to the level of PAH concentrations within these field-collected sediments. While the growth and biological biomarkers of juvenile blue crabs examined during this 31 day exposure were not significantly impacted by the various concentrations of sediment PAH exposure, that does not mean that there would not be long-term or delayed effects if exposure continued for a longer period of time as it could in the environment. The growth and molting results could be different if the exposure was extended longer to account for multiple molts in more than 17% of the total crabs. Additionally, extended exposures could be used to examine more population level impacts such as size at and time to reach maturity or impacts on reproductive success. Furthermore, this study only addresses PAHs in sediments as the primary route of exposure. Crabs living in a region of contaminated sediments would also be consuming food potentially contaminated by PAHs adding an additional route of exposure that could alter the impacts of contamination.

While juvenile blue crabs do not appear highly sensitive to PAH concentrations from oil contamination, younger life stages (e.g. zoeal stage) of blue crabs could be more susceptible to impacts from an oil spill/leak. In most species the most sensitive stages of organisms to pollution impacts are the younger life stages, such as larvae (Rand and Petrocelli, 1985). Blue crab zoea (larval stage) migrate from the spawning grounds at the mouths of estuaries to the open waters of the continental shelves where they live a

planktonic existence on the surface, particularly the upper 3m including the neuston (Epifanio, 1995; Johnson and Perry, 1999). Therefore, blue crab zoea have a potential to be impacted by oil spills and leaks as water-borne rather than sediment exposures, especially those like the DWH incident that co-occur with crab spawning.

3.8. Acknowledgments

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Chapter 4. Understanding the Sublethal Impacts of Oil, Chemical Dispersants, and Chemically Dispersed Oil Exposures on Blue Crab Larvae³

4.1. Abstract

The blue crab, *Callinectes sapidus*, is an important species to the ecology and economy of the Atlantic coast and Gulf of Mexico. The distribution of blue crab zoea on the surface of open, coastal waters can put them at risk of exposure to oil and chemically dispersed oil in an oil spill event during periods of crab spawning. Therefore, the CRRC study sought to examine the impacts of sublethal exposures to oil, chemical dispersant, and chemically dispersed oil on blue crab larvae over a 6-day exposure period. Biological endpoints from the larger CRRC study covered in this chapter include survival and biomarker endpoints for oxidative stress/detoxification pathways and DNA damage. All exposure solutions were characterized for the concentration of 50 individual polycyclic aromatic hydrocarbons (PAHs) and their summation (TPAH). Furthermore, the amount and size of oil droplets/particles were measured to define exposure concentrations of oil (and components) and to aid in assessment and correlations of their impacts on crab larval behavior. Blue crab larvae were exposed to low concentrations of TPAH that

3

This chapter includes my involvement in a larger study for which a final report has been submitted to the Coastal Response Research Center (CRRC), University of New Hampshire:

Eric J. Schott, J. Sook Chung, Carys L. Mitchelmore, Andrew Heyes (2014). Understanding chronic impacts of chemical dispersant and chemically-dispersed oil on behavior, molting success, and hormone status and of blue crab larvae: inputs for recruitment and population models. Final Report to CRRC.

1.) My involvement in the study was the prepare all of the exposure solutions, assist in the set-up and maintenance of the exposures, aided in water quality monitoring, collection of exposure solution samples for chemical analysis, assisting Dr. Mitchelmore in examining oil particles counts, conducting the Comet assay (along with FRA Maureen Strauss), and conducting the GST and MnSOD gene expression analysis.
2.) While not directly conducted by myself, the survival results (by Dr. Eric Schott) reported in this experiment and the PAH chemistry results (by Dr. Andrew Heyes and FRA, Cheryl Clark) will be reported in this chapter.

ranged from 1.53-2.76 $\mu\text{g L}^{-1}$ (low dose) and 21.0-28.2 $\mu\text{g L}^{-1}$ (high dose) for WAF exposures, while CEWAF exposure concentrations were 10 fold higher at ranges of ~12.61-28.08 $\mu\text{g L}^{-1}$ (low) and 280.77-327.21 (high) $\mu\text{g L}^{-1}$. In all exposure solutions, TPAH was dominated by alkylated naphthalenes and was practically non-detectable after 3 days. The average particle size was similar between WAF and CEWAF (4.5-14.3 μm), but the total number and volume of particles was substantially higher in CEWAF exposures. Additionally, filtration removed the majority of PAHs in CEWAF exposures to similar levels as WAF solutions. This highlights that the substantially higher TPAH and particle concentrations in CEWAF exposures was due to the increase in PAHs in the particulate phase. Increases in DNA damage was observed in all exposures except the low dispersant treatment, but only crabs in the low dose WAF had significantly higher DNA damage than controls ($p < 0.05$). Unfortunately, due to quality issues in RNA/cDNA, gene expression analysis for oxidative stress and detoxification genes could not be accurately assessed. Delayed mortality (high dose only) and temporary narcosis was also observed in CEWAF exposures. While this study shows that blue crab larvae can be impacted by sublethal concentrations of oil and chemically dispersed oil, further investigation needs to be done, particularly a successful molecular biomarker analysis, to draw more complete conclusions as to the impacts of oil and chemically dispersed oil on this sensitive blue crab life stage.

4.2. Introduction

The rising production and transportation of offshore oil has resulted in an increase in oil pollution within marine environments from multiple sources, including oil spills/leaks

with transportation and extraction processes (e.g. the Gulf of Mexico Deepwater Horizon incident). Multiple means of remediation are used to reduce the overall impacts of oil spills and leaks on marine ecosystems such as the application of chemical dispersants. Chemical dispersants increase the generation of small droplets of oil, which enter the water column by wave energy, dissipating and diluting the oil rapidly. It is often not the inherent toxicity of the dispersant itself but what it does to the oil (i.e. the dispersant:oil mixture) that is of toxic concern. These small oil droplets tend to remain suspended in the water column, especially within the top 10m, before diluting in three dimensions over time post-spill. This increases the bioavailability of oil to organisms within those systems as the oil is more likely to remain in the water column rather than return to the surface to reform oil slicks.

Chemical dispersant use in oil spill response strategies has always been controversial, particularly given the data gaps and uncertainties regarding their chronic, sublethal, and delayed impacts (NRC, 1989; NRC, 2005). The decision to use dispersants on both surface and underwater source oil is based upon minimizing the overall environmental harm of oil, coupled with human health and economic considerations. For example many of these environmental tradeoff decisions are aimed at reducing the amount of oil reaching sensitive and highly productive shoreline habitats, whilst potentially increasing oil impacts for pelagic (water column) and benthic (bottom dwelling) organisms.

Exposure to oil and its constituents can be highly detrimental to marine organisms and can result in acute toxicity (mortality) as well as sublethal and delayed effects that can impact an organism's growth, reproduction and potentially have population-level consequences (Hylland et al., 2008; NRC, 2003). While the acute toxicity of oil and

dispersed oil has been extensively studied worldwide in multiple species and life stages as reviewed in the NRC (1989, 2005) reports, large scale oiling events like the Deepwater Horizon (DWH) incident serve to highlight the numerous data gaps and uncertainties regarding long-term and sublethal impacts of oil and chemically dispersed oil, especially on ecologically and economically important species like the blue crab, *Callinectes sapidus*.

The blue crab is a keystone species for the Atlantic coast and Gulf of Mexico both ecologically and economically. Blue crabs contribute ecologically to the estuarine and coastal food webs as they serve as both prey and predators to numerous other species as summarized in Guillory et al. (2001). Blue crabs are economically important as they are one of the top ten key domestic commercial fisheries nationally as well as for many individual states, particularly Louisiana, in weight and monetary value (NMFS, 2013). As of 2012, hard blue crab U.S. landings were valued at \$186.1 million with 25 percent of the total landings coming from Louisiana (NMFS, 2013). Blue crab zoea (larval stage) disperse from the spawning grounds at the mouths of estuaries to the open waters of the continental shelves where they live a planktonic existence on the surface, particularly the upper 3m, including the neuston (Epifanio 1995; Johnson and Perry, 1999). Therefore, they are likely to be exposed to oil and chemically dispersed oil in an offshore oil spill as they live well within the pre-approved guidelines for dispersant use, especially those events like the DWH incident that co-occurred with crab spawning. Given that in many species the most sensitive period for exposure to pollution impacts is during the younger life stages (Rand and Petrocelli, 1985), it is important both for the food web and human seafood consumers to understand the impacts of oil and dispersed oil on blue crab larvae.

In spite of their importance and potential for exposure, there is limited data on the effects of oil and dispersed oil to blue crabs at any life stage, but particularly the early larval life stage. To date, only one study has been published examining the acute toxicity of Corexit 9527, oil (WAF), and Corexit 9527 dispersed oil on blue crab early life stages (Fucik et al., 1995).

Multiple risk and impact assessment regimes have been suggested for monitoring the environmental effects of oil spill events (OSPAR, 2004; Martinez-Gomez et al., 2010). The consensus for these assessments is that it is essential to use a set of biological endpoints, including biomarkers known to be responsive to PAHs, as well as other general stress or health biomarkers to examine the impacts from oil exposure. The genes of the two most common biomarkers for PAH exposure are cytochrome P450 and glutathione-*S*-transferase (GST), which are enzymes involved in the biotransformation (metabolism) and excretion of xenobiotic compounds as well as the oxidative stress pathway (van der Oost et al., 2003; Schlenk et al., 2008). Neither gene has been fully characterized in blue crabs. However, a putative GST gene (Accession # KJ925004) was used to examine GST gene expression for this study. The validation and characterization for the putative GST gene are described in Appendices 2 and 3.

Within invertebrate species, the metabolism of PAHs can lead to oxidative stress through redox cycling, which is a common metabolic route in marine invertebrates, including crabs (Livingstone, 2003; Vijayavel et al., 2004). The induction of redox cycling produces reactive oxygen species (ROS), which can interact to oxidize most cellular compartments (lipids, protein, DNA) leading to altered cell physiology and even mutations or cell death (Cadenas, 1995). Cells contain multiple antioxidant enzymes that

can neutralize these ROS and prevent oxidative stress such as superoxide dismutase (SOD). However, if these systems are overwhelmed, the oxidative stress can lead to cellular damage, including DNA strand breaks. Studies in various marine invertebrate species have shown increases in DNA damage with exposures to individual PAHs, particularly benzo(a)pyrene (BaP), or in PAH/oil contaminated sediments and water as reviewed by Mitchelmore and Chipman (1998) and more recently by Frenzilli et al. (2009). Therefore, to examine if exposure to oil and dispersed oil results in oxidative stress impacts in blue crab larvae, the gene expression levels of the mitochondrial manganese superoxide dismutase (MnSOD) and the putative GST as well as the extent of DNA damage (single-strand DNA breaks) were examined.

The CRRC study as a whole sought to examine the impacts of sublethal levels of oil, dispersed oil, and chemical dispersants during a 6-day chronic exposure to doses at or below no observed effect concentrations (NOEC) derived from the median lethal concentration (LC₅₀) tests conducted prior to the 6-day exposure experiment. Experiment endpoints included survival, growth, and photo/geo-tactic behavior with the goal of providing numerical or qualitative inputs for existing or novel crab population and recruitment models. Additional biomarker endpoints to assess impacts of exposure include endocrine and molting pathways, oxidative stress, and DNA damage. All exposure solutions were characterized for the concentration of 50 individual and total PAH (TPAH) as well as the amount and size of oil droplets/particles in order to define exposure concentrations of oil (and components) and to aid in assessment and correlations of their impacts on crab larval behavior. Based on my involvement in the project, I will be discussing the oxidative stress and DNA damage biomarker results

alongside the survival and chemical analyses results as well as the LC₅₀ tests conducted to determine NOEC to be used for the definitive sublethal experiment.

4.3. Materials and Methods

4.3.1. Blue crab culture and maintenance

The larvae used for this proposal were produced in the Aquatic Research Center (ARC) of IMET (Baltimore, MD). The blue crab hatchery process, supported by a live food chain of cultured rotifers and freshly hatched *Artemia* sp., has been previously published (Zmora et al., 2005). Larvae were reared in 1000 L tanks at 20-100 larvae per L. Disease-free adult, female broodstock crabs were brought in from the wild, tested for three pathogens: *Hematodinium*, virus, ciliates (Nagle et al., 2009; Bowers et al., 2010; Small et al., 2013), and maintained in 30 ppt salinity in long days (16L: 8D) at 22-23°C to encourage production of egg masses. In 2013, broodstock crabs were obtained from MD, VA, and FL. Extrusion/fertilization of eggs, embryonic development (~14-19 days), and hatching of larvae are synchronous. However, the subsequent growth rates of individual larvae within a single cohort or brood varies. By the third zoea molt stage (ZIII), a single brood will contain two or three different stages of development. Range-finding LC₅₀ tests were conducted using larvae at stages ZII and ZIII (ZII were from two different brood batches) and the definitive sublethal experiment was conducted using larvae at stages ZIV-ZVI from one brood batch.

4.3.2. Acquisition and weathering of crude oil

The oil used for the study was a Louisiana light crude oil (January 2013, Exxon Mobil, from Irene B 1188527) provided by the CRRC. Weathering was carried out by

modifying a rotary evaporator to allow a stream of nitrogen to pass across the oil while gently heating and stirring. This was carried out over a period of 6 days reducing the total volume by 25% as targeted. This oil was then characterized by a laboratory at LSU and the Heyes laboratory at the Chesapeake Biological Laboratory (CBL). Corexit 9500 was supplied by Nalco Inc.

4.3.3. *Preparation of test solutions*

4.3.3.1. Stock solutions

For each set of experiments, water accommodated (WAF) and chemically enhanced water accommodated fractions (CEWAF) were freshly prepared using standard methods described in Singer et al. (2000), with some modifications as suggested in Clark et al. (2001) and Baron and Ka'aihue, (2003). Briefly, stock solutions of artificial seawater (ASW) only (control), oil (WAF), dispersant (Corexit 9500) and chemically dispersed oil (CEWAF) were prepared using 4L solvent rinsed aspirator bottles with attached Teflon tubing. To each of the four aspirator bottles, 3.6 L of 0.2 μm sterile ASW (29-31 ppt) and a stir bar were added. Bottles were placed on a stir plate so that a 25% vortex was established (Rowe et al., 2009). Oil was then added at a concentration of 1 g per L ASW (for range-finding LC_{50} stock solutions) or 0.5 g oil per L ASW (for definitive sublethal test) to the WAF and CEWAF bottles. After oil addition, dispersants were added at a 1:10 dispersant to oil ratio (i.e. 0.1 g or 0.05 g Corexit 9500) to the CEWAF or dispersant only stock bottles. The exposure solutions were set-up in the darkened fume hood for 18 hours of mixing at a 25% vortex followed by a 4 hour settling period. After the settling period, the solutions were aspirated out (surface slicks not collected) and mixed to

achieve a homogeneous solution to form each of the required 4 stock solutions (ASW, WAF, CEWAF, dispersant).

4.3.3.2. Dilution preparations

For each preliminary acute range-finding test, a log-series of dilutions of a stock solution (a 1:1000 oil:water ratio and a 1:10 dispersant:oil ratio, or 1.0 g L⁻¹ oil and 0.1 g L⁻¹ dispersant) were prepared. The tests were standard invertebrate acute toxicity tests of 96 hours duration. These LC₅₀ range finding tests (Section 4.3.6.1) were used to define the suitable range for sub-lethal exposure for the definitive sublethal test.

The range-finding tests showed that the CEWAF preparations were the most acutely toxic. Therefore, all sublethal exposure test solutions were based on the highest non-toxic concentration (NOEL) for the CEWAF solutions. It is important for the sublethal exposures to consistently compare among treatments for the amount of oil and/or dispersant used despite LC₅₀ values varying across exposure regimes. Therefore, the definitive sublethal test used a high dilution (the highest level of CEWAF that demonstrated baseline mortality in the range-finding test) and a low dilution (a ten-fold lower value). This was done to insure minimal mortality in the definitive sublethal experiment.

The definitive sublethal exposures (based on LC₅₀ range-finding data) were prepared from a stock solution of 0.5 g L⁻¹ (oil:water) using a 1:10 dispersant to oil ratio on both Day 0 and Day 3. For the high dose on Day 0, the stock solution was diluted 1:5 and then further diluted 1:2 as 375 mL of this solution was added to beakers containing 375 mL of filtered seawater with larvae for a final exposure of 0.05 g L⁻¹ (50 mg L⁻¹) nominal oil

and/or 0.005 g L^{-1} (5 mg L^{-1}) dispersant. The 1:5 dilution was used to prepare the low dose on Day 0 by diluting it 1:10 with filtered seawater, which was subsequently diluted 1:2 as 375 ml of this solution was added to beakers containing 375 ml of filtered seawater and larvae for a final exposure was 0.005 g L^{-1} (5 mg L^{-1}) nominal oil and/or 0.0005 g L^{-1} (0.5 mg L^{-1}) dispersant. For the high dose on Day 3 (80% water exchange), freshly prepared stock solutions (as above) were diluted 1:10 for a 0.05 g L^{-1} (50 mg L^{-1}) nominal oil and/or 0.005 g L^{-1} (5 mg L^{-1}) dispersant exposure. The low dose on Day 3 was prepared from a 1:10 dilution of the high dose dilution for a 0.005 g L^{-1} (5 mg L^{-1}) and/or 0.0005 g L^{-1} (0.5 mg L^{-1}) dispersant exposure.

4.3.4. *Chemical and physical analysis of stock solutions*

The analysis of the PAHs in stock solutions was carried out largely following the methods outlined in Ko and Baker (1995) and Rowe et al. (2009), which involves extraction into dichloromethane (DCM) then into hexane. Extracts were concentrated to 2 mL. Samples were analyzed using an Agilent 6890 GC with a 5975 MS in selected ion monitoring mode using a J&W Scientific 128-5522 DB-5MS fused silica column with helium as the carrier gas. Surrogate PAH standards of perdeuterated PAHs (d_8 -naphthalene, d_{10} -fluorene, d_{10} -fluoranthene, and d_{12} -perylene) were added prior to analysis on GC/MS to quantify method performance, while internal standards (d_{10} -acenaphthalene, d_{10} -phenanthrene, d_{12} -benzo[a]anthracene, d_{12} -benzo[a]pyrene, d_{12} -benzo[g,h,i]perylene) were added to samples prior to extraction for quantification. Calibration standards (Supelco Separation Technologies, Bellefonte, PA) for all 50 PAHs were used. Extraction recoveries were typically 60% and above.

PAHs were identified by retention time relative to that of mixed standards. Identification was confirmed by the abundance of a secondary mass fragment relative to the molecular ion. Integrated responses of each PAH were first normalized to responses of blank (containing perdeuterated-PAH standards only) extractions. The blank-normalized integrated area responses for each PAH were then adjusted relative to that of the appropriate internal standard (refinement of EPA Method 8270D). Instrument detection limits were calculated as sum of the average integrated response and three times the standard deviation of mean for the integrated responses for an individual PAH or grouping for blank samples. Any PAH value below the detection limit or not detected with analysis was recorded as below the detection limit (BDL). Each PAH was assigned an identification number to be used in subsequent Figures (Table 4.1).

To estimate the amount of PAHs in the dissolved versus droplet particulate fraction half of each stock exposure solutions was passed through a 0.7 μm GF/F filter using methods as described in (Payne and Driskell, 2003; Mitchelmore and Baker, 2010) so that two solutions per stock were prepared and quantified for PAHs as described above. Stock (filtered and unfiltered) solutions and all biotic and abiotic exposure solutions for the definitive sublethal experiment were measured for the total volume of particulates and their average size (diameter) using a Coulter counter (Multisizer M4, Beckman Coulter).

4.3.5. *Water quality monitoring*

Temperature and dissolved oxygen were monitored daily during the definitive sublethal tests. Water samples were collected and frozen for later measurements of pH and ammonia. Ammonia tests were completed using the salicylate-based spectrophotometric

Table 4.1 PAH analyte identification numbers for the 50 PAHs measured in the study

PAH analyte	#	PAH analyte	#
Naphthalene	1	9-Methylanthracene	26
2-Methylnaphthalene	2	3,6 dimethylphenanthrene	27
1-Methylnaphthalene	3	Fluoranthene	28
Biphenyl	4	Pyrene	29
2,6 dimethylnaphthalene	5	2,3 dimethylanthracene	30
1,3 dimethylnaphthalene	6	9,10 dimethylanthracene	31
1,6 dimethylnaphthalene	7	1-methylpyrene	32
1,4 dimethylnaphthalene	8	2 methylfluoranthene	33
Acenaphthylene	9	Benzo[a]fluorene	34
1,2-Dimethylnaphthalene	10	Benzo[b]fluorene	35
1,8-Dimethylnaphthalene	11	Benz[a]anthracene	36
Acenaphthene	12	Chrysene-triphenylene	37
2,3,5-trimethylnaphthalene	13	Naphthacene	38
Fluorene	14	6 methylchrysene	39
1-Methylfluorene	15	Benzo(b)fluoranthene	40
Dibenzothiophene	16	Benzo(k)fluoranthene	41
Phenanthrene	17	Benzo(e)pyrene	42
Anthracene	18	Benzo(a)pyrene	43
2-Methyldibenzothiophene	19	Perylene	44
4-Methyldibenzothiophene	20	3-Methylcholanthrene	45
2-Methylphenanthrene	21	Indeno(1,2,3-cd)pyrene	46
2-Methylanthracene	22	Dibenz(a,c+a,h)anthracene	47
4,5-Methylenphenanthrene	23	Benzo(g,h,i)perylene	48
1-Methylanthracene	24	Anthanthrene	49
1-Methylphenanthrene	25	Coronene	50

assay (DR/2400 Spectrophotometer, HACH).

4.3.6. *Experiment exposure designs*

4.3.6.1. Range-finding LC₅₀ test experiments

Range-finding exposures followed standard US EPA toxicity testing methods for invertebrate species with concentrations ranging from 1000 mg L⁻¹ to 1 mg L⁻¹ oil (WAF and CEWAF) and 100 mg L⁻¹ to 0.1 mg L⁻¹ dispersant (CEWAF and dispersant only) as seen in Table 4.2. Concentrations were adjusted for trial 2 based on toxicity results from trial 1 to try and determine a more linear range of exposures to conduct LC₅₀ calculations. Tests were modified standard static acute toxicity test methods (e.g. US EPA 821/R-02-012) as described in Hemmer et al. (2011). Range-finding experiments were conducted in open top 20 ml baked scintillation vials filled with 10 ml test solutions lacking aeration. Based on previous similar experiments in the Chung and Mitchelmore labs, this method had less than 10% mortality in 48 hr tests (See Chapter 5). To minimize trauma to larvae, water changes were not conducted during the 96 hour preliminary tests, although water quality was adequately maintained.

Crab larvae (ZII and ZIII) were gently transferred (wide bore plastic pipette) into glass scintillation vials which were filled with 10 ml of filtered ASW treatment solutions. For trial 1, each condition consisted of 5 vials containing 3 larvae each, and trial 2 consisted of 6 vials each containing 8 larvae. Larvae were fed daily with minimal numbers of rotifers and *Artemia* sp. as described in Zmora et al (2005). Mortality was monitored daily under a dissecting microscope and recorded. Test vials were held in a temperature controlled room (23• C) and 16 hours/day fluorescent light. For range-

Table 4.2 Summary of treatment concentrations and stage of zoea used for LC₅₀ range-finding experiments

Trial #	Zoea Stage	Dispersant mg L⁻¹	WAF mg oil L⁻¹	CEWAF mg oil L⁻¹
2	2	0.1, 0.5, 1, 5, 10, 50, 100	1, 5, 10, 15, 50, 100, 500, 1000	1, 5, 10, 15, 50, 100, 500, 1000
3	2	1, 10, 20, 40, 50, 65, 100	5, 10, 100, 250, 500, 700, 850, 1000	10, 25, 50, 75, 100, 150, 200, 1000
3	3	1, 10, 20, 40, 50, 65, 100	5, 10, 100, 250, 500, 700, 850, 1000	10, 25, 50, 75, 100, 150, 200, 1000

finding experiments, a minimum of 6 dilutions of each exposure solution plus an ASW control were used. Data is presented using nominal values because detailed chemical analyses of stocks or exposure solutions were not carried out for this phase of the study. Trial 1 was conducted using stage ZII, while trial 2 was conducted using both stage ZII and ZIII, with larvae from two different broodstock females. LC_{50} values, when possible, were calculated using the probit method as described in Section (4.3.8).

4.3.6.2. Definitive sublethal experiment

Exposures of larvae to sublethal concentrations of oil and dispersant mixtures were conducted in 1 L baked or solvent rinsed tempered glass beakers with 750 mL exposure volumes. Based on the range-finding experiments, δ_{high} and δ_{low} sublethal WAF, CEWAF, and dispersant doses were defined as 50 mg l^{-1} and 5 mg l^{-1} nominal oil and/or containing 5 mg l^{-1} or 0.5 mg l^{-1} nominal dispersant, respectively. A total of 5280 larvae at stages ZIV-ZVI were harvested from a 1000 L culture system using a combination of 200 μ m mesh and individual movement by pipettors. All 5280 larvae were placed in 8 L of 0.2 μ m filtered ASW and 200 ml aliquoted into 40 individual 1 L beakers with air hoses. Because manipulating individual larvae was impractical, the number of larvae transferred to each beaker was approximate (~188 each). Four beakers were established for each of the 6 conditions (Control, WAF-high, WAF-low, CEWAF-high, CEWAF-low, dispersant-high, and dispersant-low). Beakers were housed in a temperature controlled room (23• C) under 16 hours/day fluorescent light. In addition to larval exposures, abiotic beakers (also aerated) were established in duplicate for each of the conditions.

C. sapidus undergo 7-8 zoeal stages over 20-30 days, before reaching the post-larval stage, megalopa. Therefore, exposures were conducted for 6 days, to allow for larvae to undergo the molting process at least once, to monitor developmental progress. During the 6 day, experiment, water exchanges (with freshly prepared WAF, CEWAF, and dispersant) were conducted on Day 3. The exuviae and dead larvae were removed regularly with mortality counts. The experiment was extended beyond 6 days in recovery conditions to examine development to megalopa stage; the results of which are not included in this thesis.

4.3.7. *Biological endpoints*

4.3.7.1. Mortality

For range-finding tests, mortality was recorded daily by direct inspection of vials under a dissecting microscope. Dead larvae were distinguished from simply non-motile larvae by movement of maxillipeds and opacity (dead larvae become opaque). For large-scale definitive tests, mortality was assessed by swirling beakers (without aeration) to concentrate larvae to the center of the beaker where immobile larvae will remain as live ones swim away. In a dimly lit room, settled larvae were illuminated with oblique light (LED flashlight or fiber optic source) to reveal opaque corpses. Using a wide bore plastic pipette, suspected dead larvae were moved to a dissecting microscope stage in ASW and examined for movement of maxillipeds.

4.3.7.2. DNA damage assessment

Genotoxicity (DNA damage) was assessed on four individual pools of 3-5 larvae per exposure treatment following a modified method from Mitchelmore et al. (1998) . Microscope slides were coated with 1% NMPA in PBS and allowed to dry at 37°C in the dark. These frosted slides were stored in slide boxes within plastic bags containing desiccant. The 3-5 larvae were minced with a razor blade in 100 µL of HBSS-Hepes (pH 7.6) buffer. The resulting homogenate was centrifuged at 1,500 x g for 5 minutes. All but 50 µL of the supernatant was removed by pipette. From the remaining supernatant, 10 µL was added to 100 µL of 0.6% LMPA (37°C) in HBSS-Hepes and layered over the NMPA layer on the frosted slides. Two replicate slides were made for each crab sample. Coverslips were placed onto slides and agarose allowed to polymerize for at least 5 min on metal tray over ice.

Following solidification, the coverslips were removed and slides placed into lysing solution (10% DMSO, 1% Triton X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris Base, 1% sodium sarcosinate; pH 10) for approximately 1hr at 4°C in the dark. Once removed from the lysing solution, slides were rinsed with distilled water, placed on a horizontal gel electrophoresis tray, and covered with electrophoresis buffer (0.20 M NaOH, 1 mM EDTA; pH>12) for 5 min to allow DNA to unwind. Electrophoresis was conducted at 25 V, 300 mA for 5 min. Slides were removed and placed in neutralization solution (0.4M Tris, pH 7.5) for 5 min (repeated for three washes with a total of 15 min). Slides were drained and placed in 100% ethanol for 5 min then allowed to dry in dark container overnight. Slides were permanently stored in a desiccated slide box until processing. All solutions kept at 4° C prior to use.

For analysis, each slide was reconstituted with $2 \mu\text{M mL}^{-1}$ ethidium bromide in HBSS-Hepes and examined using an epifluorescent microscope (Olympus BX50) with a green filter at 40x magnification (Q Imaging Retiga 1300 camera). The Komet 5.5 Software (Kinetic Imaging, Liverpool, UK) image analysis package was used to score the cells. From each duplicate slide, up to 100 non-overlapping cells were randomly selected for quantification. Results expressed as mean \pm standard error of the mean in terms of the percentage of DNA in the tail region (Tail % DNA).

4.3.7.3. Gene expression analysis

Three samples of 5 larvae each were collected at the start of the experiment (Day 0) prior to exposure. At Day 3 and Day 6 of the experiment, 15 larvae were collected from each beaker for all 6 treatments. All larvae collected for gene expression analysis were stored in Eppendorf tubes on dry ice and then permanently stored at -80°C until analyzed. Total RNA was extracted from the 5 or 15 pooled larvae using Qiazol (Qiagen). RNA quantity and quality was assessed by spectrophotometry (Nanodrop, Thermo Scientific) and A260/280 ratios. 1.5 to 3 μg of total RNA/sample was subjected to 1st strand cDNA synthesis using a Takara PrimeScript kit (Takara) designed for qPCR, which includes the DNase treatment step. After cDNA synthesis, the samples were diluted in water to give a 12.5 ng/ μl total RNA equivalent. The quality of the samples was tested using an end-point PCR assay with arginine kinase primers (Table 4.3) by the Chung Lab (IMET, Baltimore). Originally, arginine kinase was proposed as a reference gene in blue crabs to conduct relative quantification (i.e. the delta-CT method), but was deemed unsuitable as a reference gene for the oil contaminated sediment exposure in Chapter 3 (Appendix 1).

Therefore, gene expression levels in both Chapter 3 and this study were conducted using absolute quantification PCR.

The primers for mitochondrial manganese superoxide dismutase (MnSOD) were designed using NCBI Primer BLAST from existing blue crab mRNA sequences in GenBank (Table 4.3). The primers for a putative blue crab GST (Table 4.3) were designed from an EST hit observed by running a BLAST search with the mRNA sequence for delta-GST from the Chinese mitten crab, *Eriocheir sinensis* against a non-public EST library (Place; IMET, Baltimore).

PCR products for GST and MnSOD were run on a 1.5% agarose gel, extracted using a QIAQuick gel extraction kit (QIAGEN, Valencia, CA), and ligated into a TOPO pcR-II plasmid. This plasmid was transformed into chemically competent *E. Coli* (TOP10). The plasmids were then purified from the *E. coli* using a ZR plasmid classic mini-prep kit (Zymo Research, Irvine, CA) and the plasmid DNA quantified on a NanoDrop. The purified products and plasmids were sequenced by the BAS lab (IMET, Baltimore) and the orientation of the sense strand of product insert in relation to T7 promoter was verified. The plasmid DNA for each gene was then linearized using a restriction digest (*Hind iii*) and completion of digestion was verified by gel electrophoresis. The linearized plasmids were then appropriately diluted (30-30,000,000 copies) and qPCR run to examine qPCR efficiency (qPCR efficiencies were >95%, with R² values>0.995). Absolute quantitative-PCR was conducted using the POWER SYBRGreen reagent kit on an Applied Biosystems 7500 Fast Real-Time PCR System.

For both genes, the initial qPCR was conducted in duplicate (25 ng/well) for all samples collected on Day 0, 3, and 6 with standards in triplicate. A dissociation curve

was conducted for all samples and standards. A linear regression equation was produced for the cDNA standards of each gene and used to determine the copy number of each crab sample. The copy numbers were then normalized to the total RNA concentration (μg) and reported as copy number μg^{-1} total RNA. The initial qPCR test resulted in lower than expected copy numbers and double peaks in some of the dissociation curves (not seen in standards or previous work using these primers). Therefore, a repeat qPCR was conducted for GST using only one well per sample, due to limited cDNA volume, and standards in triplicate.

4.3.8. *Statistical analysis*

All statistical analyses were conducted using R statistical software 2.15.2. Mortality was examined by calculating the lethal concentration at which 50% of individuals exposed were dead (LC_{50}) compared to blue crabs in the controls after 48- and 96-hr exposure when possible for dispersant and CEWAF exposures. No WAF trials could be quantified for LC_{50} . The LC_{50} values and their 95% confidence intervals were calculated using a probit linked binomial generalized linear model in R using the MASS package. Plots of proportion of dead larvae over log dispersant concentrations were produced and lines of the regression model and its 95% confidence values were fitted to the data. McFadden's Pseudo R^2 was calculated for each model.

For DNA damage, the mean and standard error of the mean values of percent DNA in the tail (Tail % DNA) in larvae collected on Day 6 of exposures were calculated. The DNA damage results were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test). As both tests passed, an Analysis of Variance

Table 4.3 Primer descriptions and GenBank Accession numbers for gene expression assessment of AK, GST, and MnSOD in larval blue crabs.

Gene Target	Primer Name	Primer Sequence (5'-3')	Accession Number	Product Size (bp)
Arginine Kinase	AK-QF	CTACCACAACGACAACAAGACCTTC	AF233355.1	187
	AK-QR	ACGGGCTTCTCAATCTCGTTA		
Glutathione S-transferase	<i>GST-QF</i>	GGACTTCAGCAAGCATAACCAAC	KJ925004	103
	GST-QR	GGTGCTGAGGATTGGGGAAG		
Mitochondrial Manganese Superoxide Dismutase	<i>MnSOD-QF</i>	CAGACCTCCCCCTACGACTATG	AF264029.1	148
	<i>MnSOD-QR</i>	GAAGCCAAAGAGAAAGGTGATGT		

(ANOVA) was conducted. If a significant difference was found in the ANOVA test (p value < 0.05), then a multiple pairwise comparisons was conducted using Tukey's HSD test. Any comparisons with a p value < 0.05 were considered a significant difference between treatments.

4.4. Results

4.4.1. Range-finding LC_{50} trials

Two range-finding exposure trials were conducted on two different broodstock batches of larvae at stage II zoea (ZII) and the first broodstock batch again at stage III zoea (ZIII). For both trials, control larvae mortality was 0%. Trial 1 was conducted using the full range of concentrations (1000 mg L⁻¹ to 1 mg L⁻¹ for oil and 100 mg L⁻¹ to 0.1 mg L⁻¹ for dispersant). Neither the WAF nor CEWAF exposures for trial 1 had a linear dose-response relationship (Fig. 4.1). Therefore, LC_{50} concentrations could not be accurately established for either exposure. The trial 1 WAF exposures had minimal toxicity for all but the stock concentration (1000 mg L⁻¹ oil), while the CEWAF exposure seemed to show a threshold in toxicity between 5% and 1% (50 and 10 mg oil L⁻¹ ASW) with approximately 50% mortality at 5% and 4.2% mortality at 1% CEWAF.

Additionally, the toxicity of both WAF and CEWAF static exposures increased in most doses over time throughout the 96 hr exposure, especially in the higher concentrations.

Trial 1 dispersant only exposures did have a linear dose-response with a logarithmic transformation of dose that allowed for accurate LC_{50} concentrations to be calculated using the probit analysis (Fig. 4.2). The 48 hr LC_{50} and standard error was 44.0 ± 1.3 mg dispersant L⁻¹. As with the other two the toxicity increased as the exposure continued

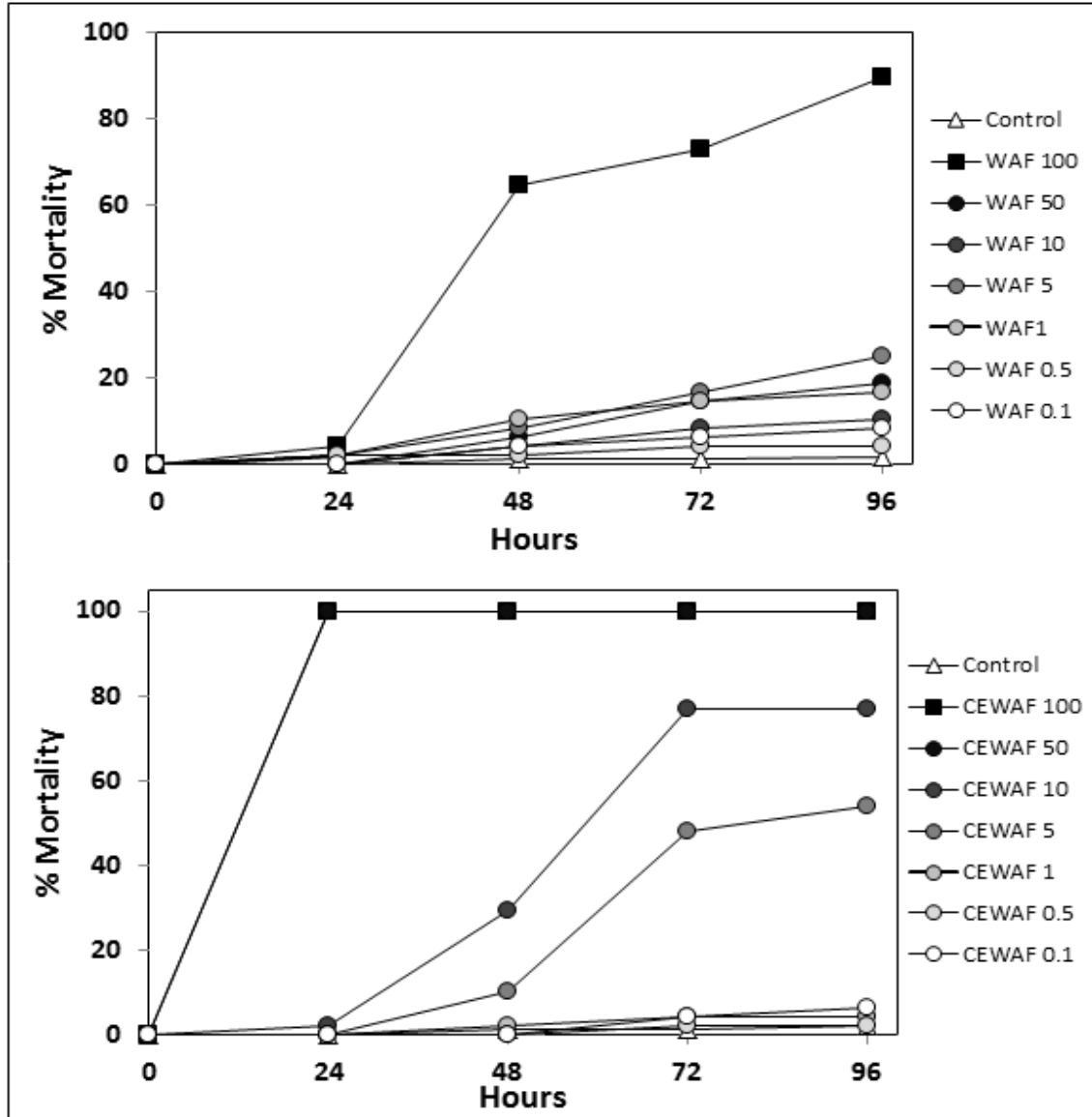


Fig. 4.1 Trial 1 mortality of ZII larvae in stocks (squares) and dilutions (circles) of WAF (top) and CEWAF (bottom) with controls (triangles) over the 96 hr exposures. Label numbers indicate the percent of stock for that exposure solution.

with 96 hr LC₅₀ concentration and standard error of 32.7.0±1.3 mg L⁻¹ with the probit model having a McFadden's Pseudo R² of 0.713 (results not shown).

Given the results observed in trial 1, the dilution concentrations examined were adjusted for trial 2 in an attempt to better capture a concentration range that could yield a linear dose-response relationship needed to quantify an LC₅₀. In trial 2, ZII larvae from a new broodstock and the zoea from same broodstock used in trial 2, now at ZIII, were examined concurrently using the same stock and dilutions preparations. WAF exposures for both ZII and ZIII larvae showed low toxicity for all concentrations, even the 100% stock, with <10% mortality in all concentrations. CEWAF exposures still appeared to be more of a threshold response between 7.5 and 15% (75 to 150 mg oil L⁻¹ and 7.5-15 mg dispersant L⁻¹).

In the CEWAF exposures, ZII larvae went from 64% mortality with the 10% dose (100 mg oil L⁻¹) to 6.3% mortality with the 7.5% dose (75 mg oil L⁻¹), while ZIII larvae went from 86% mortality with the 15% dose (150 mg oil L⁻¹) to 19% mortality with the 10% dose (100 mg oil L⁻¹). ZII larval CEWAF exposures could be examined by probit analysis with a resulting LC₅₀ and standard error of 12.1±1.0% or 121±10 mg oil L⁻¹ (Fig. 4.3).

Of the dispersant exposures, only the ZIII larvae had a linear dose-response with a logarithmic dose transformation that could be examined by probit analysis as ZII larvae dispersant only exposures did not have a dose below the stock that had >50% mortality. The ZIII larvae dispersant only exposure resulted in a LC₅₀ equivalent to that of ZII larvae from trial 1 with a LC₅₀ and standard error of 42.5±1.3 mg dispersant L⁻¹. Based on the results from both trials it was determined that nominal exposure concentrations of

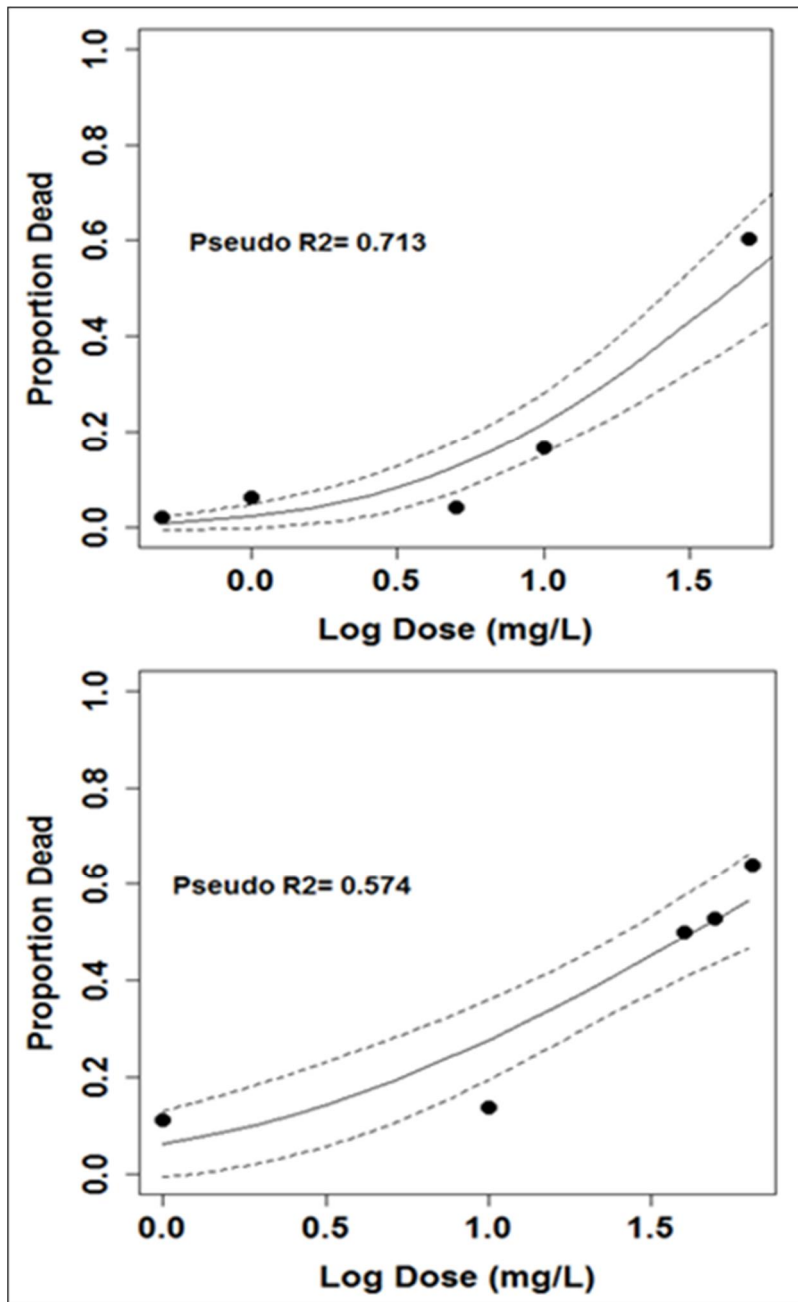


Fig. 4.2 Nominal concentration mortality response curves for larvae from same batch in trial 1 at ZII (top) and trial 2 at ZIII (bottom) exposed to Corexit 9500 only. The probit linked binomial generalized linear model (solid line) and the 95% confidence intervals (dashed lines) for each model are shown for each trial. McFadden's Pseudo R² value is included for each model.

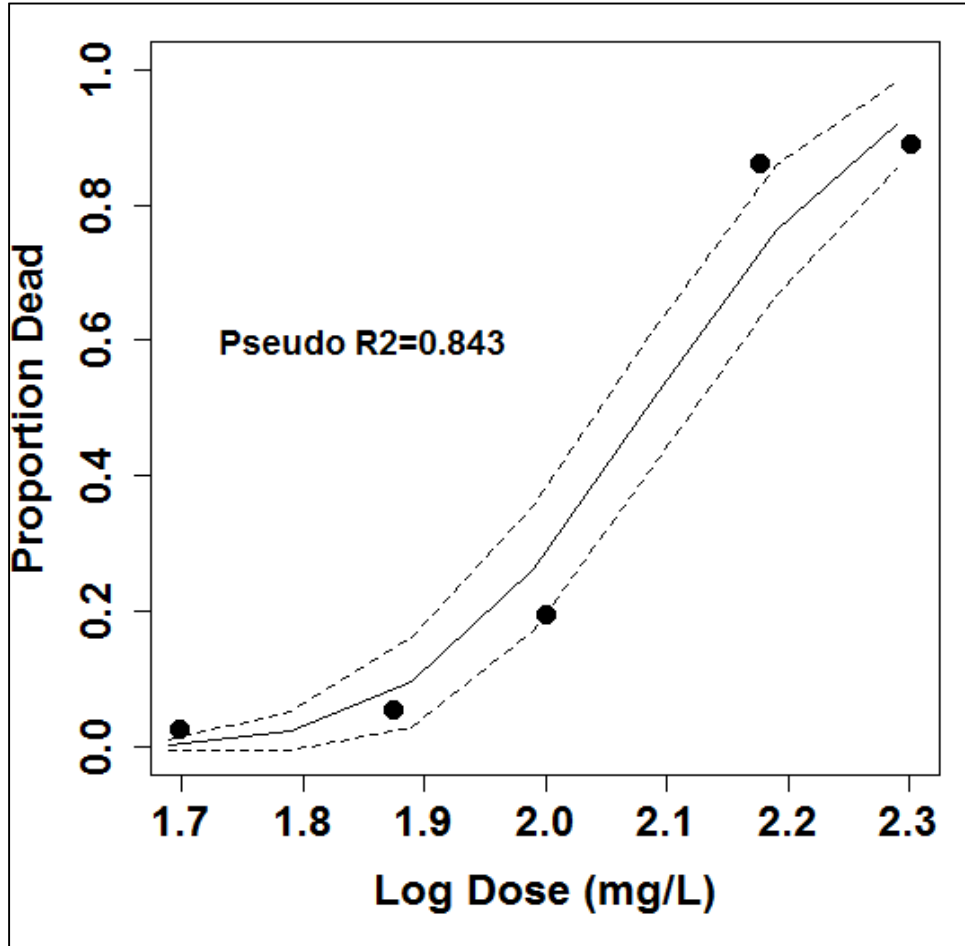


Fig 4.3 Nominal oil concentration mortality response curve for ZIII larvae in trial 2 exposed to CEWAF. The probit linked binomial generalized linear model (solid line) and the 95% confidence intervals (dashed lines) for each model are shown. McFadden's Pseudo R^2 value is included for the model.

50mg oil L⁻¹ and 5 mg oil L⁻¹ for the WAF and CEWAF and 5 mg dispersant L⁻¹ and 0.5 mg dispersant L⁻¹ for CEWAF and dispersant only would be used for the definitive chronic, sublethal exposures.

4.4.2. *Definitive sublethal test*

4.4.2.1. Chemistry analysis of stock solution

The stock solutions of WAF, CEWAF, dispersant only, and ASW were analyzed for PAHs as well as particle concentration and size analyses. During the 6 day exposure, stocks were prepared fresh on Day 0 and Day 3. Understanding the form (i.e. dissolved versus particulate fractions) of the PAH/oil that organisms are being exposed to is equally as important as the overall concentration in determining exposure with mixed solutions. To account for this, the stock solutions for analysis were split in half and one of the subsamples was filtered through a 0.7µm GF/F filter. The unfiltered stocks represent the total pool of PAHs, but there is no distinction between what is freely dissolved in solution and what remains in the particulate (e.g. oil droplet) phase. However, filtration of the stocks allows for the analysis of primarily the freely-dissolved fraction of PAHs present as filtration through 0.7µm GF/F filter removes the majority of oil particles present. A mass balance calculation can then be conducted to assess the amount of PAHs in the oil particulate phase. Fully defining the exposure solution this way can be critical in a toxicity experiment so as to not over- or under-estimate toxicity as toxicity can be specific to route of uptake of the organisms examined.

There were no PAHs detected above a molecular weight of 216; therefore, higher mass compounds were removed from subsequent tables and figures. Individual PAHs are

denoted in figures using the numbering system assigned in Table 4.1. Dispersant and seawater (ASW) stock solutions were analyzed for PAHs. As expected, few to no PAHs were detected above detection limits for either stock. ASW solutions had TPAH concentrations that were below detection limit (BDL) and 300 ng L^{-1} for Day 0 and Day 3, respectively. Unfiltered dispersant only stocks had TPAH concentrations that were $2,566 \text{ ng L}^{-1}$ (Day 0) and 922 ng L^{-1} (Day 3), and filtered TPAH concentrations of 73.4 ng L^{-1} (Day 0) and BDL (Day 3).

As seen by Figures 4.4 and 4.5, the TPAH concentrations for both WAF and CEWAF varied between the 2 stock preparations. The WAF stocks varied by less than a factor of 3 between preparations (Fig. 4.4). However, given that the filtered WAF of the Day 3 stock resulted in uncharacteristically higher PAH concentrations than the unfiltered WAF, it is likely that there was a loss in the lower molecular weight PAHs in Day 3 unfiltered WAF stock sample, which could have happened in transport or processing. Subsequent high and low dilution PAH concentrations and particle analysis results for the Day 3 WAF preparation provides further evidence of a loss of PAHs in the stock sample (see Table 4.5). The CEWAF stocks varied by less than a factor of 2 between preparations with the biggest difference between Day 0 and Day 3 preparations being in the concentrations of 2,6-dimethylnaphthalene and 1,3-dimethylnaphthalene (Fig. 4.5). Such variability in stock preparations, particularly CEWAF solutions, is not uncommon.

While the TPAH concentrations differed between CEWAF and WAF preparations, the distribution of percent of TPAH for the individual PAHs was similar between the different oil preparations. Overall, alkylated naphthalenes accounted for 79.3-85.7% of

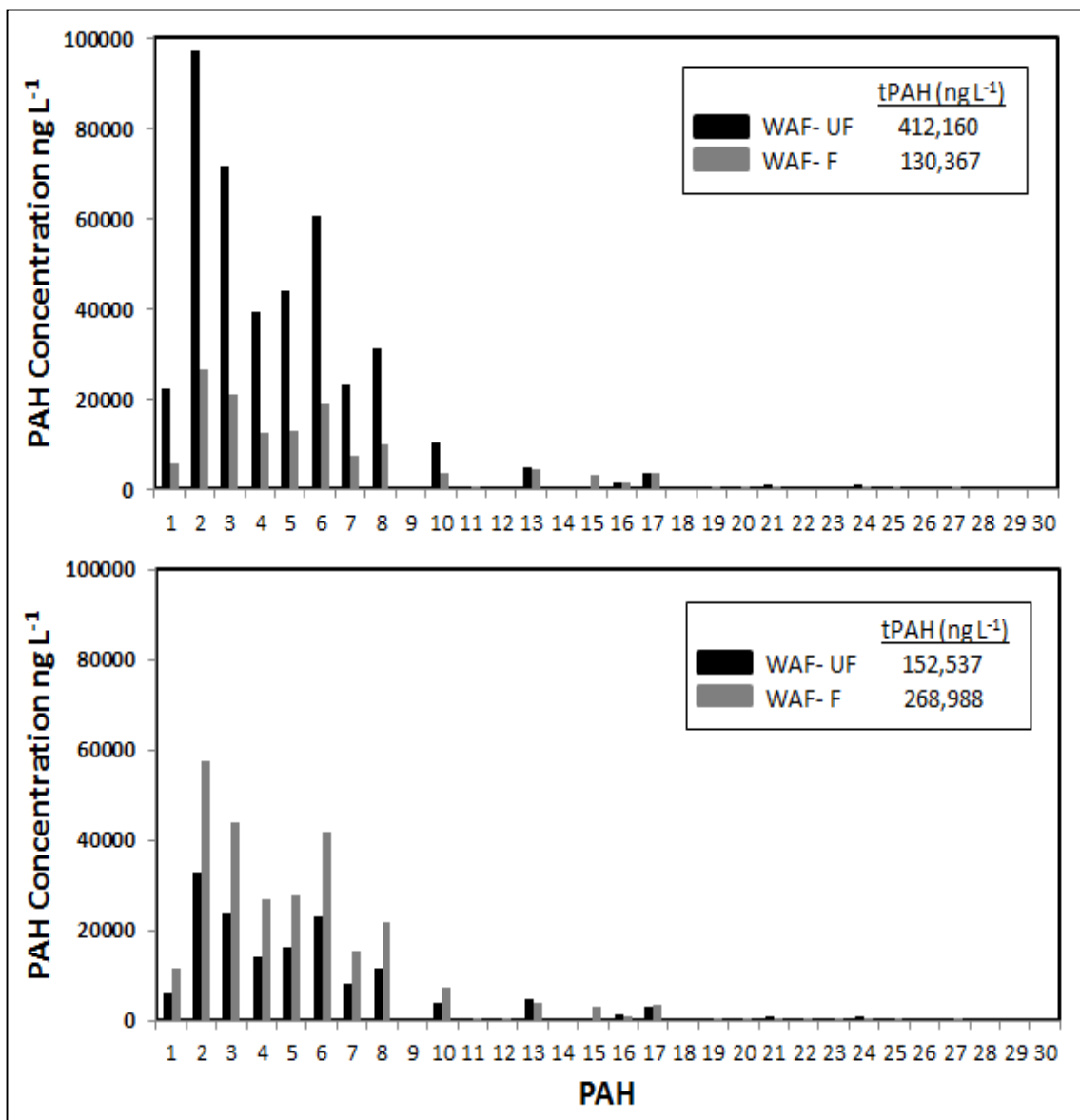


Fig. 4.4 Individual PAH concentrations (ng L⁻¹) for unfiltered (UF) and filtered (F) WAF stocks prepared on Day 0 (top) and Day 3 (bottom). Total PAH (TPAH) concentrations (ng L⁻¹) included for each unfiltered and filtered stock preparation.

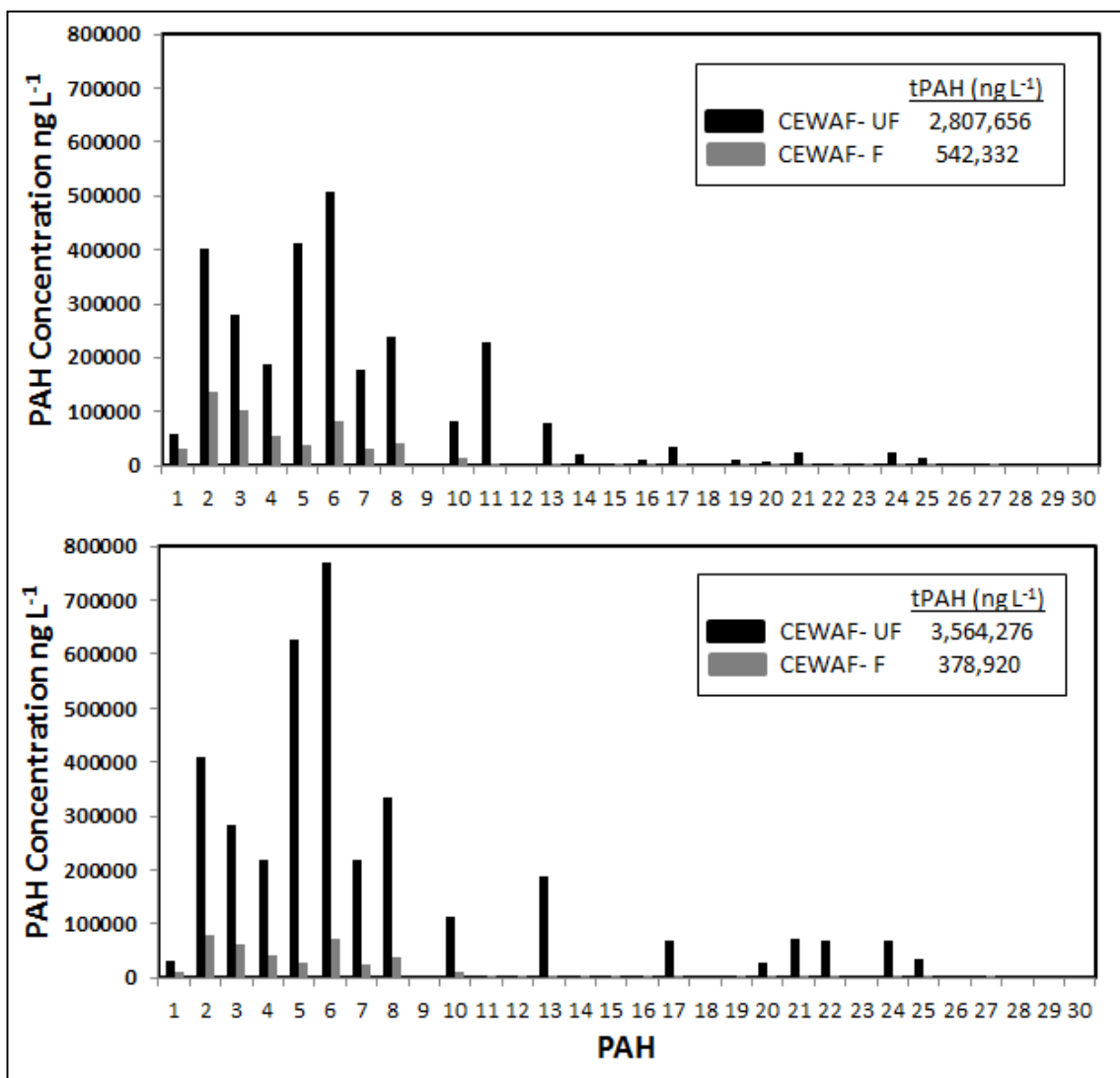


Fig. 4.5 Individual PAH concentrations (ng L^{-1}) for unfiltered (UF) and filtered (F) CEWAF stocks prepared on Day 0 (top) and Day 3 (bottom). Total PAH (TPAH) concentrations (ng L^{-1}) included for each unfiltered and filtered stock preparation.

TPAH for all preparations of WAF and CEWAF both filtered and unfiltered. However, proportions of individual alkylated naphthalenes differed between the WAF and CEWAF preparations. The individual PAH distribution was similar between the two stock preparations of WAF, both filtered and unfiltered, with the most prevalent PAHs being 2-methylnaphthalene, 1-methylnaphthalene, and 1,6-methylnaphthalene, which accounted for 20.4-23.5%, 15.7-17.3%, and 14.3-15.5% of TPAH, respectively. Both unfiltered CEWAF stock preparations were also similar in their individual PAH distributions. In the unfiltered CEWAF stocks, 2,6-dimethylnaphthalene and 1,3-dimethylnaphthalene were the predominant PAHs at 14.7-17.6% and 18.1-21.6% of TPAH, respectively. However, the filtered CEWAF stocks showed a more similar distribution to the WAF preparations with the most prevalent PAHs being 2-methylnaphthalene, 1-methylnaphthalene, and 1,3-dimethylnaphthalene at 20.9-25.4%, 16.1-18.6%, and 15.3-18.9% of TPAH, respectively.

Other than the Day 3 WAF preparation, filtration did indeed reduce the concentration of PAHs by removing the oil particulate fraction. This was most prominent in the CEWAF stocks, which had an 80.7% and 89.6% reduction in TPAH with filtration for Day 0 and Day 3, respectively. While the Day 0 WAF preparation did have a reduction in TPAH with filtration, it was not nearly as prominent as the CEWAF. This serves to highlight the fact that WAF preparations have much less oil in the particulate phase than CEWAF preparations. The small amount of oil particulates observed in WAFs could also be in part due to the filtration method as it could result in either the enhancement of dissolution of PAHs (i.e. dissolved) or the formation of very small colloidal fractions, which are essentially dissolved and could potentially pass through the GF/F. While the

two different preparations varied by an order of magnitude in the amount of oil in the particulate phase, they had similar proportions of dissolved PAHs as seen in the TPAH concentration of filtered samples (Fig. 4.4 and 4.5). Although, the filtered CEWAF stocks had slightly higher concentrations of dissolved PAHs than the filtered WAF stocks.

The reduction of oil particulates with filtration was also confirmed in the particle analysis (Table 4.4). Both WAF and CEWAF stocks had a reduction in total particle volume and total number of particles with at 0.7 μ m filtration. This reduction was more pronounced in the two CEWAF stock preparations. Interestingly the average size of particles increased after filtration for all WAF and CEWAF preparations, which was again more pronounced in the two CEWAF stock preparations. This increase in particle size could just be due to the filtration method causing physical changes to the particles such as aggregation of smaller particles into larger ones.

4.4.2.2. Chemical analysis of exposure waters

The high and low dose exposures were prepared from stocks on Day 0 and Day 3 as described in Section 4.3.3.2. The TPAH concentrations for the high and low dose dilutions are described in Table 4.5. The stock concentrations are also included as a reference. The expected concentrations of each dilution in this table are based on what concentrations would be expected from each dilution from the stock, assuming a linear-response in TPAH. As the Day 0 high and low dose dilutions were prepared at a 2x higher concentration than what was in the exposures, the actual and expected exposure concentrations are shown in Table 4.5 as a 2-fold reduction from the measured and expected concentrations of TPAH and total particle volume. This was not necessary for

Table 4.4 Particle analyses of unfiltered (UF) and filtered (F) stock solutions using a Coulter counter for the Day 0 and Day 3 WAF, CEWAF, and ASW stock preparations.

Day	Stock solution	Total particle volume ($\mu\text{l l}^{-1}$)	Average size of particles (μm)	Total number of particles ($\times 10^3$) l^{-1}
0	ASW UF	0.01	NA	NA
0	ASW F	0.00	NA	NA
0	WAF UF	0.42	8.782	28,585
0	WAF F	0.11	10.814	2,212.5
0	CEWAF UF	30.63	5.551	2,342,000
0	CEWAF F	0.01	7.922	229.5
3	ASW UF	0.01	NA	NA
3	ASW F	0.00	NA	NA
3	WAF UF	0.24	5.906	16,378.5
3	WAF F	0.04	28.336	378.5
3	CEWAF UF	72.74	5.043	6,266,480
3	CEWAF F	0.03	26.656	328.5

Table 4.5 Comparison of TPAH and particle numbers measured between stock solutions and diluted (high and low dose) preparations. Actual concentrations measured are compared to the expected concentrations (italicized) and final exposure concentrations in Day 0 are included.

Stock Solutions	TPAH Day 0 ($\mu\text{g L}^{-1}$)		Day 0 Total Particle Volume ($\mu\text{l L}^{-1}$)		TPAH Day 3 ($\mu\text{g l}^{-1}$)	Day 3 Total Particle Volume ($\mu\text{l l}^{-1}$)	
	Measured	Conc. in Exposures	Measured	Conc. in Exposures			
WAF UF STOCK	412.2*	NA	0.42	NA	152.5#	0.24#	
WAF HIGH	Actual	42.05*	21.03	0.14	0.07	28.18	0.26
	<i>Expected</i>	<i>82.40</i>	<i>41.22</i>	<i>0.08</i>	<i>0.04</i>	<i>15.25</i>	
WAF LOW	Actual	5.51*	2.76	0.09	0.045	1.53	0.18
	<i>Expected</i>	<i>8.24</i>	<i>4.12</i>	<i>0.01</i>	<i>0.005</i>	<i>1.53</i>	
CEWAF UF STOCK	2,807.7	NA	30.63	NA	3,564.3	72.74	
CEWAF HIGH	Actual	22.41*#	11.21	6.89	3.45	327.21	8.67
	<i>Expected</i>	<i>561.54</i>	<i>280.77</i>	<i>6.13</i>	<i>3.065</i>	<i>356.43</i>	<i>7.27</i>
CEWAF LOW	Actual	7.93*#	3.97	0.67	0.335	12.56	0.75
	<i>Expected</i>	<i>56.15</i>	<i>28.08</i>	<i>0.61</i>	<i>0.305</i>	<i>35.64</i>	<i>0.73</i>

*Measured concentrations in Day 0 are 2x the actual exposure concentrations

#Values measured are much lower than expected

the Day 3 high and low dose dilution concentrations as the measured concentrations were the actual exposure concentrations.

WAF TPAH concentrations in exposures were 1.53-2.76 $\mu\text{g L}^{-1}$ for the low dose and 21.03-28.18 $\mu\text{g L}^{-1}$ for the high dose. The CEWAF TPAH concentrations in exposures were approximately 10-fold higher at 12.56-28.08 $\mu\text{g L}^{-1}$ for the low dose and 280.77-327.21 $\mu\text{g L}^{-1}$ for the high dose. The actual exposure values are reported for all exposure solutions except CEWAF Day 0 high and low, which are the expected exposure values due to the fact that measured values for both are an order of magnitude lower than expected from the unfiltered CEWAF STOCK. This was particularly evident in a severe depletion of the three lowest molecular weight PAHs (naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene), which only accounted for 1% of the total PAH in the Day 0 high dose as compared to 21% of TPAH in the Day 3 high dose and 26% of the Day 0 CEWAF stock.

Prior studies in our lab and other results in this report highlight a direct linear relationship with variable dilution and TPAH concentrations. Furthermore, the total particle volumes results for the Day 0 CEWAF high and low doses were very similar to the expected volumes based on a linear-dose response from the unfiltered CEWAF stock. This suggests that there was likely a loss of PAHs prior to analysis of these solutions for PAH concentrations that did not occur in the subsample collected for particle analysis. Additionally, the TPAH concentrations and total particle volumes measured in the Day 3 high and low doses serve to confirm that there was likely a loss in PAHs in the Day 3 WAF stock sample(s), especially as the total particle volume in the stock was lower than that in the WAF high dose. Another explanation for this could be poor mixing of the

stock solution prior to collection or analysis making the sample not indicative of actual exposure.

With the exception of the Day 0 CEWAF high and low doses, the distribution of percent of TPAH for the individual PAHs was similar between the high and low dose of the two WAF preparations and the Day 3 CEWAF high and low doses and their respective stocks. WAF high and low dose for both days had 13.9-24.5%, 15.1-18.0%, and 14.3-20.8% of TPAH for 2-methylnaphthalene, 1-methylnaphthalene, and 1,3-dimethylnaphthalene, respectively. The biggest difference in the WAF exposure solutions was the lack of naphthalene (below detection limit) in the Day 3 WAF low dose. The CEWAF low dose for both days and Day 0 CEWAF high dose were also below the detection limit for naphthalene. The CEWAF low and high dose for both days had concentrations of 2,6-dimethylnaphthalene and 1,3-dimethylnaphthalene that were 18.3-23.8% and 10.9-16.9% of TPAH, respectively.

The TPAH concentrations were measured in pooled aged solutions collected from exposure containers after 3 days before replacing with new solutions on Day 3 and at the end of the experiment on Day 6. All 3 day aged solutions of WAF and CEWAF high and low doses had TPAH concentrations that were below or close to the detection limit (Fig. 4.6). The highest TPAH concentrations were the two CEWAF high doses, which were 0.085 and 0.403 $\mu\text{g L}^{-1}$ for Day 3 and Day 6, respectively. This highlights the rapid removal of PAH components over the duration of exposure, which is expected given that the oiled solutions contain mostly low molecular weight PAHs, which are more easily lost to the atmosphere with volatilization. Additionally, there was no appreciable differences in TPAH between 3 day aged biotic and abiotic solutions collected on Day 3

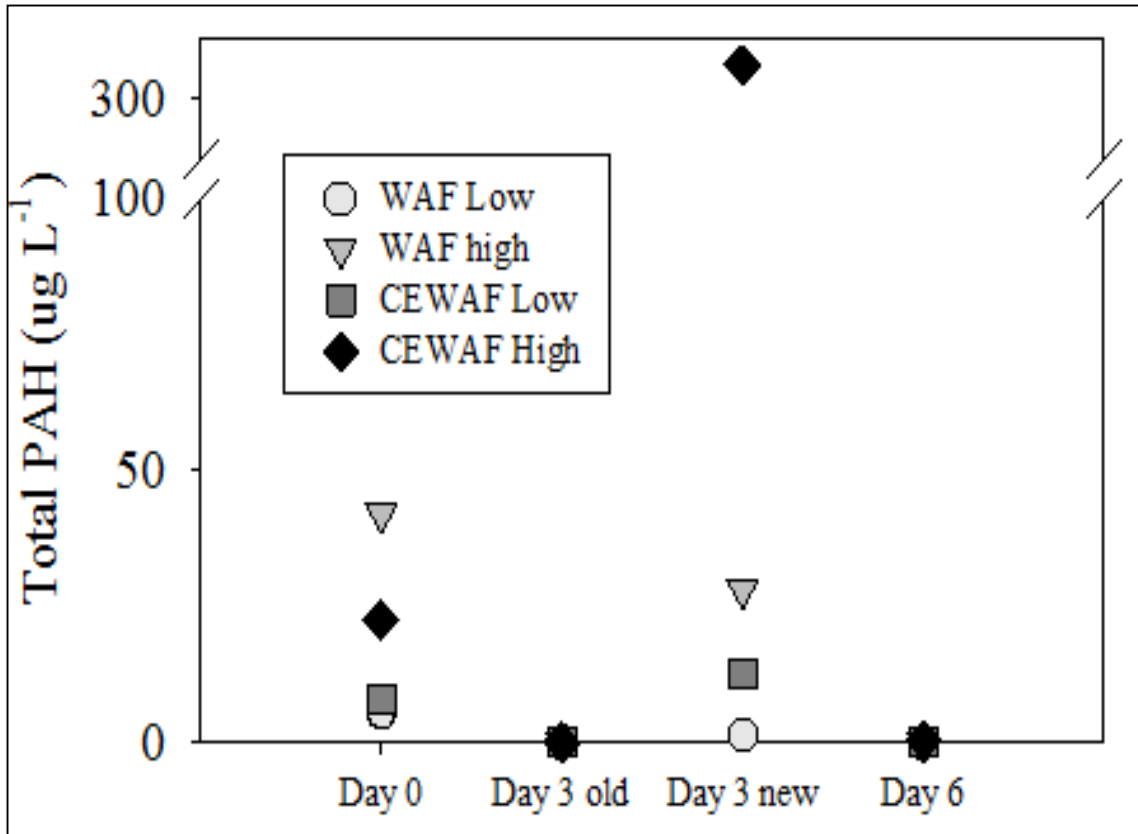


Fig. 4.6 Total PAH concentrations in fresh (Day 0 and Day 3 new) and 3 day aged (Day 3 old and Day 6) exposure waters during the 6 day sublethal experiment.

Table 4.6 Particle analyses of biotic and abiotic exposure solutions using a Coulter counter. Actual exposure volumes and number of particles for Day 0 preparations are in parenthesis.

Day Collected	Exposure Solution	Total droplet volume ($\mu\text{l L}^{-1}$)	Average size particle (μm)	Total Number of particles ($\times 10^3$) L^{-1}
0	CEWAF high	6.89 (3.45)	4.594	575,142 (287,571)
0	CEWAF low	0.67 (0.335)	4.54	60,636 (30,318)
0	WAF high	0.14 (0.07)	4.808	21,420 (10,710)
0	WAF low	0.09 (0.045)	14.324	5,790 (2,895)
3	CEWAF high	8.67	5.459	657,015
3	CEWAF low	0.75	4.479	70,272
3	WAF high	0.26	7.309	19,647
3	WAF low	0.18	9.724	7,128
3	CEWAF high Day 0 Abiotic	1.78	8.181	85,558
3	CEWAF low Day 0 Abiotic	0.5	7.568	33,627
3	WAF high Day 0 Abiotic	0.66	7.989	28,591
3	WAF low Day 0 Abiotic	0.53	8.444	17,635
6	CEWAF high Day 3 Abiotic	3.26	7.025	257,274
6	CEWAF low Day 3 Abiotic	0.23	6.78	10,843
6	WAF high Day 3 Abiotic	Nd	Nd	Nd
6	WAF low Day 3 Abiotic	0.13	5.929	9,656

*Nd: not determined due to food contamination of beakers

and Day 6. All the abiotic WAF and CEWAF solutions collected were below or close to the detection limit for TPAH with the highest TPAH concentrations again in the CEWAF high dose at 0.41 and 1.75 $\mu\text{g L}^{-1}$ for Day 3 and Day 6, respectively.

The particle analysis of exposure solutions (Table 4.6) shows a consistent linear response in total droplet volume and total number of particles between high and low dose with all Day 0 and Day 3 preparations (except Day 0 WAF) having an approximately 10-fold difference between low and high doses. Day 0 WAF, however, appears to have closer to a 5-fold difference between the low and high dose in total droplet volume and total number of particles. These particle results also highlights the fact that CEWAF exposures have much higher particle volumes and number of particles than WAF exposures even though both preparations have the same linear dilutions from stock oil preparations with the same oil loading rates. CEWAF solutions have at least an order of magnitude higher particle volumes and number of particles than their respective WAF solution dilutions.

The abiotic exposure solutions were also examine for particle analysis in 3 day aged solutions collected prior to the water change on Day 3 and at the end of the experiment (Table 4.6). The exposure solutions with larvae collected prior to the water change at Day 3 and at the end of the exposure could not be examined for particle analysis as well as PAH analysis due to the high biotic material in the solutions, particularly with the addition of larval food to the exposure solutions that interfered with Coulter counter analysis. Abiotic exposures of the WAF high dose were accidentally öfedö the rotifers and *Artemia* sp., which prevented particle analysis of those solutions at the end of the

experiment. The loss of PAHs over the 3-day time period was also observed in with the particle analysis, especially for the CEWAF exposures.

Generally, the abiotic exposure in the CEWAF high and low dose collected on Day 3 and Day 6 showed a 2-3 fold reduction in both total droplet volume and total number of particles from their subsequent dilution stocks after 3-days. The exceptions to this were abiotic CEWAF low collected on Day 3, which had a slight increase in total droplet volume from the dilution stock, and abiotic CEWAF low collected on Day 6, which had close to a 7-fold reduction in total number of particles. The abiotic WAF collected on Day 3 and Day 6, however, showed similar or slightly higher total droplet volumes and total number of particles than their respective dilution stocks. Additionally, all CEWAF and WAF abiotic and biotic exposures had average size of particles that were 10 μm or less, with the exception of the Day 0 WAF low solutions.

4.4.2.3. Mortality and narcosis

Larval mortality was low during the entire 6 day period with all exposures having 0% mortality (Fig. 4.7) and no more than 1% mortality per day, on average, from Day 0 to Day 6. During the continued 3 day recovery period, the mortality did not increase substantially in most treatments with the exception of the CEWAF high treatment, which doubled to 12% mortality by day 9.

Additionally, a temporary narcosis was observed on Day 3 with the addition of fresh treatment solution in the CEWAF high and low treatments. The narcosis was more prominent in the CEWAF high treatment with the majority of larvae in all 4 beakers sinking to the bottom and becoming relatively immobile, while less than half of the

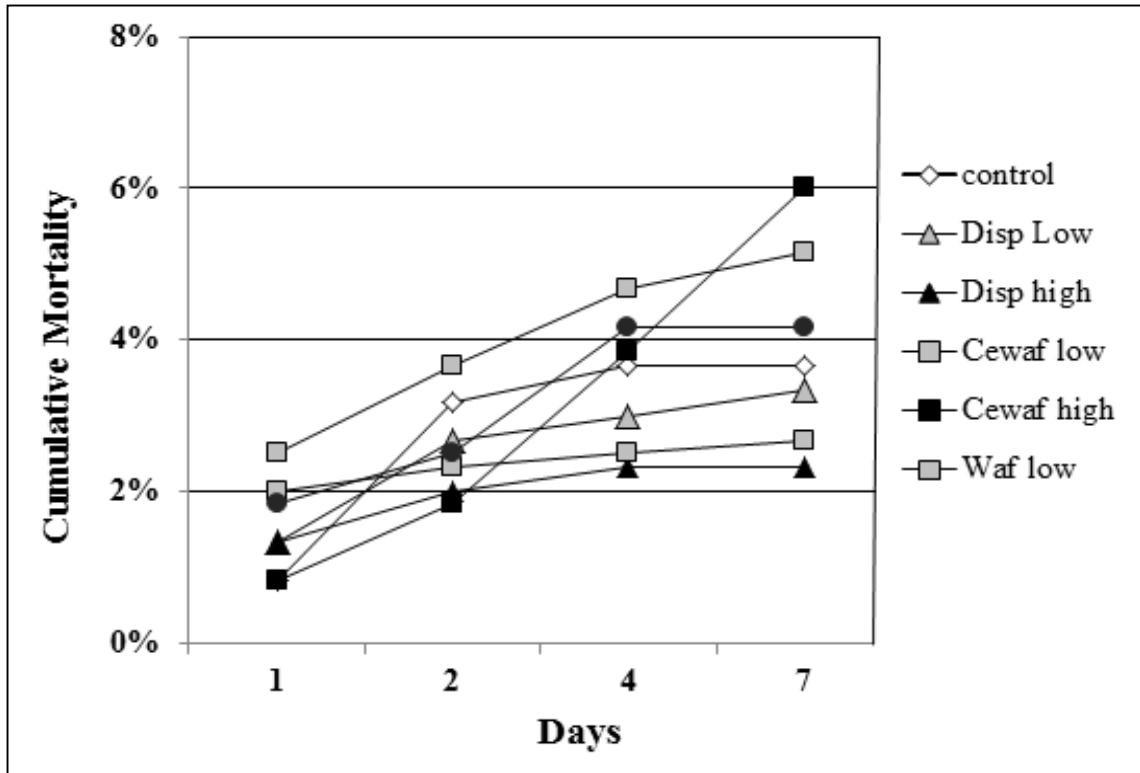


Fig. 4.7 Cumulative mortality (sum of mortality in all 4 beakers for each condition) of larvae is shown for the 6 days of exposures and one day recovery (Day 1 to Day 7) for the control and all exposure treatments.

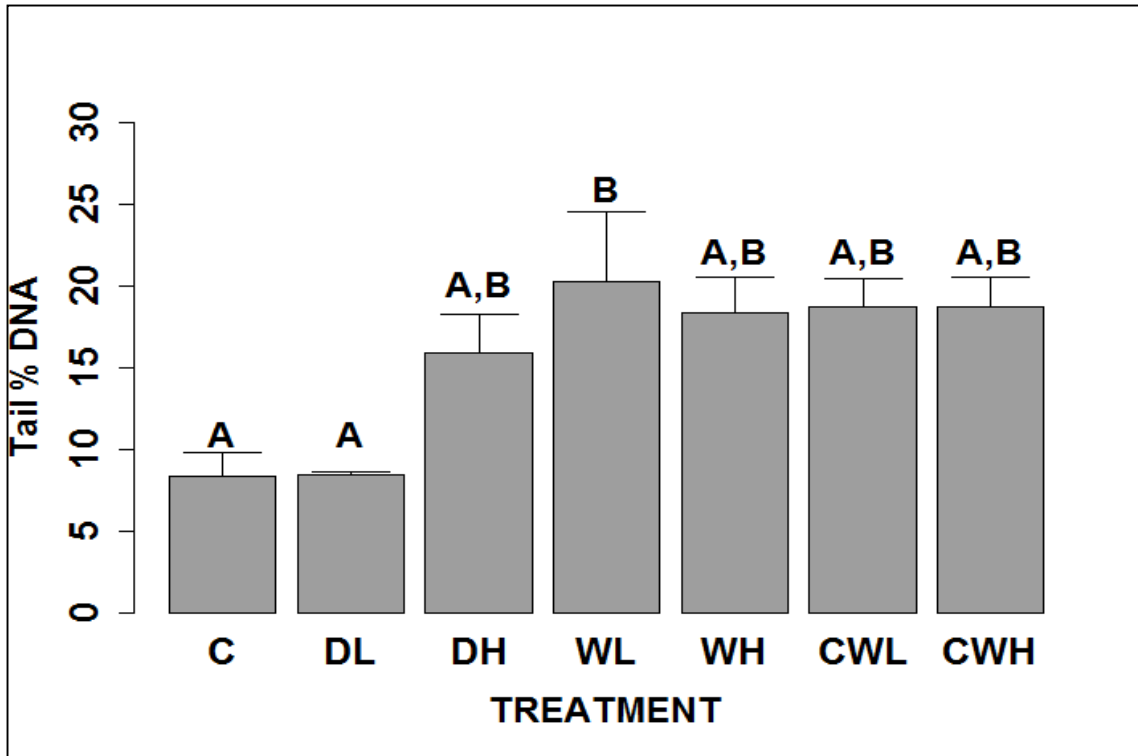


Fig. 4.8 Whole larvae DNA damage as percent DNA in tail shown as tail % DNA (mean + SEM). Different letters signify significant differences ($p < 0.05$).

larvae in the CEWAF low beakers sank to the bottom. However, within 6 hours there was no obvious difference in mobility between the CEWAF and the other treatments. This narcosis response was not observed on Day 0, but as we were not specifically scoring for this response it might have occurred without being noticed. Additionally, the TPAH concentrations were higher in the Day 3 CEWAF preparations than those from Day 0, which could potentially account for this response only being observed on Day 3.

4.4.2.4. DNA damage

The preparation of the Comet slides resulted in very few cells per slide, so all possible cells from a slide were scored for every duplicate slide for each of the $N=4$ samples (one sample per treatment beaker). Given the time constraints to examine this, only Day 6 results were analyzed for whole body larvae. The percent of DNA in the tail (tail % DNA) was examined as the most useful endpoint. The mean and standard error of mean for tail % DNA for each treatment is shown in Figure 4.8. Only larvae from WAF low doses had significantly higher tail % DNA from the control larvae ($p<0.05$). However, both WAF and CEWAF high and low doses had over a 2-fold increase in tail % DNA from the control larvae.

4.4.2.5. Gene expression

The standard curve for the putative blue crab GST primers and linearize plasmid standards (3-3,000,000 copies) shows high qPCR efficiency with a slope of -3.34 and an efficiency of 99.2% with each standard run in triplicate (Fig. 4.9). The standard curve for MnSOD primers and linearized plasmid standards (30-3,000,000 copies) also shows high

qPCR efficiency with a slope of -3.32 and an efficiency of 100% (Fig. 4.9) with each standard run in triplicate. In juvenile blue crabs, this putative GST gene is expressed close to 1,500 copies per ng of RNA (1,500,000 copies μg^{-1} RNA) and the MnSOD gene expression levels are close to 7,000 copies per ng RNA (7,000,000 copies μg^{-1} RNA), in pre-exposure baseline levels within hepatopancrease tissue (see Chapter 3). However, neither gene has been examined yet in blue crab larvae.

Unfortunately, the results for both GST and MnSOD gene expression could not be accurately quantified. One of the biggest problems observed in the gene expression results was the presence of primary or secondary peaks with lower than expected melting temperatures in the dissociation curves. These incorrect peaks were not observed in even the lowest of the standards, previous qPCR runs with these genes in juvenile blue crabs (Chapter 3), or in the no-template controls. The MnSOD gene expression results from Days 0, 3, and 6 had almost entirely primary or secondary dissociation peaks with melting temperatures lower than expected based on the standards. Therefore, the MnSOD gene expression could not be quantified. While these secondary peaks were still present in some of the GST samples, there were enough GST qPCR results from Day 6 with only peaks of the correct melting temperature to warrant further examination.

The linear-regression determined by the linearized plasmid standard curve was used to calculate the GST copy number within each sample for the different treatments, which was subsequently normalized to μg of RNA (Table 4.7). This first qPCR run was conducted in triplicate, but not all replicates had single dissociation peaks to use for the analysis. Therefore, the results in Table 4.7 for the first qPCR run represent one or the average of two replicates only. Many of the results were below the lowest copy number

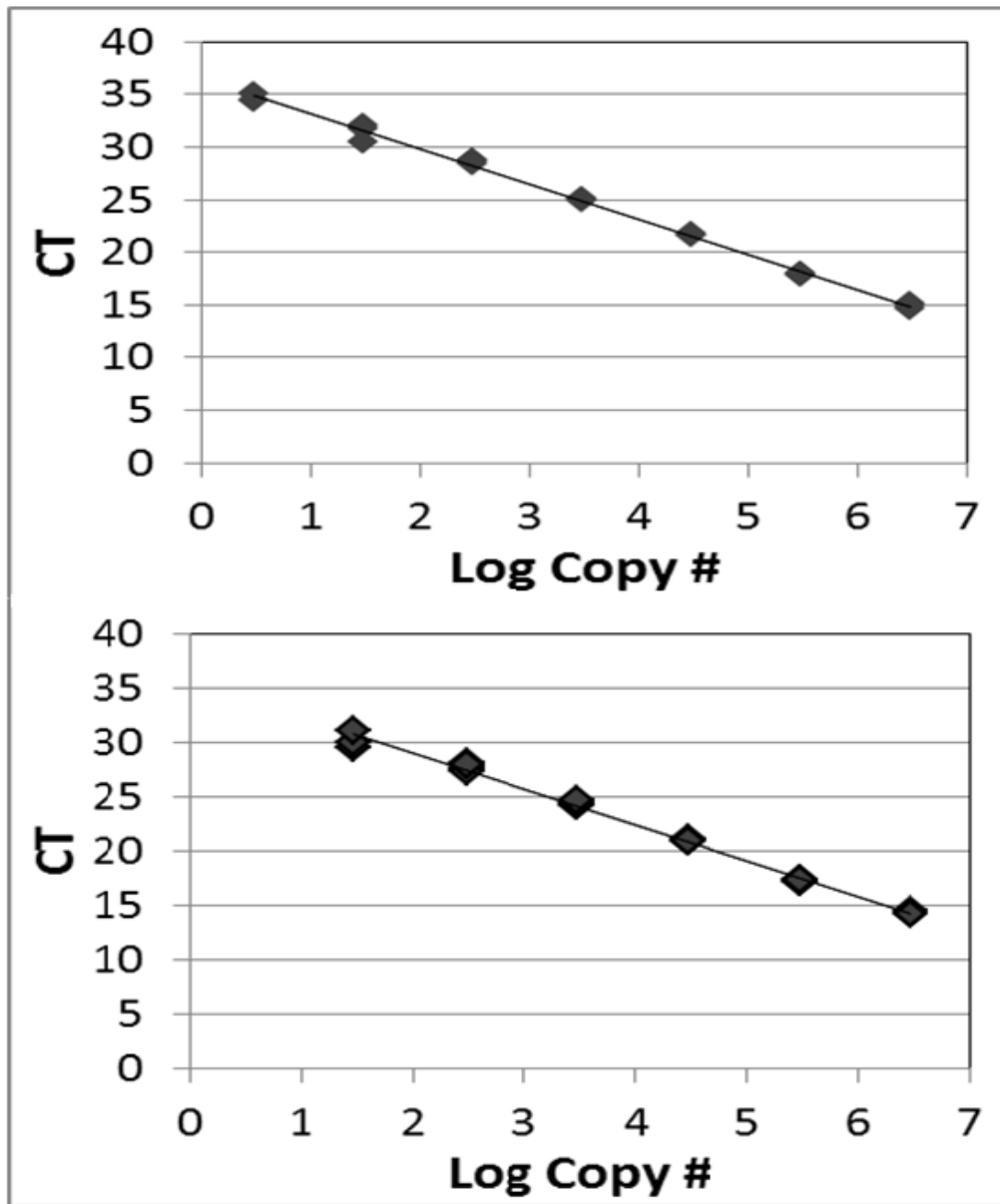


Fig. 4.9 Standard curve for GST (top) and MnSOD (bottom) linearized plasmid serial dilution from 3-3,000,000 copies for GST and 30-3,000,000 copies for MnSOD

Table 4.7 Whole larvae GST gene expression from larvae collected on Day 6 as copy number and copy number normalized to RNA (μg) for the two qPCR trials.

Treatment		qPCR Run-1		qPCR Run-2	
Name	Beaker #	Copy #	Copy # μg^{-1} RNA	Copy #	Copy # μg^{-1} RNA
Control	1	4.09	163.41	NA	NA
	2	1.07	42.79	0.42	8.40
	3	0.63	25.07	NA	NA
	4	NA	NA	NA	NA
Dispersant Low	1	0.71	28.38	0.31	6.20
	2	2.12	84.90	NA	NA
	3	NA	NA	4.24	84.80
	4	1.53	61.37	NA	NA
Dispersant High	1	3.56	142.37	1.80	36.00
	2	2.58	103.04	0.84	16.80
	3	1.47	58.92	0.64	12.80
	4	0.75	29.99	NA	NA
WAF Low	1	1.27	50.98	2.52	50.40
	2	1.48	59.33	0.25	5.00
	3	2.01	80.35	NA	NA
	4	NA	NA	NA	NA
WAF High	1	NA	NA	0.55	11.00
	2	NA	NA	249.14	4982.80
	3	6.30	251.97	2.36	47.20
	4	NA	NA	1.56	31.20
CEWAF Low	1	5.20	208.00	2.90	58.00
	2	2.23	89.31	0.47	9.40
	3	NA	NA	NA	NA
	4	NA	NA	NA	NA
CEWAF High	1	8.03	321.10	NA	NA
	2	2.04	81.46	1.14	22.80
	3	14.33	573.23	15.22	304.40
	4	NA	NA	0.35	7.00

*NA means that there was a double peak or a peak at the wrong T_m in the dissociation curve

used in the standard curve (3 copies) and some were even below 1 copy, which does not make sense biologically. This suggests that there might be something either wrong with the RNA/cDNA quality or something inhibitory in the reaction.

4.5. Discussion

The blue crab, *C. sapidus*, is an important species to the ecology and economy of the Atlantic coast and Gulf of Mexico. In spite of this, very little is known about the impacts of oil or chemically dispersed oil on early life stage blue crabs. The distribution of blue crab zoea on the surface of open, coastal waters (Epifanio, 1995; Johnson and Perry, 1999) would put them at risk of exposure to high concentrations oil and chemically dispersed oil in an oil spill event. Blue crab recruitment and subsequent fishery abundance depends on the survival of larvae in the coastal, open ocean. Therefore, it is imperative to understand the biological impacts of such chemicals on early stage zoea.

To account for this critical data gap, this study sought to examine the sublethal impacts of two doses each of WAF (1.53-2.76 and 21.03-28.18 $\mu\text{g TPAH L}^{-1}$), CEWAF (12.56-28.08 and 280.77-327.21 $\mu\text{g TPAH L}^{-1}$), and dispersant (0.5 mg L^{-1} and 5 mg L^{-1}) over a 6 day exposure period. The doses for all WAF and the CEWAF low exposures were within environmental TPAH concentrations (84.8 $\mu\text{g L}^{-1}$) observed in the surface waters near the MC252 wellhead following the DWH incident (Diercks et al., 2010). While the CEWAF high doses were 3- to 4-fold higher than this surface TPAH concentration, the exposure to these high concentrations would have been relatively short given the rapid loss of TPAH observed.

The doses of WAF, CEWAF, and dispersant were chosen to be below the approximate LC₅₀ values observed in preliminary tests. The final nominal oil concentration chosen for the high dose (50mg oil L⁻¹ ASW) was over 2-fold lower than the LC₅₀ dose for CEWAF (121 mg oil L⁻¹) in the latest stage larvae examined with definitive results. Additionally, the final nominal dispersant concentration for the high dose (5 mg dispersant L⁻¹ ASW) was almost 8-9-fold lower than the LC₅₀ dose for Corexit 9500 (42.5 mg oil L⁻¹) in the latest stage larvae examined with definitive results.

Overall, low mortality (6%) was observed in all exposures over the 6 day exposure period. However, the CEWAF high dose doubled to 12% mortality after 3 days of recovery, which could indicate a delayed toxicity. This highlights the need to extend future studies longer to fully understand the long-term impacts of acute exposures on larval development. While the mortality was low during the 6 days of exposure, a strong narcotic response was observed in larvae in the CEWAF treatments, especially in the high dose, with the addition of fresh exposure solutions on Day 3. Narcosis is generally considered to be one of the primary contributors of acute toxicity for organisms exposed to oil as many of the lower molecular weight PAHs (and the more volatile mono-aromatic hydrocarbons) are considered type I narcotic chemicals with an additive toxicological effect (DiToro et al., 2000). The arrested activity resulting from exposure to narcotic chemicals can be reversible, as was seen in this study, but can also lead to direct mortality if the concentrations of total narcotic chemicals exceeds a threshold in target lipids as is suggested in the narcosis target lipid model (McGrath et al., 2004).

However, even if the concentrations of narcotic chemicals are low enough for the response to be reversible, the temporary immobility could still indirectly remove effected

individuals from the population. Larval blue crabs display vertical migrations that allow them to optimize feeding and predator avoidance as well as swimming behaviors that result in their movement from coastal zone into estuarine waters at the larval-megalopa transition (Epifanio, 1988; Blaxter and Ten Hallers-Tjabbes, 1992). While this narcosis impact on larval mobility might not directly result in mortality (as the larvae recovered mobility within 6 hours), it could disrupt this vertical migration and the ability to maintain position in the water column. This disruption could result in decreases in feeding rates and increased predation as well as ultimately disrupting their ability to persist in the coastal zone or recruit into nursery habitat. This narcosis response was also observed in blue crab larvae exposed to Corexit 9500 only solutions (Chapter 5). However, this occurred in nominal concentrations higher than the 5 mg L⁻¹ high dose used in this study.

To address sublethal impacts of oil, dispersed oil and dispersants on blue crab larvae, molecular biomarkers for genotoxicity and oxidative stress were examined. The Comet assay demonstrated at least a 2-fold increase in larval DNA damage for all the CEWAF and WAF exposures from controls, but only the low dose WAF exposure was significantly higher. The dispersant high dose also had about 1.5-fold higher DNA damage than controls. A more substantial difference might have been observed if slides had higher cell counts to allow for a more thorough analysis. In invertebrates exposed to oil or PAH, DNA damage is considered to be primarily a result of the production of ROS through the metabolism and activation of PAHs, like benzo[a]pyrene (BaP), involved in redox cycling (Livingstone 2003). Studies in various marine invertebrate species (primarily bivalves) have shown increases in DNA damage with exposures to individual

PAHs, particularly BaP, or in PAH/oil contaminated sediments and water as reviewed by Mitchelmore and Chipman et al. (1998) and more recently by Frenzilli et al. (2009).

Unfortunately, due to inhibition in the cNDA and/or possible poor quality RNA template, no reliable data could be obtained for the biomarker genes involved in detoxification and oxidative stress (GST and MnSOD) from this experiment. Therefore, we could not make any statements or speculate regarding a possible link between oil exposures and levels of MnSOD or GST gene expression. While results examining changes in GST gene expression or GST enzyme activity in crustaceans exposed to xenobiotic compounds, particularly oil and PAHs, is limited, a few field studies have examined the relationship between GST and PAHs (Lee 1988, Martín-Díaz et al. 2007, 2008, and Morales-Caselles et al., 2008). The study by Lee (1988) showed increases in hepatopancrease GST activity in field collected shore crab, *Carcinus maenas*, with increases in tissue PAH concentrations. However, studies examining 28 day exposures of *C. maenas* to contaminated sediments collected from Spanish ports in the laboratory or in field cages did not show any correlations between PAH concentrations and various biomarkers of oxidative stress and metabolism of xenobiotic compounds in hepatopancrease, including glutathione *S*-transferase (GST), glutathione reductase (GR), ethoxyresorufin O-deethylase (EROD), and glutathione peroxidase (GPx) activities (Martín-Díaz et al., 2007, 2008). Additionally, another 28 day exposure of *C. maenas* to contaminated sediments collected from different areas around the Spanish coast in the laboratory did show significant correlations between PAH concentrations and EROD activity, but not GST activity (Morales-Caselles et al., 2008). Other marine crustaceans, particularly marine copepods, have shown inductions of GST gene expression with

exposure to naphthalene and water accommodated fractions of oil (Hansen et al. 2008, Hansen et al. 2011).

While not all of the molecular endpoints were able to provide definitive results for understanding the sublethal impacts of oil and dispersed oil on blue crab larvae, the PAH chemistry and particle analysis conducted with the experiment serves to highlight how essential it is to fully characterized exposure solutions. Filtration of the stock solutions allowed us to estimate and define the amount of PAHs in the dissolved and particulate fractions in order to compare the influence of these routes of exposure to blue crab larvae. Even though the CEWAF and WAF exposures were prepared using the same oil loading rates and linear dilutions for final sublethal exposures, the addition of chemical dispersants in the CEWAF preparations resulted in greater than an order of magnitude higher TPAH concentrations than in the WAF preparations without the addition of dispersant. Without the use of filtration to examine the nature of this increase, it would be unclear whether the increase in TPAH in CEWAF was a result of increased oil droplets (i.e. particulate phase) or a result of enhanced dissolution of PAHs into the dissolved phase. The filtration results conducted indicate that the increase in TPAH in CEWAF preparations is primarily due to enhancement of oil in the particulate phase as there was not an appreciable difference in the TPAH of filtered samples between WAF and CEWAF preparations. The enhancement of the oil particulate phase in CEWAF preparations was further confirmed by the substantial reduction of particles with filtration of CEWAF preparations in the particle analysis.

The difference in dissolved versus particulate phase is particularly important for invertebrate species that can have different routes of exposure based on the extent of oil

each phase. Generally, the higher the solubility of a PAH in water (i.e. lower K_{ow}), the more bioavailable it is to impact marine organisms as dissolved hydrocarbons are more likely to diffuse across gills, skin, or other exposed membranes (NRC, 2005). Whereas, the particulate oil tends to impact marine species more through physical coating of gills or other bodily surfaces. However, ingestion of particulate oil can be an important route of exposure to PAH for marine invertebrates like filter-feeding bivalves or larval stages of species that feed on prey ranging from a few to a couple hundred μm sizes. A few studies have examined the consumption of phytoplankton by portunid crab larvae in field and laboratory conditions (Harms et al., 1994; Johnson and Allen, 2013). However, the prey preference and selection of zoeal stage larvae is not well studied in wild crab species. Hatchery raised blue crab larvae feed on range of prey sizes, including phytoplankton ($<10 \mu\text{m}$), rotifers (100-290 μm), and artemia ($>450 \mu\text{m}$). The size of prey the larvae can consume increases as the zoea continue to molt with the earliest zoeal stages unable to consume prey as large as *Artemia* sp. nauplii. However, the exact gape size of zoea larvae at their different molt stages has not been reported in the literature. This would suggest that it is likely that the larvae used in this experiment were capable of consuming oil particles from both WAF and CEWAF exposures as the average particle sizes ranged from 4.5-14.3 μm . The larvae exposed to the CEWAF high and low doses could be more impacted by this as they had substantial higher oil particles than the WAF exposures. However, it is unclear how much oil in the droplet/particulate phase was ingested by larvae.

Additionally, the loss of PAHs observed over the two three-day periods was important to consider. The rapid loss during the experiment could be due to the WAF and

CEWAF preparations being enriched in low molecular weight PAHs, particularly alkylated naphthalenes, which tend to be more volatile. This loss is not unusual as lower molecular weight hydrocarbons tend to be removed rapidly from the environment during oil spill events as the oil weathers (McAuliffe, 1989). Examining this exchange of PAHs across the air-water interface is still the focus of ongoing research (Fang et al., 2012). While the ability to measure PAHs in the frequency needed to examine this loss more fully was not possible for this study, other studies have shown the loss of PAHs in oil and dispersed oil exposures to occur most rapidly within 24hr in open exposures (Fucik et al., 1995). Additionally, short term static exposures like this study that mimic short term pulses might be more representative of environmental exposures (Coelho et al., 2013), as compared to continuous flow-through exposures.

In general, the particle counts and volumes followed a similar reduction over time as TPAH with a more pronounced reduction (2- to 3-fold reduction) in CEWAF exposures. The TPAH concentrations and particle counts in the abiotic solutions for each treatment showed similar reductions over time to the biotic solutions indicating that there was no appreciable change in PAH concentrations due to the addition of larvae. It is interesting to note that significant levels of particles were still detectable in the CEWAF high solutions after 3 days, while TPAH levels were very low to non-detectable. It is possible that the particles are oil, but the LMW PAHs (i.e. naphthalene and alkylated naphthalenes) that account for the majority of TPAH concentrations have partitioned out into the dissolved fraction of the exposure water and were ultimately lost from the exposure solution due to the atmosphere.

4.6. Conclusion

Overall, the results of this study demonstrate that early life stage blue crabs are impacted by sublethal concentrations and short-term exposure durations of oil and dispersed oil. Exposure to oil and dispersed oil for 6 days showed elevated levels of DNA damage and the larvae exposed to dispersed oil displayed a temporary narcosis response. This narcosis and the delayed mortality observed in the highest CEWAF exposures may have implications for the long-term survival and fitness of larvae in situations where dispersants are used in oil-spill remediation.

Additionally, this study highlights the need to consider the specific route of exposure of potentially impacted species to PAHs when determining the appropriate response options following an oil spill as the use of chemical dispersants on oil substantially increased the amount of PAHs in the particulate phase. Therefore, careful consideration should be taken before dispersants are used in blue crab spawning areas during this critical early stage of development.

While this study laid the ground work to understanding the impacts of oil and dispersed oil on early life stage blue crabs, additional studies should be conducted to further examine the impacts of oil and dispersed oil on detoxification and oxidative stress biomarkers in larval blue crabs, particularly a repeat of the molecular analysis attempted in this study. Furthermore, the impacts of oil and dispersed oil should be examined over the full range of larval stages (younger zoea to megalopa). Future studies should be also conducted for longer exposure times to assess growth and delayed responses in order to more fully assess the impacts to such an ecologically and economically important species.

4.7. Acknowledgments

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Chapter 5. Determining the acute toxicity of current and alternative oil spill chemical dispersants to early life stage blue crabs (*Callinectes sapidus*).⁴

5.1. Abstract

The aim of this study was to examine the acute toxicity of five oil spill chemical dispersants on the ecologically and economically important coastal and estuarine species, blue crab *Callinectes sapidus*. Static, non-renewal 48 hr acute toxicity tests were performed on stage-II blue crab zoea. The median lethal concentration (LC₅₀) was calculated for each dispersant at 24 hr and 48 hr using nominal concentrations for each dispersant tested. The 48 hr LC₅₀ values from the most to the least toxic ranged from 10.1mg L⁻¹ for Dispersit SPC 1000 to 76.5 mg L⁻¹ for Orca. For all dispersants, the swimming activity and mobility of larvae decreased with increasing dispersant concentration within 24 hr of exposure and reached relative immobility at concentrations below LC₅₀ values. These results show that the dispersants examined in this study are only slightly toxic after 48 hr exposure to the earliest life stage of blue crabs that might likely be exposed to dispersants in the environment, with the exception of Dispersit SPC 1000 that bordered between slightly and moderately toxic. Although the dispersants themselves appear to not cause substantial acute toxicity, sublethal and potentially delayed impacts, such as, reduced mobility or food source availability could indirectly remove larvae from the population and need to be further examined. Furthermore dispersants are not released into the environment in isolation and so the impact of

⁴ Hannah V. Pie, Carys L. Mitchelmore. In preparation for submission.

dispersed-oil using these dispersant formulations needs to be investigated to translate into real-world situations.

5.2. Introduction

The rising demand for petroleum-based products over the last few decades has resulted in an increase in oil pollution within marine environments from multiple sources, including oil spills/leaks with transportation and extraction processes (e.g. Exxon Valdez and Deepwater Horizon incident). Exposure to oil and its constituents can be highly detrimental to marine organisms and can result in acute toxicity (mortality) as well as sublethal (chronic) and delayed effects that can impact an organism's growth, reproduction and potentially have population-level consequences (Hylland et al., 2008; NRC, 2003). Multiple means of remediation are used to reduce the overall impacts from oil spills and leaks on marine ecosystems. While the primary means of clean-up remains mechanical containment and recovery of the oil slick, chemical dispersants are used as a secondary mechanism to reduce the environmental and economic impact of an oil spill. Chemical dispersant use in oil spill response strategies has always been controversial, particularly given the data gaps and uncertainties regarding their chronic, sublethal, and delayed impacts (NRC, 1989; NRC, 2005). The decision to use dispersants on both surface and underwater source oil is based upon minimizing the overall environmental harm of oil, coupled with human health and economic considerations. For example many of these environmental tradeoff decisions are aimed at reducing the amount of oil reaching sensitive and highly productive shoreline habitats whilst potentially increasing oil impacts for pelagic (water column) and benthic (bottom dwelling) organisms.

Dispersants are generally pre-approved for use in waters beyond 3 miles of the shoreline and water depths greater than 10m (NRC, 2005). Dispersants increase the generation of small droplets of oil, which enter into the water column by wave energy, dissipating and diluting the oil rapidly. These oil droplets remain suspended in the water column and are bioavailable to water column organisms especially within the top 10m before they dilute in three dimensions over time post-spill.

Corexit 9500 and the older formulation, Corexit 9527, have been the primary dispersants utilized in the U.S. and their toxicity has been extensively studied worldwide in multiple species and life stages as reviewed by the NRC (1989, 2005) reports. However, very little is known about the impacts of oil, chemically dispersed oil, or chemical dispersants on blue crabs, *Callinectes sapidus*, especially at the early larval stages. To date, only one study has been published examining the acute toxicity of Corexit 9527, oil, and Corexit 9527 dispersed oil on blue crab megalopa (Fucik et al., 1995).

The blue crab is a keystone species for the Atlantic coast and Gulf of Mexico both ecologically and economically. Juvenile and adult blue crabs are benthic dwelling organisms that live in lower salinity estuaries and coastal rivers with brackish water (Tagatz, 1968a), which are not pre-approved areas for dispersant use. Therefore, adult and juvenile blue crabs would be less likely to be exposed to dispersants and chemically dispersed oil. However, larval blue crab zoea migrate from the spawning grounds at the mouths of estuaries to the open waters of the continental shelves (within pre-approved regions) where they live a planktonic existence on the surface, particularly the upper 3m including the neuston (Epifanio, 1995; Johnson and Perry, 1999). Therefore, blue crab

zoea have a potential to be impacted by the use of chemical dispersants in an oil spill event, especially those like the Deepwater Horizon (DWH) incident that co-occur with crab spawning. Furthermore, in many species the most sensitive periods to pollution exposure are the early, larval life stages (McKim, 1995).

Given the extensive use of dispersants (Corexits 9500 and 9527) during the DWH incident and EPA's call for BP to use a less toxic alternative than Corexit 9500 (US EPA, 2010), there has been an increased emphasis on the potential use of less toxic, but equally or more effective dispersant alternatives. While Corexit 9500 (and previously Corexit 9527) is the most commonly used dispersant, numerous alternative dispersants listed under the EPA's National Contingency Plan (NCP) Product Schedule are also available (US EPA, 2014). However, very few toxicity studies have been conducted on these alternatives beyond the data directly supplied to the EPA from the manufacturers (US EPA, 2014) and the EPA's recent toxicity tests by Hemmer et al. (2010).

Considering the potential impacts of Corexit products and more importantly, oil dispersed by Corexit products on many invertebrate species as reviewed in the NRC (2005) report, it is important to fully investigate the toxicity of alternative dispersants from the NCP Product Schedule to know if any of these dispersants are less harmful to blue crabs; and therefore, could be more suitable alternatives to use in remediation of oil spills.

Therefore, this project sought to examine the acute toxicity of multiple chemical dispersants used for oil spill remediation on early life stage blue crabs, *Callinectes sapidus*, in comparison to Corexit 9500. The overall goal of this study was to provide data on the sensitivity of early life stage blue crabs to the currently used chemical dispersant, Corexit 9500, in comparison to four alternative chemical dispersants,

including one microbially-based dispersant, to determine if a less toxic option might be available for use in oil spill remediation in areas containing larval blue crabs. Examining acute toxicity of dispersants is a crucial first step in understanding the impacts of dispersants, oil, and dispersed oil on larval blue crabs. For a full study other longer-term sublethal, delayed and indirect impacts of these dispersants need to be investigated and toxicity trade-offs (if any) compared with effectiveness of the formulations.

5.3. Materials and Methods

5.3.1. Study design

In this study, the acute toxicity of five chemical dispersants to blue crab, *Callinectes sapidus*, larvae was examined. The larvae used for the experiment were stage II zoea (ZII). ZII larvae were utilized as they are the earliest life stage of larvae found predominantly in the open ocean environment along the continental shelf (Epifanio, 1995). Stage I zoea are found in substantial numbers at the mouths of estuaries and coastal areas where spawning occurs and are then transported out to open waters of the continental shelf (Epifanio, 1995). The blue crab larvae used for testing were provided by the Institute of Marine Environmental Technology-Aquatic Research Center (IMET-ARC)'s blue crab hatchery. The larvae were hatched and raised in-house under standard conditions until the experiment.

Three of the dispersants were selected based upon their current availabilities as well as their acute toxicities (48 hr LC₅₀ values) to juvenile mysid shrimp as detailed in the NCP Product Schedule (US EPA, 2013) and the study by Hemmer et al. (2010). The dispersants used in this study include Corexit 9500 (Nalco), Sea Brat #4 (Alabaster

Corp.), and Dispersit SPC 1000 (US Polychemical Corp.). Two additional dispersants were provided by Alabaster Corp. for analysis including a microbially-based dispersant/surface washing agent, Petro-Clean, and a new formula dispersant, Orca. Orca is not on the NCP Product Schedule nor was it tested in the study by Hemmer et al. (2010). Petro-Clean is a pH-neutral blend of nonionic surfactants and emulsifiers used for petroleum bioremediation, but is classified by the NCP Product Schedule as a surface-washing agent (US EPA, 2013). Petro-Clean contains naturally-occurring, non-pathogenic microorganisms used to aid in the degradation process and is the least toxic surface-washing agent listed on the NCP Product Schedule (US EPA, 2013).

The experiment was a standard 48 hr static acute toxicity test following the EPA 821-R-02-012 method (US EPA, 2002). The exposure waters were non-renewable, given the sensitivity of the blue crab larvae to mechanical stress, and spanned a log-range of dispersant concentrations that were prepared with filtered artificial seawater (ASW) as well as a control of ASW only. The ASW used in the experiments was prepared by the ARC facility staff and adjusted to 30 ppt with charcoal filtered municipal water then filtered with a 0.2 μm filter. Exposures were carried out in 20 mL glass scintillation vials with each vial containing 10 mL of test exposure solution and 5 total larvae. There were 8 replicate scintillation vials ($N=40$ zoea) for each dispersant test solutions and 9 replicate scintillation vials ($N=45$ zoea) for ASW only control. Scintillation vials were cleaned and baked at 500°C for 4 hours prior to use.

The vials were not sealed during the experiments, but lightly covered with plastic wrap to allow light penetration and air transfer. No water change was carried out (as mentioned above) and water quality parameters, including dissolved oxygen (D.O.), pH,

salinity, ammonia and temperature, did not change appreciably over the 48 hr period in preliminary trials. Organisms were fed daily with 10 μL of concentrated rotifers (*Brachionus* sp.). Scintillation vials were maintained at 21-23°C under a photoperiod of 14:10 hr light:dark. Water quality parameters were monitored at time 0 hr and 48 hr.

Dispersant solutions were made by dilution from a stock of the highest concentration tested using filtered ASW at 30ppt. The highest dispersant stock concentration was prepared using a glass syringe to add the dispersant to a glass aspirator bottle with 1 or 2 L ASW stirring (in the dark) with a standard 25% vortex for 18 hours. The exact loading rate of each dispersant was determined by pre-weighing an empty glass syringe, weighing the syringe with dispersant added, re-weighing the syringe after adding the dispersant to the filtered seawater, and then calculating the mass difference. The experiments were conducted using a range of concentrations based on previous work done by the Mitchelmore lab with blue crab larvae exposed to Corexit 9500, the LC_{50} concentrations of blue crab larvae exposed to Corexit 9527 (Fucik et al., 1995), and the LC_{50} concentrations of the alternative dispersants on mysid, *Americamysis bahia* (Hemmer et al., 2010). The concentrations used for the experiment are detailed in Table 5.1. It is important to note that nominal concentrations are reported as mg L^{-1} in this experiment as compared to $\mu\text{L L}^{-1}$ in other experiments. The specific gravity of the dispersants used in this experiment range from 0.95 for Corexit 9500 to 1.02 for Orca and Petro-Clean, which means the results reported in the different units above should be comparable, but not exactly equivalent.

Table 5.1 Nominal concentrations of chemical dispersants used in experiments

Dispersant Type	Concentrations (mg L⁻¹)
Corexit 9500	10, 25, 50, 75, 100, 125, 150
Seat Brat #4	10, 50, 75, 100, 125, 150, 175, 200, 225
Orca	10, 50, 75, 100, 150, 175, 200
Petro-Clean	10, 25, 50, 75, 100
Dispersit SPC 1000	0.5, 1, 10, 25, 50, 75, 100, 125, 150

5.3.2. *Mortality and Mobility*

Mortality of zoea in each treatment was recorded at 24 hr and 48 hr by examining the larvae under a dissecting microscope at 20x magnification without removal from the scintillation vials. The time spent under the light of the microscope was minimized to reduce stress on the larvae. A blue crab zoea was designated dead if there was no longer movement in maxillipeds or a visible heart-beat. All deceased larvae were removed from the vials as soon as possible to minimize impacts on water quality. Mobility and general swimming activity were also observed and the concentration at which most larvae were immobile was determined for each dispersant.

5.3.3. *Statistical analysis*

The lethal dispersant concentration at which 50% of individuals exposed are dead (LC_{50}) compared to the blue crabs in the controls after 24 hr and 48 hr exposures were determined for all five dispersants. No mortalities occurred in the control (ASW only) zoea, so the mortalities of crabs in exposure treatments did not need to be normalized to the control mortalities. The LC_{50} values and their 95% confidence intervals were calculated using a probit linked binomial generalized linear model in R using the MASS package. Plots of proportion of dead larvae over log dispersant concentrations were produced and lines of the regression model and its 95% confidence values were fitted to the data. McFadden's Pseudo R^2 was calculated for each model.

Table 5.2 Median lethal concentration (LC₅₀, mg L⁻¹) values and 95% confidence intervals in brackets for *C. sapidus* 24 hr and 48 hr static acute toxicity tests with five dispersants

Dispersant	LC ₅₀ mg L ⁻¹ [95% Confidence Interval]	
	24 hr	48 hr
Corexit 9500	105.6 [91.7-121.7]	55.0 [47.5-63.8]
Dispersit SPC 1000	41.0 [30.6-55.0]	10.1 [6.2-16.4]
Orca	169.7 [148.0-193.4]	76.5 [66.4-88.1]
Petro-Clean	>100*	52.0 [46.5-58.2]
Sea Brat #4	105.2 [96.1-115.4]	41.0 [33.6-50.1]

*Exact LC₅₀ could not be determined

5.4. Results

Control performance (ASW only) met all criteria for acceptable toxicity test exposure ($\times 90\%$ survival) with 100% survival of larvae for 48 hr exposure. All water quality parameters were within acceptable ranges. The 24 hr LC_{50} values ranged from 41.0 mg L⁻¹ for Dispersit SPC 1000 to 167.7 mg L⁻¹ for Orca (Table 5.2). The concentrations tested for Petro-Clean exposures were not within the range to determine a 24 hr LC_{50} value. The highest concentration tested for Petro-Clean was 100 mg L⁻¹ which resulted in only 47.5% mortality with 24 hr exposure. Concentration-response curves with regression models and McFadden's Pseudo-R² values for 24 hr exposures are presented in Figure 5.1. Using the US EPA toxicity classification system for aquatic organisms (US EPA, 2012), all dispersants would be classified as practically non-toxic, except Dispersit SPC 1000, which was slightly toxic to *C. sapidus* larvae with 24 hr exposure.

In addition to examining mortality, a decrease in swimming activity and mobility of larvae was observed with increasing dispersant concentrations for all five dispersants after 24 hr of exposure. The control larvae and the larvae exposed to the lowest concentrations of dispersants were highly active and moved throughout the water column. This active swimming decreased with increasing dispersant concentrations until an almost complete immobility was reached, which was below the 24 hr LC_{50} values for all 5 dispersants. At this point, larvae were inactive on the bottom of the exposure containers as opposed to moving throughout the water column, but were still alive as determined by movement of the maxillipeds or visible heat beat under a microscope. This immobility point at 24 hr occurred at 10 mg L⁻¹ for Dispersit SPC1000, 75 mg L⁻¹ for Corexit 9500, Sea Brat #4, and Petro-Clean, and 100 mg L⁻¹ for Orca. It is also interesting to note that

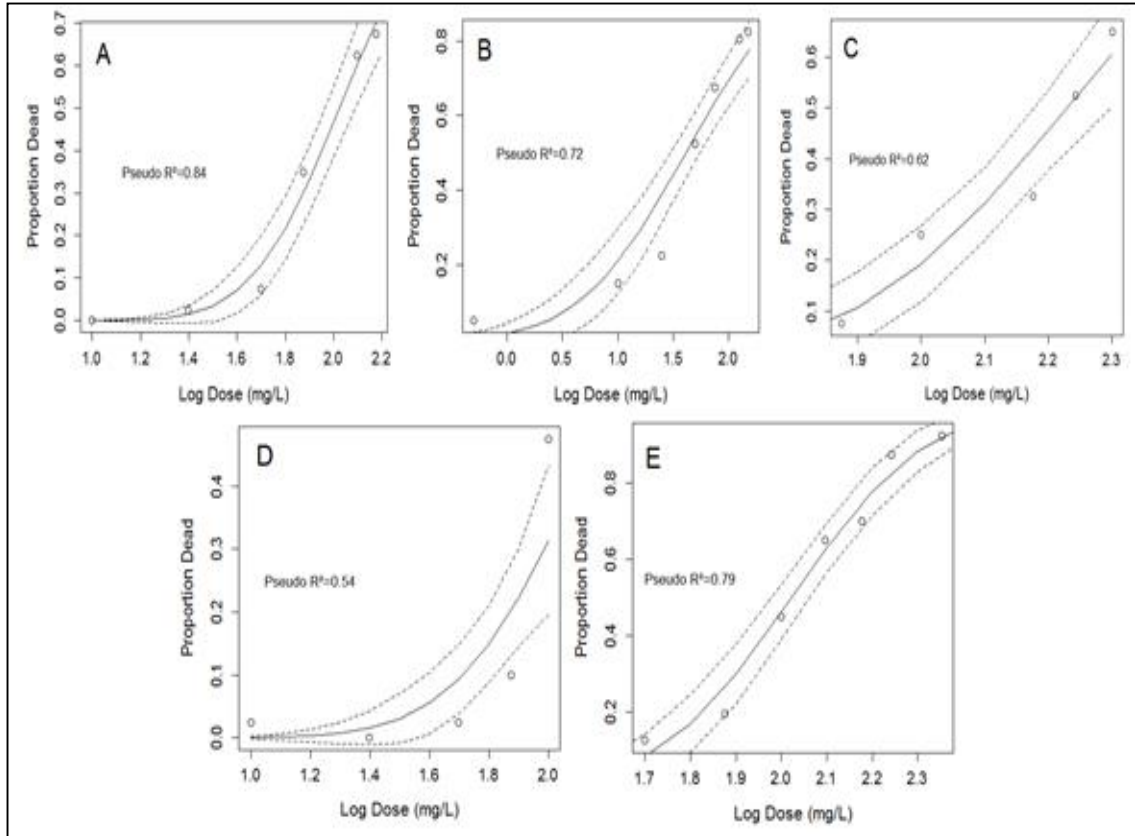


Fig. 5.1 Concentration-response curves for blue crab, *Callinectes sapidus*, ZII larvae exposed for 24 hr to Corexit 9500 (A), Dispersit SPC 1000 (B), Orca (C), Petro-Clean (D), and Sea Brat #4 (E). The probit linked binomial generalized linear model (solid line) and the 95% confidence intervals (dashed lines) for each model are shown for each dispersant. McFadden's Pseudo R^2 value is included for each model.

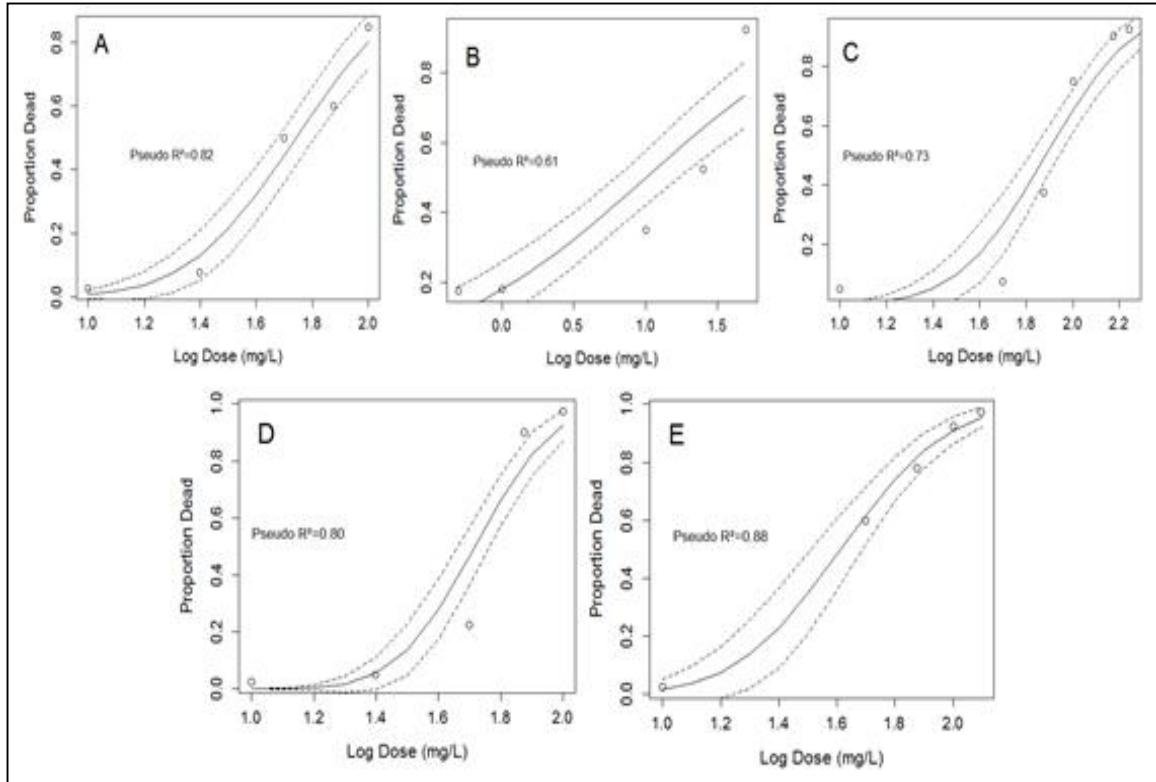


Fig. 5.2 Concentration-response curves for blue crab, *Callinectes sapidus*, ZII larvae exposed for 48 hr to Corexit 9500 (A), Dispersit SPC 1000 (B), Orca (C), Petro-Clean (D), and Sea Brat #4 (E). The probit linked binomial generalized linear model (solid line) and the 95% confidence intervals (dashed lines) for each model are shown for each dispersant. McFadden's Pseudo R^2 value is included for each model.

the rotifers (*Brachionus* sp.) that were not consumed by larvae were either dead or immobile at all but the lowest concentrations examined for each dispersant within 24 hr.

The 48 hr LC₅₀ values were lower for all dispersants than the 24 hr LC₅₀ values and ranged from 10.1 mg L⁻¹ for Dispersit SPC 1000 to 76.5 mg L⁻¹ for Orca (Table 5.2). Concentration-response curves with regression models and McFadden's Pseudo-R² values for 48 hr exposures are presented in Figure 5.2. Using the US EPA toxicity classification system (US EPA, 2012) all dispersants would be classified as slightly toxic, except Dispersit SPC 1000, which bordered between slightly and moderately toxic to *C. sapidus* larvae. By comparing LC₅₀ values and 95% confidence intervals, the rank order toxicity (most to least toxic) of the dispersants to *C. sapidus* larvae after 48 hr exposure was Dispersit SPC1000 > Sea Brat #4 > Corexit 9500 > Petro-Clean > Orca.

5.5. Discussion/Conclusions

The blue crab, *C. sapidus*, is an important species to the ecology and economy of the Atlantic coast and Gulf of Mexico. In spite of this, very little is known about the impacts of oil, chemically dispersed oil, or chemical dispersants on any stage of the blue crab life cycle. The distribution of blue crab zoea on the surface of open, coastal waters (Epifanio et al., 1995; Johnson and Perry, 1999) would put them at risk of exposure to potentially high concentrations of dispersants and chemically dispersed oil in an oil spill event. Blue crab recruitment and subsequent fishery abundance depends on the survival of larvae in the coastal, open ocean. Therefore, it is imperative to understand the biological impacts of such chemicals on early stage zoea.

Similar to Hemmer et al. (2010), the toxicity of Corexit 9500 was essentially equivalent to most of the dispersants tested with the exception of Dispersit SPC 1000, to which the blue crab larvae showed increased sensitivity. The new formula dispersant, Orca (Alabaster Corp.), was the least toxic of the dispersants tested (48 hr $LC_{50}=76.5 \text{ mg L}^{-1}$). Additionally, the dispersants used in this study, except Orca, were more acutely toxic to blue crab zoea than Corexit 9527 (96-hr $LC_{50}=77.9\text{-}81.2 \text{ mg L}^{-1}$) was to blue crab megalopa (Fucik et al., 1995).

With the exception of Corexit 9500, very few studies have examined the toxicity of the other four dispersants on aquatic organisms. Currently, Gulf mysid shrimp, *Americamysis (Mysidopsis) bahia*, is the most closely related species to compare the toxicity of the dispersants tested in this study with blue crab larvae. Hemmer et al. (2010) examined the acute toxicity (48 hr static exposures) of eight commercial oil dispersants, including Corexit 9500, Sea Brat #4, and Dispersit SPC 1000, on mysid shrimp (*Americamysis bahia*). The 48 hr LC_{50} values with 95% confidence intervals for *A. bahia* exposed to Corexit 9500, Dispersit SPC 1000, and Sea Brat #4 were $42 \mu\text{l L}^{-1}$ (38-47 $\mu\text{l L}^{-1}$), $12 \mu\text{l L}^{-1}$ (10-14 $\mu\text{l L}^{-1}$), and $65 \mu\text{l L}^{-1}$ (57-74 $\mu\text{l L}^{-1}$), respectively (Hemmer et al., 2010). These results were similar to those found on the NCP Product Schedule results except for Sea Brat #4, which showed high toxicities that were closer to Dispersit SPC 1000 (US EPA, 2013). The NCP Product Schedule results' 48 hr LC_{50} values for Corexit 9500, Dispersit SPC 1000, and Sea Brat #4 were $32.2 \mu\text{l L}^{-1}$ (26.5-39.2 $\mu\text{l L}^{-1}$; 95% CI), $16.6 \mu\text{l L}^{-1}$ (14.1-19.6 $\mu\text{l L}^{-1}$; 95% CI), and $14.0 \mu\text{l L}^{-1}$ ($\pm 10.4 \mu\text{l L}^{-1}$; SE), respectively (Hemmer et al., 2010). Additional studies by Clark et al. (2001) resulted in a 96-hr LC_{50} value of 35.9 mg L^{-1} (32.2-41.3 mg L^{-1} , 95% CI) for *Mysidopsis bahia* continuously

exposed to Corexit 9500. The Z II blue crab larvae in this study show similar sensitivity to Dispersit SPC 1000 and slightly less sensitivity to Corexit 9500 than Gulf mysid shrimp. The sensitivity of larval blue crab to Sea Brat #4 was between the two study results above for Gulf mysid shrimp.

Overall, the dispersants examined in this study are only slightly toxic to the earliest life stage of blue crabs that might likely be exposed to dispersants in the environment, with the exception of Dispersit SPC 1000 that bordered between slightly and moderately toxic. However, the LC_{50} values for all the dispersants are well above the estimated and measured concentrations of Corexit 9500 applied in the DWH (Nalco, 2010; Gray et al., 2014) and all but Dispersit SPC 1000 are above the maximum measured field concentration of dispersants, $13 \mu\text{l L}^{-1}$, described in George-Ares and Clark (2000). Dispersant concentrations in DWH applications were estimated to be 30 ppb to a depth of 10 m (Nalco, 2010). The highest surface concentration of dioctyl sodium sulfosuccinate (DOSS), a major surfactant component of Corexit 9500, measured in ocean waters during the DWH incident was $229 \mu\text{g L}^{-1}$ at one location with the rest of the sites examined having concentrations ranging from below the detection limit of $<0.25 \mu\text{g L}^{-1}$ to $24.5 \mu\text{g L}^{-1}$ (Gray et al., 2014). Additionally, previous studies have indicated that Corexit 9500 is expected to decrease in concentration by dilution below detection limits within hours of application (NRC , 2005; George-Ares and Clark, 2000). Therefore, ZII blue crab larvae are unlikely to be exposed to concentrations of dispersants that will result in extensive acute toxicity.

Although the dispersants themselves appear to not cause substantial acute toxicity, we did observed sublethal impacts on the swimming activity and mobility of the larvae with

concentrations well below the LC₅₀ values. Larval blue crabs display vertical migrations that allow them to optimize feeding and predator avoidance, as well as swimming behaviors that result in their movement from coastal zone into estuarine waters at the larval-megalopa transition (Epifanio et al., 1988; Blaxter and Hallers-Tjabbes, 1992). While impacts of larval mobility might not directly lead to mortality, they could disrupt this vertical migration and the ability to maintain position in the water column, which could result in decreases in feeding rates and increased predation as well as ultimately disrupting their ability to persist in the coastal zone or recruit into nursery habitat. Furthermore, an additional indirect impact of chemical dispersant use on larval blue crabs could be a reduction of food source availability. The rotifers, *Brachionus* sp., used for feeding in this study appeared to be much more sensitive to dispersant exposure than the blue crab larvae with all but the lowest concentrations of dispersants resulting in mortality or immobility. A recent study by Rio-Martínez et al. (2013) examined the acute toxicity of 5 *Brachionus plicatilis* strains to Corexit 9500 and saw 24 hr LC₅₀ values between 0.447-14.25 mg L⁻¹, which are much lower than those observed for blue crab larvae in this study. Therefore, impacts to larval blue crab survival and eventual recruitment back to nursery habitat could occur at dispersant concentrations below which acute mortality is observed.

While sublethal impacts on mobility were detected, this study did not address other sublethal or delayed impacts from acute or chronic dispersant exposures, particularly impacts on growth, molting and behavior, which could also result in the removal of blue crab larvae from the population. Additionally, chemical dispersants are not released into the environment in isolation, but in response to oil contamination. Therefore, the impacts

of dispersed-oil using these dispersant formulations should also be investigated, especially as it is generally not the dispersants alone, but the oil/dispersed oil that is the driving factors in acute toxicity when chemical dispersion is used in oil spill remediation (NRC, 2003). For *A. bahia* exposed to the chemically enhanced water accommodated fraction (CEWAF) of dispersant-Louisiana Sweet Crude (LSC) oil mixtures, the 48 hr LC₅₀ values ranged from 1.4-5.4 mg total petroleum hydrocarbons (TPH) L⁻¹ for Corexit 9500-, Dispersit SPC 1000-, and Sea Brat #4-LSC mixtures, resulting in these dispersant-LSC mixtures being classified as moderately toxic (Hemmer et al., 2010; US EPA, 2012). The WAF of LSC oil alone resulted in a 48hr LC₅₀ value of 2.7 mg TPH L⁻¹ (2.5-3.0 mg TPH L⁻¹, 95% CI) for *A. bahia*, which would also be considered moderately toxic (Hemmer et al., 2010; US EPA, 2012). It is also important to note that the effectiveness of the dispersant in dispersing oil should also be considered along with the toxicity of a dispersant and dispersed-oil. For instance, if different dispersants have similar toxicity when dispersing oil but different effectiveness, the more effective dispersant should be used for remediation as less of it would be needed to fully disperse an oil slick. This present study only addresses the acute toxicity of chemical dispersants, but a study including examining the impacts of oil and chemically dispersed oil as well would need to be conducted to have a more complete understanding of the impacts of using chemical dispersion in oil spill remediation on blue crab larvae.

5.6. Acknowledgements

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Chapter 6. Overall Conclusions

The Northern Gulf of Mexico continental shelf is one of the most ecologically productive and economically important regions in North America, but it is also an area with potential for high natural and anthropogenic exposure to oil and petrogenic PAHs. While the impacts of oil spill events on marine ecosystems have been well studied over the past half-century, disasters like the DWH incident serve to highlight how much we still need to learn and what data gaps remain in our understanding of the impacts of oil exposure and the use of chemical dispersants in oil spill remediation. This dissertation strove to address two such data gaps. The first is the lack of baseline biomonitoring data of offshore organisms in areas with exposure to oil and dispersed oil following an oil spill event within the Northern Gulf of Mexico oil field. The second is the lack of acute or sublethal effects data with exposure to oil, chemically dispersed oil, and chemical dispersants for one of the most important fisheries species within the Gulf of Mexico, the blue crab, *Callinectes sapidus*.

6.1. Biomonitoring Oil Exposures Using Oil Rig Structure Fouling Invertebrates

One of the important data gaps this dissertation endeavored to address is the lack of baseline data and the potential use of a novel resource like oil rig structure fouling invertebrates for offshore biomonitoring of the impacts from oil and petroleum PAHs. Accumulations of contaminants like PAHs have been routinely monitored in invertebrate bivalves throughout coastal wetlands around the United States, including the Gulf of Mexico, through the NOAA Mussel Watch Program. However, it is equally important to

understand the exposure and impacts of oil to organisms living offshore, especially when the application of chemical dispersants moves oil from the surface water into the pelagic and ultimately also benthic ecosystems. Vertical artificial reef systems on oil rig structures can be useful for biomonitoring in these offshore regions because they offer a variety of stationary organisms, like oysters and corals, essentially living in the water-column that can be used to examine the exposure to and impacts of oil during a spill as well as to monitor the recovery following a spill.

These vertical artificial reefs on offshore oil rigs (active or inactive) have become habitat for many reef-dependent taxa (Gallaway and Lewbel, 1982; Dokken et al, 2000) as well as habit and a food source for numerous fish species of recreational and commercial importance such as red snapper (NRC, 1996; Gallaway et al., 2009). Densities of fish near an oil and gas platform can be 20 to 50 times higher than nearby open waters with estimated total number of fish at a platform as high as $28,138 \pm 5,532$ (Stanley and Wilson 1997). Furthermore, the sensitive early life stages of many ecologically and economically important species like blue crab, shrimp, and fish are in high abundance in offshore waters of the continental shelf within the oil fields (Epifanio, 1988; Rogers et al., 1993; Lyczkowski-Shultz, 2007). Therefore, baseline biomonitoring data on the ecological (species and diversity), biological health, and chemical state of resident organisms in the offshore region is critical to be able to identify where and the extent organisms might be exposed to oil as well as how such important and/or sensitive species are impacted by it during an oil spill event.

Biomonitoring data can help determine the extent of recovery and the effectiveness of remediation options in an offshore region after an oil spill event. This type of

biomonitoring has been utilized in the oil and gas fields of Europe to examine the impacts of oil and natural gas extraction activities with mostly caged bivalves (Gorbi et al., 2008; Hylland et al., 2008; Sundt et al., 2011, 2012), with the exception of one study examining native fouling mussels (Gomiero et al. 2011). In US waters, the Gulf of Mexico Offshore Operations Monitoring Experiment (GOOMEX) study examined tissue contamination and sublethal detoxification biomarker responses in fish and invertebrates living near oil rigs off the coast of Texas (Kennicutt et al. 1996, McDonald et al. 1996). However, other than this study there has been no consistent monitoring to date of contaminate levels in offshore organisms on or near oil rigs and none on rigs within the Northern Gulf of Mexico near Louisiana.

The sessile invertebrates of oil rig fouling, artificial reef communities offer a unique and promising ecosystem that can be used for biomonitoring in the offshore areas of the Northern Gulf of Mexico. From the study in Chapter 2, oysters and corals on oil rigs showed relatively low baseline accumulation of PAHs for organisms collected in April, compared to bivalves collected near the shoreline and coastal wetlands (Wade et al., 1988; NCCOS, 2011). Additionally, organisms collected in May had higher TPAH concentrations than those collected in April from similar rig locations. This increased bioaccumulation response could be due to the Mississippi River flooding event that May, which delivered extensive suspended sediment loads into the Gulf of Mexico. Furthermore, the highest accumulation levels in both species occurred in the oil rig closest to the Mississippi River Delta. Organism collected from this rig in May were the only ones to have a predominant petrogenic source input, while all other organisms indicated either predominantly pyrogenic source inputs or a mixture between the two.

Diagenic inputs were evident in May samples only as indicated by the high concentrations of perylene in oysters.

One potential complication to conducting a baseline survey of blue water oil rig organisms off the coast of Louisiana could have been that organisms were collected 9-months post DWH incident and some (but not all) of the rigs were in the observed path of the surface plume for many days (Optical Oceanography Laboratory, 2010). However, no spatial trends in PAH concentrations were apparent in either species in relation to exposure and duration of exposure to the surface oil plume. Given the low baseline accumulation of PAHs and the quick accumulation response of PAH to a potential contamination event, fouling invertebrates on oil rig structures could make excellent biomonitoring tools to examine the exposure to and recovery from oil and petroleum PAHs in the offshore Northern Gulf of Mexico. Furthermore, this study highlights the necessity of examining multiple species to be used for biomonitoring as there were differences in accumulation and distribution of PAHs between the oysters and corals collected. This could be particularly important when attempting to assess diagnostic ratios for PAH sources as the differences in accumulation by ring size for a given species might bias the diagnostic analysis, especially when examining ratios that span more than one ring size like alkylation homolog to parent PAH or high molecular weight to low molecular weight.

6.2. Impacts of Oil, Dispersed Oil, and Chemical Dispersants on Larval and Juvenile Blue Crabs

The second data gap this thesis strove to address was the lack of information on the acute or sublethal toxicity of oil exposures on an important ecological and economic species, the blue crab, particularly sensitive early life stages potentially exposed in the pelagic zone following the DWH incident. Many toxicity studies use model organisms like Gulf mysid shrimp, *A. bahia*, to understand the toxicity of oil and dispersed oil exposures to early life stage crustacean species (NRC, 2005; Hemmer et al., 2010). This is often because many native species, especially the early life stages of many non-model species, can be more difficult (if not impossible) to culture in the laboratory or collect in sufficient quantities within the field. While information gained from these species can be helpful in risk assessment decisions during an oil spill event, it does not guarantee that other important non-model invertebrate species native to the Gulf of Mexico, like blue crabs, will respond in the same or similar way to oil and dispersed oil exposures. Therefore, it is important to also examine the impacts of these exposures to native non-model species whenever possible, which the blue crab hatchery in ARC allowed us to do for the three studies within this dissertation (Chapters 3-5).

The difference in distribution of blue crabs throughout their life stages can alter the potential exposure routes to oil and PAHs with offshore oil spill events. Larval blue crabs in the zoea stage (II-VII) are planktonic and mainly distributed in the surface waters offshore along the continental shelf (Epifanio, 1995; Johnson and Perry, 1999), while juvenile crabs are benthic dwelling and distributed in the shallow, lower salinity waters of estuarine marsh and seagrass beds (Orth and van Montfrans 1987, Thomas et al. 1990).

As a benthic species, juvenile crabs live and feed within coastal wetlands and estuarine sediments; and therefore, would be more likely exposed to weathered, sedimented oil that reached the coastal wetlands and estuaries. However, blue crab zoea reside in the offshore surface waters well within pre-approved areas for dispersant use. Therefore, they are likely to be exposed to oil and chemically dispersed oil in an offshore oil spill, especially those like the DWH incident that coincided with blue crab spawning.

Additionally, larvae live within the top 3m of the water column, which means they will be exposed to high concentrations of hydrocarbons with chemical dispersion before the oil can dilute with dispersion to less harmful concentrations. As blue crab are translucent during the larval stage and live in the photic zone, phototoxicity of oil components like PAHs from UV light could possibly enhance the toxicity resulting from chemical dispersion of oil. Many standard laboratory experiments do not account for this; and therefore, could underrepresent toxicity when used as surrogates to predict impacts on blue crabs. Therefore, given their importance to Gulf of Mexico environment and economy and the likelihood of exposure to oil during a large oil spill event, three studies (Chapters 3-5) in this dissertation sought to address the critical data gaps regarding acute and sublethal impacts of oil, chemically dispersed oil, and chemical dispersants to blue crabs at the zoea and juvenile stages.

Juvenile blue crabs (Chapter 3) did not appear to be acutely impacted by exposure to sediment bound PAHs. There was no treatment mortality observed in juvenile blue crabs exposed to sediment TPAH concentrations as high as $2,196,658 \mu\text{g kg}^{-1}$. While the sediment TPAH concentrations were high in the juvenile study, the water-borne concentrations were low as exposure waters in the treatment with the highest sediment

PAHs only had an initial TPAH of $65.3 \mu\text{g L}^{-1}$, which decreased to $4.9 \mu\text{g L}^{-1}$ by the end of the 31 day experiment. This suggests desorption of PAHs from the sediments into the aqueous (dissolved) phase was limited, even with the occasional resuspension of sediment particles observed with crab movements. Generally, the toxicity and bioavailability of hydrocarbons is related to their solubility in water as dissolved (water-borne) hydrocarbons can readily diffuse across the gills or other exposed membranes of aquatic organisms.

Furthermore, a study by Payne and Driskell (2003) examining PAH accumulation in Dungeness crabs and mussels exposed to the *M/V New Carrisa* oil spill suggests that crab accumulation with oil exposure predominately comes from direct uptake of the dissolved phase, compared with the dissolved and particulate fractions in the filter feeding bivalves. In this study, $0.7 \mu\text{m}$ GF/F filtration (similar to methods in Chapter 4) was used to determine the extent and distribution of PAHs within the dissolved and oil droplet/suspended particulate material (SPM) phases of water samples of physically dispersed oil, which was then compared to the PAH distributions within crab or mussel tissues (Payne and Driskell, 2003). Dungeness crabs accumulated mostly lower molecular weight PAHs from the dissolved phase, particularly naphthalenes, while mussels accumulated primarily intermediate molecular weight PAHs suggesting accumulation from both dissolved and oil droplet/SPM phases. Therefore, as the majority of PAHs remained bound to particulate matter within sediments and very few partitioned out in the dissolved phase, the sediment PAHs were potentially less bioavailable for uptake by the juvenile crabs. While juvenile crabs were occasionally observed to feed off of the larger sediment matter (e.g. twigs) in exposures, ingestion of PAHs would probably be a more

limited route of exposure. In the environment, ingestion of contaminated prey could be an important route of exposure for juvenile crabs during an oil contamination event, but not for this laboratory study as crabs were fed uncontaminated squid. Analysis of PAH concentrations within tissues of juvenile crabs from this study would have needed to be conducted to determine the full extent of bioavailability and accumulation of PAHs by juvenile crabs.

Ingestion of particulate oil/PAHs could be an significant exposure route for larval blue crabs as marine invertebrates like filter-feeding bivalves or the larval stages of most species feed on prey ranging from a few to a couple hundred μm sizes (i.e. range of oil droplet sizes). While the prey preference and selection of zoeal stage larvae is not well studied in wild crab species, hatchery raised blue crab zoeae feed on range of prey sizes, including phytoplankton ($<10\mu\text{m}$), rotifers (100-290 μm), and artemia ($>450\mu\text{m}$). Therefore, zoea in this study (Chapter 4) were capable of consuming oil particles from both WAF and CEWAF exposures as the average particle sizes ranged from 4.5-14.3 μm . Even though the concentration of dissolved PAHs was relatively similar between WAF and CEWAF with the same oil loading rates, CEWAF solutions had orders of magnitude higher concentrations of PAHs in the particulate phase. Therefore, consumption of or exposure to particulate PAHs could be one of the reasons acute toxicities of CEWAF were much higher than WAF exposures.

The sublethal endpoints examined Chapter 3 also indicated that juvenile blue crabs were not impacts by PAH contamination in sediments over the 31 day exposure. Neither the growth nor molting of juvenile crabs was negatively impacted by PAH concentrations in the sediments. Additionally, none of the biomarkers of early biological stress were

correlated with PAH concentrations. However, growth and molting results might be different with a longer study that accounted for multiple molts in all the crabs, as compared to the only 17% of total crabs that molted twice in this study.

In contrast to juveniles, blue crab larvae did show sublethal impacts during a 6 day exposure to oil and chemically dispersed oil. Larvae had over 2-fold increases in DNA damage (tail % DNA) with exposure to both high and low doses of WAF and CEWAF. Unfortunately, due to either poor RNA quality or an inhibitory factor within cDNA, the gene expression results for oxidative stress and detoxification genes could not be examined for this study. Another sublethal effect observed in blue crab larvae, was the temporary narcosis response in larvae exposed to both CEWAF treatments, especially in the high dose, with the addition of fresh exposure solutions on Day 3. Narcosis is generally considered to be one of the primary contributors of acute toxicity for organisms exposed to oil as many of its LMW components are considered type I narcotic chemicals with an additive toxicological effect (Rand et al., 1995). This narcosis response was also observed in blue crab larvae exposed to Corexit 9500 and four alternative dispersants solutions (Chapter 5), but at nominal concentrations higher than the 5 mg L⁻¹ high does used in the Chapter 4 study. Even though the concentrations of narcotic chemicals in CEWAF exposures were low enough to be reversible, the temporary immobility could still indirectly remove effected individuals from the population in the environment, by disrupting their vertical migration and ability to maintain position in the water column. This disruption could also result in decreases in feeding rates and increased predation as well as ultimately disrupting their ability to persist in the coastal zone or recruit into nursery habitat.

Another indirect impact of chemical dispersion of oil and chemical dispersant use near larval blue crabs could be a reduction of food source availability. In Chapter 5, the rotifers, *Brachionus* sp., used for feeding appeared to be much more sensitive to dispersant exposure than the blue crab larvae with all but the lowest concentrations of dispersants causing mortality or immobility. A recent study by Rico-Martínez et al. (2013) examined the acute toxicity of 5 neonate *Brachionus plicatilis* strains to WAF and CEWAF of Macondo sweet crude oil as well as Corexit 9500 only. The 24 hr LC₅₀ values were highest for WAF exposures (2.47-19.33 mg L⁻¹) and lowest for the three ratios of CEWAF (1:10, 1:50, and 1:130 dispersant:oil) at 0.21 (0.17-0.27, 95% CI), 0.23 (0.19-0.28, 95% CI), and 0.40 mg L⁻¹ (0.27-0.59, 95% CI), respectively. The 24 hr LC₅₀ values for Corexit 9500 alone ranged from 0.447-14.25 mg L⁻¹. All of these LC₅₀ concentrations are much lower than those observed for blue crab larvae and would even be below the lowest observed effect concentration for acute mortality (Chapters 4 and 5). Therefore, impacts to larval blue crab survival and eventual recruitment back to nursery habitat could occur at sublethal concentrations of oil, dispersed oil, and dispersant concentrations due to reductions in prey availability.

The study in Chapter 5 also shows that in general the chemical dispersants tested had similar toxicity to Corexit 9500 for blue crab larvae. Many alternative dispersants on NCP Product Schedule have higher percent effectiveness at dispersing oil than Corexit 9500 for South Louisiana Crude (US EPA, 2013), including Sea Brat #4 and Dispersit SPC 1000. Corexit 9500 only has 54.7% effectiveness, while Sea Brat #4 and Dispersit SPC 1000 have 60.65% and 100% effectiveness for dispersing Southern Louisiana Crude (US EPA, 2013). Therefore, alternative chemical dispersants with similar toxicity, but

more effectiveness at dispersing oil might be a better choice to use in oil spills within offshore Northern Gulf of Mexico where the larvae of blue crabs and other species like shrimp and fish predominate. With such a combination, less concentrations of dispersant would be needed to achieve the same extent of dispersion; therefore, potentially reducing toxicity to organisms living near the surface of the water column. However, to determine if such alternatives would ultimately be less toxic to blue crab larvae, the impacts of dispersed oil using these dispersant formulations should also be investigated, especially as it is generally not the dispersants alone, but the oil/dispersed oil that is the driving factors in acute toxicity when chemical dispersion is used in oil spill remediation.

6.3. Future Considerations

For biomonitoring in the Northern Gulf of Mexico, the sampling regime in Chapter 2 was limited in the number of samples per rig and the total rigs examined in both a spatial and temporal context compared to the amount of rigs within the region. For a successful biomonitoring program, a more thorough sampling of fouling species on offshore oil rigs should be conducted to have a more comprehensive understanding of baseline levels of PAH accumulation in the offshore Gulf of Mexico. Additionally, future studies should also incorporate sublethal biomarkers of PAH exposure and effects such as physiological stress, DNA damage, oxidative stress, and detoxification pathways to understand the baseline health of fouling organism. This would allow for the biomonitoring of not only exposure to oil and PAHs, but also the potential biological effects of such contamination. Additionally, baseline ecological (e.g. species diversity) of oil rig fouling communities

could also be used to determine ecological impacts from oil exposure such as population shifts or alterations in recruitment.

While these three studies on blue crabs represent a crucial foundation for this species, much more work is needed to fully understand the impacts of oil, chemically dispersed oil, and chemical dispersants to the early life stages of blue crabs. Another study examining sublethal exposure to WAF and CEWAF should be conducted to examine the impacts of oil and dispersed oil on the various proposed molecular endpoints described in Chapter 4 and in the main CRRC report. In addition to the oxidative stress and detoxification genes (MnSOD and GST) attempted in Chapter 4, other genes for peptide hormones regulating growth and molting were to be examined in the study to determine the extent of endocrine disruption with oil and dispersed oil exposures. The loss of RNA/cDNA quality in this study was a major shortcoming and a repeat of the experiment would be worth conducting to assess these multiple gene expression endpoints. Additionally, a repeat of this study with a modified Comet assay method that produces larger cell counts on slides might further elucidate the impacts of oil and dispersed oil on DNA damage in blue crab larvae. Further validation and characterization of the identified putative cytochrome p450 gene or other possible CYP genes in blue crabs should be conducted for future biomarker assessment of oil and PAH exposure. Furthermore, the narcosis response observed in these studies with dispersed oil and chemical dispersants should be further examined in both larval blue crabs and their prey to determine what concentrations lead to narcosis and how long the response lasts if the organisms are able to recover from it.

Furthermore, it could be beneficial to extend the exposures time to fully assess impacts to molting and growth and delayed responses from zoea through megalopa and into the first instar of the juvenile stage. This could potentially serve to further highlight the delayed mortality observed in the CEWAF high dose exposures after 3 days of depuration. Acute and sublethal toxicity studies with WAF and CEWAF exposures should also be conducted on megalopa as blue crabs in this stage could also be exposed to oil and dispersed oil as they remain near the surface waters while returning to estuaries before settling into the benthos (Epifanio, 1995). Moreover, acute toxicity studies with blue crab larvae examining the toxicity of dispersed oil using multiple alternative dispersants with higher dispersion effectiveness than Corexit 9500 should also be conducted. This could help determine a more suitable dispersant to be used in the offshore of the Gulf of Mexico, particularly in another oil spill that co-occurs with blue crab spawning.

With the juvenile sediment exposures, another study could be conducted with an extended exposure period to account for multiple molting periods in all of the crabs as well as potential reproductive success if carried out to maturity. Additionally, the enzyme activities of oxidative stress and detoxification biomarkers like superoxide dismutase, glutathione *S*-transferase, and cytochrome p450 (EROD assay) within hepatopancreas tissues could be conducted in place of gene expression analysis. Often times, gene expression levels can alter quickly with contaminant exposures, but have a more limited window of induction, while increases in enzyme activity might be delayed following exposure, but can remain high for longer periods of time. Therefore, it is possible that increases in enzyme activities might be observed in juveniles exposed to sediments with

higher PAH concentrations, even though gene expression levels did not show a correlation to PAHs. Moreover, examining the PAH accumulation within sediment exposed crabs with a future study could help elucidate the route of exposure and bioavailability of oil associated PAHs for uptake by crabs living within contaminated sediments.

6.4. Final Conclusions

Managers, responders, and remediators for recovery face a difficult task when trying to decide the best course of action for response options, damage assessments, and recovery strategies following an oil spill event. Often times they are forced to make decisions without adequate scientific data on the unique details for a given oil spill or what/how resident species might be impacted by relying on best estimate models or standard laboratory species toxicity results. Therefore, this dissertation aimed to address observed data gaps in response options, damages assessments, and remediation strategies relevant to the Northern Gulf of Mexico. Namely by describes a novel resource for potential offshore biomonitoring with the use of oil rig fouling invertebrates to assess exposure to and impacts of oil during and recovery following an oil spill event and by seeking the address the lack of acute and sublethal toxicity information for an important native Gulf of Mexico species, the blue crab. Furthermore, this dissertation highlights the need for more consistent and thorough chemical characterization of exposure mediums to allow for better comparison between toxicity tests and more complete understanding of observed toxicities, especially in relation to species specific method of uptake.

Appendix 1. Arginine Kinase as a Putative Reference Gene for qPCR

A1.1 Introduction

Initially, the results from the study in Chapter 3 were to be examined by relative quantification (i.e. the delta-CT method), which compares the changes in target gene expression relative to the gene expression of a reference gene. For this method to be utilized, a proper reference gene must be shown to be stable in various tissue types examined and not display a relationship between expression levels and intended exposure concentrations (i.e. regulated by the treatment). Finding an appropriate unregulated reference gene for non-standard model species, like blue crabs, without a fully characterized genome can be challenging. Other common housekeeping genes such as 18S rRNA and beta-actin have been discarded for use in blue crabs as they were either too variable in certain tissue types, beta-actin, or too highly expressed to conduct proper qPCR normalization, 18S rRNA (personal communication with Dr. Sook Chung, IMET).

Arginine kinase (AK) was originally chosen as a reference gene for this study as it has previously been used as a putative housekeeping gene in the literature for blue crabs and the shore crab, *Carcinus maenas*, (Chung and Webster, 2003; Jayasundara et al., 2007; Zmora et al., 2007; Chung et al., 2012). AK is an enzyme involved in buffering the energy supply through catalysis of the reversible transfer of a phosphoryl group from a phosphagen to ADP producing an ATP molecule (Kotlyar et al., 2000). Phosphagen kinases like AK might also function as an energy shuttle between the mitochondria and cytosol (Bessman and Carpenter, 1985).

To be acceptable as a reference gene, AK levels need to be consistent for each treatment when normalized to RNA concentrations within the tissue type examined. While no quantitative values for different tissue types were comparable in the literature, Chung (2008), showed visual AK expression from endpoint PCR in 12 different tissue types in adult male and female blue crabs following gel electrophoresis. Her results showed that for adult females most tissues appeared to have similar expression with the exception of eyestock and the y-organ, which were both much lower than expression than other tissues. In males, the AK expression appeared to be much more variable between tissue types. However, hepatopancrease AK expression appeared to be similar between adult males and females. It is important to note that these expressions were in adult crabs and expression could be different at an earlier life stage. In Chung et al. (2012), expression levels for superoxide dismutase genes were normalized to AK following immune challenge. However, to date, no studies have examined how AK gene expression levels are influenced by polycyclic aromatic hydrocarbons. Therefore, AK gene expression was examined in hepatopancrease tissues of juvenile crabs exposed to sediments collected from the Pass a Loutre WMA for 31 days (Chapter 3) to determine if AK could be used as a reference gene for qPCR in this study.

A1.2 Methods

Following the methods outlined in Section 3.4.6, primers for blue crab arginine kinase (Accession # AF233355.1) were designed using NCBI primer BLAST. The resulting amplicon product was 187 bp using the forward (5'-CAATGGCTGACGCTGCTAC-3') and reverse (5'-TCCAGGTTCTCAACACCAGA-

3 ϕ primers. The subsequent product was ligated into a plasmid, which was linearized by restriction digest and appropriately diluted (30-3,000,000 copies). These standard dilutions were run on qPCR to examine efficiency (90.2%, R^2 value=0.996). The plasmid inserted products were transcribed into RNA. A six-point RNA serial dilution with a known copy number was produced and reverse transcribed to cDNA (5,000-500,000,000 copies). Absolute qPCR was conducted using the POWER SYBRGreen kit on an Applied Biosystems 7500 Fast Real-Time PCR System. For AK hepatopancrease gene expression analysis, all the crab cDNA samples from hepatopancrease tissues were run on qPCR in triplicate and in junction with the six-point cDNA standards and no template controls, also in triplicate. A linear regression equation was produced for the AK cDNA standards and used to determine the copy number of each crab sample. The copy numbers were then normalized to the total RNA concentration (ng) and reported as copy number ng⁻¹ total RNA. The mean and standard error of AK gene expression for each treatment and the crabs collected prior to the start of the experiment (T0) are shown in Figure A3.1. An exploratory correlation analysis was conducted in order to examine relationships between the chemical contaminants and AK gene expression, as was conducted for other biological endpoints (Section 3.4.8).

A1.3 Results

Crab hepatopancrease tissue from sediment treatments D and E had significantly higher arginine kinase gene expression than crabs from treatments B, C, F, and T0 (p value<0.05). Crab hepatopancrease tissue from sediment treatment A has significantly higher AK gene expression than crabs from T0 (p-value<0.05). There was no apparent

relationship between AK gene expression and hydrocarbon (PAH, TEH, or SH) concentrations in sediments or water PAH concentrations. However, AK gene expression did have a positive correlation with each individual metal examined and the summation of all metals ($p < 0.01$).

A1.4 Discussion

Arginine kinase gene expression appeared to be responsive to the sediment treatments used in this study, particularly the concentrations of metals within the sediments. While the exact mechanism for this induction of AK expression with increasing metal contaminations is unknown, one possible explanation could be altered energy metabolism to allow for detoxification of accumulated metals. Given that AK expression was not consistent between treatments, but inducible by metal concentrations in sediments, it could not be used as reference gene for this study. Therefore, future studies examining the impacts of contaminated sediments or metal exposures on blue crabs should consider finding another reference gene for their analyses.

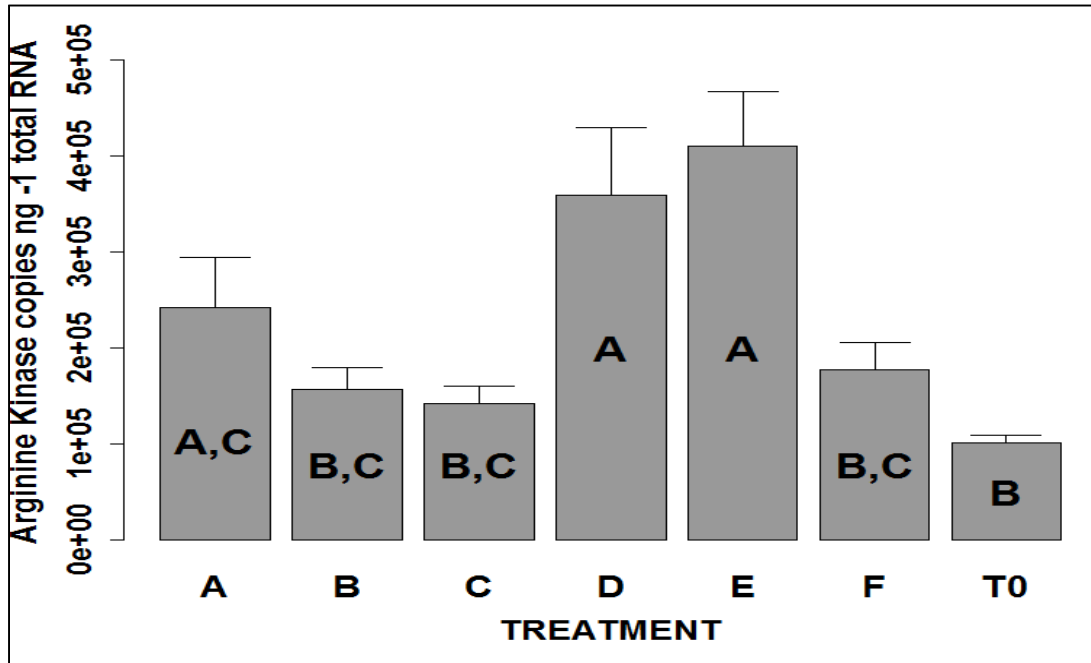


Fig. A1.1 Hepatopancrease arginine kinase (AK) gene expression levels in juvenile blue crabs for the different sediment treatments and the time 0 collected crabs. Different letters signify significant differences ($p < 0.05$).

Appendix 2. Characterization of Putative Blue Crab Glutathione *S*-Transferase and Cytochrome p450 Genes

A2.1 Introduction

The genes of the two most common biomarkers for PAH exposure involved in the metabolism and detoxification of PAHs, cytochrome P450 and glutathione-*S*-transferase (GST), have not yet been characterized in blue crabs in published literature or on NCBI's GenBank. Other studies have shown high GST enzyme activity in blue crab hepatopancrease tissue (Keeran and Lee, 1987; Lee, 1988). Additionally, Keeran and Lee (1987) purified and isolated two isoenzymes of GST from the hepatopancrease of blue crabs. However, the proteins sequences for these two isoenzymes have never been reported on NCBI's protein database. Even cytochrome p450 enzyme activity with xenobiotic contamination has not been well examined in blue crabs. One study examined EROD (i.e. cytochrome p450 enzyme activity) activity in blue crabs exposed by feeding to tributyltin (TBT), but CYP1A was not induced by exposure (Oberdörster et al. 1998).

While no genes for either GST or CYP have been fully characterized in blue crabs, gene sequences have been characterized in similar crab species and/or other crustaceans. Currently, the only GST mRNA or EST sequence on GenBank for a crab species is a delta-class GST from the Chinese mitten crab, *Eriocheir sinensis* (Zhao et al., 2010). The only other decapod crustacean GST mRNA sequences on GenBank are in the Pacific white shrimp, *Litopenaeus vannamei* (Accession # AY573381.2), and the ridgetail prawn, *Exopalaemon carinicauda* (Accession # KF430648.1). Zhao et al. (2010) examined GST

levels in various tissues and temporally in hemocytes exposed to a bacterial immune challenge. This GST was detectable in the hepatopancrease and did respond to the immune challenge with induction at 6 hr. The induction of GST from immune challenge was considered due to the scavenging of reactive oxygen species produced from immune challenge, not xenobiotic metabolism.

It is unclear whether or not GST in blue crabs is inducible with PAH exposure. One study by Lee (1988) showed that in field collected crabs, GST activity in hepatopancrease tissue was significantly higher with correspondingly higher tissue PAH concentrations in shore crabs, *Carcinus maenas*, from two polluted sites than crabs from reference sites. Other marine crustaceans, particularly marine copepods, have shown inductions of GST gene expression with exposure to naphthalene and the water accommodated fractions of oil (Hansen et al., 2008; Hansen et al., 2011). However, other studies conducting 28 day exposures of *C. maenas* to contaminated sediments did not show any correlation between PAH concentrations and GST enzyme activity (Martín-Díaz et al., 2007, 2008; Morales-Caselles et al., 2008).

Cytochrome p450 gene expression has been well characterized in the shore crab, *Carcinus maenas*, (Dam et al., 2006; Dam et al., 2008), American lobster, *Homarus americanus* (Snyder et al., 1998), and spiny lobster, *Panulirus argus* (James et al., 1996). Furthermore, Dam et al. (2008) showed that CYP2(1) isoform (full length CYP379A1) in adult *C. maenas* was inducible with exposure to benzo[a]pyrene, particularly in the hepatopancrease. Another study examining EROD activity in *C. maenas* exposed to contaminated sediments for 28 days showed significant correlations between PAH concentrations and EROD activity (Morales-Caselles et al., 2008).

Therefore, the full-length mRNA sequence for *C. maenas* CYP379A1 (Accession # FJ266093), and the full-length mRNA sequence for *E. sinensis* delta-GST (Accession # FJ610337) were used to identify possible cytochrome p450 (CYP) and glutathione *S*-transferase (GST) gene sequences in blue crabs within a non-public EST library (Place; IMET, Baltimore). From this EST library, one promising hit was obtained for each gene. These putative blue crab genes for CYP and GST were further validated to determine if they could be used for oil and PAH exposure studies.

A2.2 Methods

A2.2.1 Localized alignment searches and translation to protein sequences

A basic localized alignment search was conducted for both the *C. maenas* CYP379A1 and *E. sinensis* delta-GST mRNA sequences within a non-public EST library (Place; IMET, Baltimore). To further validate the putative blue crab genes hits from the EST library for CYP and GST, these sequences were each run through an NCBI tblastx (translated nucleotide database with a translated nucleotide) search using the entire nucleotide database and then specifically for crustaceans, taxa: 6657 (CYP only).

The factors from the BLAST results used to compare similarities between sequences for this study, include E value, percent query cover, and maximum score. The smaller the E value means the less likely that random chance led to the observed alignment; and therefore, the more significant the alignment. Percent query cover is the percent of the total sequence length that is included in the alignment segments, while maximum (max.) score is the highest alignment score between the query sequence and the database sequence segment. Scores are calculated based on the number of gaps and substitutions

associated with each alignment sequence. Higher scores correspond to higher similarities between the sequences.

The mRNA sequence for the both putative GST and putative CYP were subsequently translated using ExPASy translation software into a protein sequence in the correct reading frame, which was verified with the *E. sinensis* and *C. maenas* protein sequences (Accession # ACU46011.1 and ACI94903.1, respectively). A localized alignment between the putative GST and the *E. sinensis* delta-GST for both protein (Fig. A2.1) and mRNA was conducted using LALIGN pairwise sequence alignment software and CLC Sequence Viewer 7.0 to visualize. A localized alignment was also conducted between the blue crab putative CYP and the *C. maenas* CYP for both protein (Fig. A2.3) and mRNA. The factors from the localized alignments used to compare similarity between each pairing for this study include E value, percent identity, and percent similarity. The percent identity includes only exact matches in nucleotide or amino acid sequences, while similarity also accounts for conservative changes at specific positions in the amino acid (or nucleotide) sequences that preserve the physio-chemical properties of the original residue.

A2.2.1 Primer design and testing

For the putative blue crab GST, two sets of primers were designed using the NCBI Primer BLAST. One set produced a 674bp amplicon covering most of the translated region of the sequence with only the last eight 3' end amino acids not being included, which was subsequently inserted into a plasmid to make the calibration standards. The second primer set was to be used for qPCR as it produced a 103bp amplicon within the

first primer's product region (Table 3.2). Preliminary tests were conducted on standard endpoint PCR to confirm the ability of both primers to produce their intended amplicon products with gel electrophoresis verification using juvenile blue crab hepatopancrease cDNA from non-exposed control crabs.

For the putative blue crab CYP, two primer sets were designed using NCBI's Primer BLAST (amino acids as shown in Fig. A2.3). The first set (light gray) produced a 421bp product that spanned the region containing the stop codon and the second set (black) for qPCR produced a 115bp product, which was after the stop codon. Initial endpoint PCR analysis of the putative CYP gene expression of both primer sets in juvenile blue crab hepatopancrease cDNA from time 0 and the treatment F (sediment with highest PAH concentrations) in Chapter 3 was conducted and verified by gel electrophoresis. These tests did not yield products of the expected size, or bands in general, on the gels for either primer set.

Therefore, additional tests were conducted using adult blue crab genomic DNA to examine if this putative CYP gene was indeed present within the blue crab genome. Expression of the putative CYP gene in adult genomic DNA was examined using the original two primer sets as designed and with a mixture of the two sets (i.e. forward primer of set 1 with reverse primer of set 2 and vice versa). The mixtures were conducted to determine the potential interference of the stop codon that occurs right before the aspartic acid residue (highlighted in light gray in Fig. A2.3), which is 83 amino acids from the start of the translated sequence and 41 amino acids from the start of the first coding region. The genomic DNA product from the second original primer set (115 bp product) was extracted from the gel and sequenced.

A2.3 Results

A2.3.2 Validation of putative blue crab GST

The tblastx results for the putative GST EST hit showed high similarity with delta-GST of the Chinese mitten crab, *Eriocheir sinensis* (Accession # FJ610337) and the GST of the ridgetail prawn, *Exopalaemon carinicauda* (Accession # KF430648.1) as described in Table A2.1. The protein sequence appears to contain the full coding region for the protein which is comprised of 216 amino acids. In comparison, the GST protein for *E. sinensis* (Accession # ACU46011) is also 216 amino acids in length. The localized alignments between the putative GST and the *E. sinensis* delta-GST for both protein (Fig. A2.1) and mRNA sequences showed high similarity between the two species. The protein alignment had a 78.9% identity (94.4% similarity) in a 213 amino acid overlap with an E-value $< 3.4e^{-105}$. The mRNA alignment had a 77.3% identity (82.8% similarity) in a 675 nucleotide overlap with an E-value $< 6.9e^{-21}$.

Furthermore, the testing of the two primer sets with non-exposed crabs showed that both sets indeed produced the intended amplicon products (674bp and 103bp) with thick, visible bands on the gel indicating high gene expression. These bands were cut out of the gel, purified, and further validated by sequencing. The sequenced bands contained the correct full length products with only three non-coding differences in the mRNA sequences (compared to the EST library sequence) in the 5' Nö codon of two serine amino acids and a C to T transition in the 5' Yö codon of a leucine.

Table A2.1 BLAST results for putative blue crab GST and CYP using the NCBI nucleotide database

Gene	Species	Accession #	Query Cover (%)	Max. Score	E Value
GST	<i>Eriocheir sinensis</i>	GQ325712.1	84%	266	2e ⁻¹¹⁵
	<i>Exopalaemon carinicauda</i>	KF30648.1	79%	333	2e ⁻⁸⁸
CYP	<i>Pediculus humanus</i>	XM_002429951.1	92%	196	4e ⁻⁴⁷
	<i>Sogatella furcifera</i>	KC579454.1	86%	187	3e ⁻⁴⁵
	<i>Laodelphax striatella</i>	JX876514.1	92%	189	4e ⁻⁴⁵
CYP*	<i>Carcinus maenas</i>	FJ266093.1	60%	53.3	8e ⁻¹⁶
	<i>Carcinus maenas</i>	AY328466.1	68%	49.2	2e ⁻¹⁵
	<i>Panulirus argus</i>	U44826.1	24%	52.8	1e ⁻⁰⁶

*BLAST search specific for crustaceans (taxa: 6657)



Fig. A2.1 Localized protein alignment between putative blue crab GST protein sequence (GST-protein) and *E. sinensis* protein sequence (Accession #ACU46011.1). Non-identical residues highlighted in gray.

A2.3.2 Validation of putative blue crab CYP

The tblastx results for the entire database showed high similarity to the cytochrome p450 genes of various insect species with the top three included in Table A2.1. The closest two hits for the tblastx results for the crustacean specific search were two of the cytochrome p450 gene identified in *Carcinus maenas* (CYP379A1 and CYP330A1) and the spiny lobster, *P. argus* (Table A2.1).

The mRNA sequence for the putative CYP was subsequently translated into a protein sequence in the correct reading frame (Fig. A2.2), which was verified with the *C. maenas* CYP379A1 protein sequence. A localized alignment was conducted between the putative CYP and the *C. maenas* CYP (CYP379A1) for the entire 200 amino acid protein sequence (Fig. A2.3) and the entire mRNA sequence. The protein alignment had a 29% identity (65% similarity) in 183 amino acid overlap with an E-value $< 1.3e^{-22}$, while the mRNA alignment had a 56.8% identity (56.8% similarity) in a 366 nucleotide overlap with an E-value $< 6.3e^{-15}$.

The PCR tests examining mixtures of the two primer sets showed distinct, thick bands visible on the gel for all the primer groupings that occurred after the stop codon, but none that occurred using the primer on the 5' side of it. The sequencing results for the smaller primer set confirmed the expected 115bp putative CYP product was produced and contained a 125bp intron in the middle of it. The only change in the sequence from what was expected based on the EST hit was one C to T transition in the middle of the reverse primer. Therefore, it appears that at least the second coding product from this putative CYP gene is contained within the blue crab genome.

```
RDPADVEDLVDACFANLLTTNEEEKWDWQTILYIVEDLLGGSMAL  
NIVMRLLAHILQNPVMDALRAEIDEKIGRERTPT_StopDRHEMLYS  
AVLYEVLRVTSPPVPHVATEDSSVGGYAVKKGSIVFLNNFEMNS  
SLWDEPNKFMPEFLKDGCIKKPEYFIPFSTGKRSLRGLQDGGQHR  
HGSHHSAAALQHRHG_StopP
```

Fig. A2.2 Translated protein sequence for putative blue crab CYP. Coding region(s) highlighted in gray

	10	20	30	40	50
BC_CYP	DPADVEDLVDACFANLIT	TNEEEKW---	DWQTILYIVEDLLGG	SMASNI	VMRLLAHILQ
	::
CM_CYP	DPDNPRDLIDSYL	VEMEAKKDDPETTY	SEWDLVFILFDM	FFAGSETTVNT	FTWLCCYLAA
	260	270	280	290	300 310
	60	70	80	90	100 110
BC_CYP	NPHVMDALRAEIDEK	IGRERTPTL-D	RHEMLYSQAVLYE	VLRVTS--	SPVPHVATEDSS
	::... :.
CM_CYP	NPQAQLKLQAEI	DEVLPNGALPTL	MEKSRMPYTEAV	INEVMRVCALV	NFGLQHMAANNT
	320 330	340 350	360 370		
	120	130	140	150	160 170
BC_CYP	VGGYAVKKGSI	IVFLNNTFEMNS	SPSLWDEPNK	FMPERFLKDG--	CIKKPEYFIPFSTGKRS

CM_CYP	LGGYTIPKGAVL	SATVTSIQYDNR	YWDQPKFKPER	WLDDNGKFSMT	KEGFLPFGVGKRV
	380	390	400	410	420 430
BC_CYP	LRG				
	:				
CM_CYP	CVG				

Fig. A2.3 Cytochrome p450 protein alignment between putative blue crab (BC_CYP) and *C. maenas* (CM_CYP). Primer sets are highlighted in light and dark grey and potential stop codon in right before the aspartic acid in black

A2.4 Discussion

A2.4.1 Putative blue crab GST

The BLAST and localized alignment results show high homology between the putative blue crab GST sequence and the GST of various crustacean and insect species. Additionally, when compared to the *E. sinensis* GST protein sequence, it appears that the putative blue crab GST identified contains the full coding region for the protein. Furthermore, both primer sets produced the correct products with endpoint PCR as validated by gel and sequencing.

Given the high homology in both protein and mRNA sequences between the putative blue crab GST and *E. sinensis* GST as well as the successful primer testing, we felt confident to continue with further validations of expression of the putative GST for use as a biomarker of oil/PAH exposure in juvenile blue crabs for Chapter 3 and larval blue crabs in Chapter 4. An additional validation study was also conducted to examine the inducibility of the putative blue crab GST with exposure to benzo[a]pyrene (Appendix 3).

A2.4.1 Putative blue crab CYP

The BLAST and localized alignment results do not show high homology with the green shore crab, *C. maenas*, which is not surprising given that low homology was also observed between *C. maenas* and the spiny lobster, *P. argus* with only 45% mRNA and 41% protein identity between the two species. This is not uncommon as the identity between cytochrome p450 proteins are often below 20%, with high conservation generally only in residues involved in the structural fold of the protein (Graham and Peterson, 1999). The numerous BLAST results that were cytochrome p450 (or like) genes and the similarity in *C. maenas* does suggest that the putative CYP is a possible

cytochrome p450 enzyme in blue crabs; and therefore, deserved further examination to determine expression in juvenile blue crabs.

This putative CYP gene, however, was not expressed in visible levels using the qPCR primer set within juvenile blue crabs from the study in Chapter 3, even those with the highest sediment PAH exposure. One possible explanation for this is that this putative CYP expression is age depended and is not being expressed in juveniles. CYP expression or enzyme activity in crustaceans has been shown to be molt stage and sex dependent (Singer and Lee, 1977; Dam et al., 2008), but no studies, to our knowledge, have examined age dependence in relation to xenobiotic metabolism. There are studies that have examined the ability of different cytochrome p450 isoforms to metabolize xenobiotic and/or regulate natural ecdysteroids associated with molting in crustaceans and insects (Spiegelman et al., 1997; Snyder, 1998; Rewitz et al., 2003). In fish, however, CYP1A1 activity is generally expressed (Andersson and Förlin, 1992) and has been shown to be inducible with exposure to PAHs or oil (Couillard et al., 2005) in early life stages.

Furthermore, it does not appear that the full mRNA sequence was characterized for this putative CYP. The full amino acid length of the translated mRNA sequence for the putative blue crab CYP is only 200 amino acids as compared to the *C. maenas* CYP379A1 protein (Accession # ACI94903.1), which is 492 amino acids in length. Furthermore, the localized alignment does not begin with the *C. maenas* sequence until the 255th amino acid, further highlighting that the putative blue crab CYP sequence is most likely only a part of the full CYP protein sequence.

Overall, the above results demonstrate that additional validation and characterization of the putative blue crab CYP is needed before it can be used as a biomarker for oil or PAH exposures. Therefore, the putative blue crab CYP gene was not used for the juvenile and larval blue crab exposure studies (Chapters 3 and 4). Additional work would need to go into examining the problems in expression observed in this study before this putative CYP gene's function and expression are fully understood. A direct exposure to an inducible PAH like benzo[a]pyrene in adult and juvenile blue crabs, preferably post-molt stage, would need to be conducted to verify if this putative CYP gene is even involved in metabolism of xenobiotics and if there is a difference in expression based on developmental stage. Other cytochrome p450 genes could also be identified within the non-public EST library (Place; IMET, Baltimore) or from the Marine Genome Project database for further analysis to identify a PAH inducible CYP gene to be used as a biomarker with oil exposure.

Appendix 3. Expression of Putative Blue Crab Glutathione S-Transferase in Adult Blue Crabs Exposed to Benzo[a]pyrene

A3.1 Introduction

The study in Chapters 3 using the putative blue crab GST as a biomarker shows that this identified isoform of GST is expressed in measureable levels within hepatopancrease tissues of juvenile blue crabs and is potentially inducible by metal concentrations. However, it is still unknown whether this particular isoform is inducible by hydrocarbon exposure as there did not appear to be a correlation between GST expression levels and PAH concentrations in sediments or water for that study. If this putative GST gene is to be used for future studies as a biomarker for oil exposures, induction in expression levels must be shown with exposure to hydrocarbons.

Numerous other studies have shown induction in GST gene expression in various marine species following water-borne or injection exposures to hydrocarbons or individual PAHs like benzo[a]pyrene or naphthalene (Boutet et al., 2004; Hansen et al., 2008; Nahrgang et al., 2009; Xu et al., 2010; Zhang et al., 2012). This induction appears to be dose and time dependent and varies between species as well as the specific isoform of GST examined. At least 15 different isoforms of GST (alpha, beta, delta, epsilon, zeta, theta, kappa, lambda, mu, pi, rho, sigma, tau, phi, and omega) have been identified in mammalian and non-mammalian organisms as reviewed by Hayes et al. (2005) and Blanchette et al. (2007), but not all of these isoenzymes have been shown to be inducible in marine organisms by exposure to hydrocarbons (Zhang et al., 2012). The putative blue crab GST and the Chinese mitten crab, *Eriocheir sinensis*, GST protein sequences have

conserved domains that show homology to the delta and epsilon GST class subfamilies. Delta and epsilon GST isoenzymes have been mostly identified and characterized within insect species. Very little information is known about their detoxification potential beyond their relation to pesticide and insecticide resistance (Enayati et al., 2005).

Therefore, this study sought to determine if the putative GST isoform identified in blue crabs for this dissertation was inducible by exposure to a PAH, benzo[a]pyrene (BaP). Adult, male blue crabs (*Callinectes sapidus*) from the Chesapeake Bay were injected with two doses of BaP and the gene expression levels of the putative blue crab GST were examined after 48hr for each dose exposure and compared to control (vector only injected) crabs.

A3.2 Materials and Methods

A3.2.1 Animals

Adult, male blue crabs were collected by Coveside Crabs (Dundalk, MD) from the Chesapeake Bay on April 26th, 2013. Crabs were temporarily stored at 4°C prior to retrieval to minimize damage to organisms. Crabs were transported to the Aquatic Research Center of the Institute of Marine and Environmental Technology (IMET) and placed in individual tanks with artificial seawater (ASW) adjusted to 15 ppt. Crabs were acclimated for 4 days prior to the experiment during which the water was changed and crabs fed pieces of shrimp (2-3g) daily. After 1 hr, food remaining in tanks was removed to minimize impacts on water quality. The wet weight and carapace width of each crab were measured 24 hr prior to the start of the experiment to determine concentrations of benzo[a]pyrene (BaP) to use for injections.

A3.2.2 Exposures

Crabs were randomly distributed to three different treatment groups: control ($N=7$), low BaP dose ($N=8$), or high BaP dose ($N=8$). BaP was dissolved in 0.5% bovine serum albumin (BSA) and 0.9% saline. The doses of BaP for injection were standardized to the weight of each crab with a high dose of $0.01 \text{ mg BaP g wet-weight}^{-1}$ and low dose of $0.001 \text{ mg BaP g wet-weight}^{-1}$. The crabs in the high dose received an equivalent concentration of BaP (1.07-1.44 mg BaP) to *C. maneus* exposures in Dam et al. (2008) that were injected with 1.2 mg of BaP. Individual BaP solutions were prepared for each crab from the stock ($0.01438 \text{ mg } \mu\text{L}^{-1}$). After surface sterilizing the injection site with isopropanol, 100 μL of each BaP solution was injected into each respective crab at the base of the swimming leg using a 23 gauge needle. The control crabs were injected with 100 μL the 0.5% BSA, 0.9% saline vector.

Each individual crab tank was aerated and covered with a plastic lid to secure the crab within the tank. Water quality parameters (dissolved oxygen, ammonia, pH, temperature, and salinity) were measured at the start of the experiment, prior to the water change at 24 hr, and at the end of the experiment (48 hr). Light cycle was held at 14h light/10h dark. A full water change was conducted 24 hr-post injection with fresh 15ppt ASW. After the water change (24 hr post-injection), crabs were fed pieces of shrimp (2-3g) and food consumption was recorded. Any excess food was removed after 1 hr to minimize impacts on water quality.

A3.2.2 Dissection and collection of tissue

The crabs were placed on ice for at least 5 minutes until torpid before dissecting. All subsamples were collected from the left side (dorsal view) of the crab except if the crab was missing either the fourth pereopod or swimming leg on the left side, which was then taken from the right side. The full length of the carapace from the outer orbital tooth to the lateral spine on the left side of each crab was removed to extract a standard subsample of hepatopancrease tissue. A muscle tissue subsample was collected from the merus of the fourth pereopod. The entire testis from the left side of the crab was extracted. Additionally, the third most posterior gill was extracted from each crab. All tissue subsamples collected were placed into *RNAlater* (at least 10X volume). After allowing the *RNAlater* (QIAGEN, Venlo, Limburg) to permeate the tissues overnight at 4°C, the *RNAlater* was removed and the cryovials stored at -80°C. Only the hepatopancrease tissue was used for gene expression analysis.

Furthermore, the left side swimming leg was removed from each crab to use for molt-stage analysis. Swimming legs were stored in individually labeled bags overnight at 4°C until analysis the following day. The molt-stage of each crab was determined by examining the swimming leg under a dissection microscope following the descriptions outlined in Smith and Chang (2007).

A3.2.3 RNA preparation and cDNA synthesis

The hepatopancrease tissue samples from all crabs were homogenized with 425-600 µm acid-washed glass beads (Sigma-Aldrich, Saint Louis, MO) using a bead beater (Savant FastPrep[®] ; Bio101) and the mRNA extracted as using an *RNAqueous* kit (Life

Technologies, Grand Island, NY). The extracted mRNA for each crab was DNase treated using a TURBO DNase Free kit (Life Technologies) to remove any genomic DNA contamination and then reverse transcribed (RT) to cDNA using a High Capacity RNA-cDNA kit (Life Technologies) for absolute qPCR analysis. For each sample, a No Reverse Transcription (No RT) control was also conducted in parallel to the RT reaction to examine genomic DNA contamination in the cDNA sample remaining after DNase treating.

A3.2.4 Quantitative real-time PCR

The primers for a putative blue crab GST used for this study were the same as those in Chapters 3 and 4 (Table 3.3) were designed from an EST hit observed by running a basic local alignment search (BLAST). The reversed transcribed cDNA standard dilutions (500-50,000,000 copies) described in Section 3.4.6 were used for the qPCR quantification. Absolute quantitative-PCR was conducted using the POWER SYBRGreen kit on an Applied Biosystems 7500 Fast Real-Time PCR System. All the crab cDNA samples for the two BaP dose treatments and control crabs were run on qPCR in triplicate and in conjunction with the six-point cDNA standards and no template controls, also in triplicate. The No RT crab cDNA samples were run in duplicate to examine genomic DNA contamination. A dissociation curve was conducted for all samples and standards. A linear regression equation was produced for the cDNA standards of each gene and used to determine the copy number of each crab sample. The copy numbers were then normalized to the total RNA concentration (ng) and reported as copy number ng⁻¹ total RNA.

A3.2.5 Statistical analysis

Statistical analyses were conducted using R statistical software 2.15.2. The mean and standard error of the mean values for all treatment groups were compared for GST hepatopancrease gene expression levels (copy number per ng total RNA). GST gene expression levels were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test). If one or both tests failed, a log transformation was conducted and the tests redone. If both tests passed with (or without) the transformation, an Analysis of Variance (ANOVA) was conducted to determine if there were any statistically significant differences between any of the treatments ($p < 0.05$).

A3.3 Results

All crabs were either in the intermolt (C) or very early premolt (D_0) stages with either a green or early white sign in the distal edge of the merus of the swim paddle, respectively. No mortalities occurred during the 48 hr following injection. Most water quality parameters did not change appreciably throughout the 48hr period with temperatures of 19.6 ± 0.54 °C, pH of 8.06 ± 0.07 , and dissolved oxygen 7.87 ± 0.71 mg L^{-1} (mean \pm standard deviation of mean). Salinity was kept at 15ppt. Ammonia levels were less than 1 mg L^{-1} at the start of the experiment and immediately after the water change, but rose to 2.0 - 4.0 mg L^{-1} within 24 hr before and after the water change.

A reduction in GST gene expression levels was observed in the high dose crabs (1011.8 ± 287.6 copies ng^{-1} RNA) compared to the low dose (1765.0 ± 403.8 copies ng^{-1}

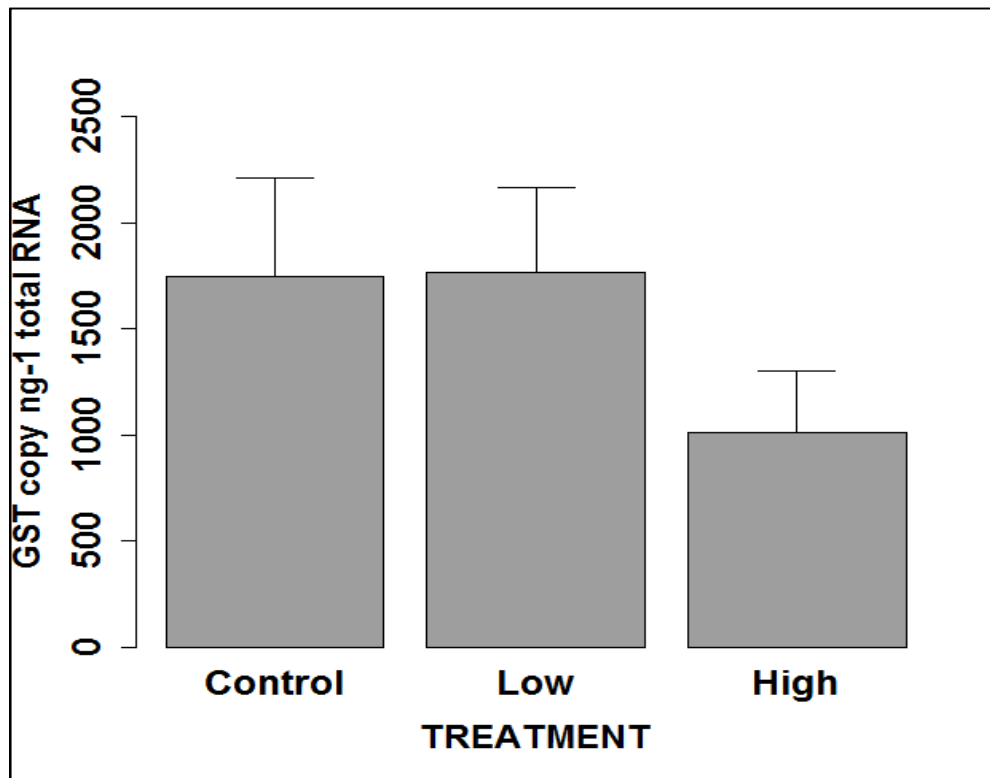


Fig. A3.1 Hepatopancrease glutathione *S*-transferase (GST) gene expression levels in adult, male blue crabs injected with low or high doses of benzo[a]pyrene compared to control crabs injected with vector only (mean + SEM, $N=7-8$ crabs).

RNA) and control crabs (1749.2 ± 462.9 copies ng^{-1} RNA) as seen in Figure A3.1.

However, there were no statistically significant differences in hepatopancrease GST gene expression levels between either the low or high dose BaP exposed crabs and the control crabs.

A3.4 Discussion

This study sought to determine if exposure to BaP resulted in up-regulation of the putative blue crab GST gene expression levels in order to validate the use of this gene for biomarker analysis with oil exposures. Unfortunately, the gene expression levels of the putative GST were not up-regulated with BaP exposure in this 48 hr study. In fact, the high BaP dose exposure crabs had the lowest overall GST gene expression.

This lack of induction could indicate that this particular isoform of GST is not inducible by hydrocarbon exposure. To my knowledge, there have been no studies examining gene expression levels of a delta or epsilon isoform of GST in an organism exposed to hydrocarbons. While many isoforms of GST (e.g. omega, pi, and sigma) have been shown to be inducible in marine organisms exposed to hydrocarbons (Boutet et al., 2004; Hansen et al., 2008; Nahrgang et al., 2009; Xu et al., 2010; and Zhang et al., 2012), a study by Zhang et al. (2012) examining 7 different isolated GSTs (5 isoform classes) in clams, *Venerupis philippinarum*, indicated that not all the isoforms were inducible over the 96 hr period with water-borne BaP exposure. Three of the isoforms even showed significant reductions from controls after 24 hr exposure (Zhang et al., 2012).

The lack of induction observed in this study, however, does not necessarily mean that this isoform of GST is not inducible by PAHs. This study had a limited sampling design

with gene expression levels only examined 48hr after exposure in a limited number of crabs. While some studies showed significant induction within 48 hr following exposure to PAHs (Hansen et al., 2008; Zhang et al., 2012), other studies did not observed significant induction till 3 days (Xu et al., 2010) or 2 weeks (Nahrgang et al., 2009). Therefore, due to limited sampling of this study, the timing of induction of GST expression could have been missed. Furthermore, although the highest concentration of BaP used in this study was high enough to result in CYP induction in green crabs (Dam et al. 2008), they could not have been high enough to result in induction of GST in blue crabs.

While this study does not serve to validate the use of the putative GST for biomarker analysis with oil/PAH exposures, additional studies are needed to definitely say whether this GST isoform is or is not inducible by PAH exposure. Another study examining the expression of the putative blue crab GST should include multiple collection time points to examine expression over a wider range of time. Furthermore, additional dose concentrations and types of exposures should be examine, particularly water-borne exposures as they are more environmentally realist.

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